MicroRNA Regulation of Th17 Cell Differentiation

by

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Contributions of Co-Authors to Presented Work

Chapter 2 of this thesis is as yet unpublished. The authors on this manuscript are Misty M. Montoya, Julia Maul, Priti B. Singh, K. Mark Ansel, and Dirk Baumjohann. M.M. performed and analyzed most of the experiments under the supervision of K.M.A. and D.B.. J.M., P.B.S., and D.B. performed and analyzed some of the experiments. M.M., K.M.A., and D.B. designed the experiments, interpreted the data, and wrote the manuscript. Although not a co-author, our collaborator at the Memorial Sloan Kettering Cancer Center, Dr. Andrea Ventura, kindly provided the miR-18a$^{\Delta/\Delta}$ mice used in this study.

MicroRNA Regulation of Th17 Cell Differentiation

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Abstract

MicroRNAs (miRNAs) are small noncoding RNAs that regulate gene expression at the post-transcriptional level. They have been shown to play important roles in the development of B and T lymphocytes, and are powerful modulators of mature helper T cell differentiation and function in immunity. The miR-17~92 cluster of miRNAs regulates the differentiation and function of several subsets of T helper cells, including T helper (Th)17 cells. Th17 cells are a subset of CD4 helper T cells that characteristically produce the pro-inflammatory cytokine IL-17. They are defined by expression of the transcription factor RORγt, and are commonly identified by the chemokine receptor CCR6. Th17 cell responses orchestrate immunity against extracellular pathogens, but also underlie autoimmune disease pathogenesis.

We used conditional deletion of the entire cluster and manipulation of individual miRNAs within the cluster to dissect miR-17~92 control of Th17 cells. These experiments uncovered a distinct and critical role for miR-18a in limiting Th17 cell differentiation. In fact, miR-18a was the most dynamically upregulated miRNA in activated T cells among miR-17~92 cluster miRNAs. Targeted deletion of miR-18a enhanced $CCR6^{+}ROR\gamma t^{+}$ Th17 cell differentiation *in vitro* and increased the frequencies of Th17 cells in the lung expressing CCR6, RORγt and IL-17A in airway inflammation models *in vivo*. Furthermore, inhibition of miR-18 increased CCR6 and RORγt expression in mouse and

human $CD4^+$ T cells, revealing functional conservation. miR-18a directly targeted *Smad4*, *Hif1a*, and *Rora,* all key transcription factors in the Th17 cell gene expression program. Together these findings indicate that activating signals influence the fate of T helper cell differentiation via differential regulation of individual miRNAs within a common cluster.

To expand our work, we also sought to determine the global miRNA regulation of Th17 cell differentiation. Using a reliable and reproducible screening method in primary T cells, we examined the scope of miRNA regulators of both mouse and human Th17 cells. Our work supported previously identified miRNAs known to modulate Th17 cells and importantly revealed new miRNAs that both enhance and inhibit Th17 cells. These exciting results open opportunities for new projects to define the specifics of their biology.

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Chapter 1

Introduction

T-helper 17 (Th17) Cells

T cells orchestrate immune responses. Through lineage specification, as well as the choice to respond to stimuli, T cells make critical decisions for both protective immunity and tolerance to self. Although T cells are only one part of the immune system, T cell biology is a major focus of study because T cells also influence the response of many other immune cells. Several distinct types of T helper cells are derived from naïve $CD4^+$ T cells according to their surrounding cytokine milieu when they are activated to participate in an immune response. Each subset has its own set of transcription factors and produces characteristic cytokines. Based on those specifications, the T helper-17 (Th17) subset was identified as a distinct lineage separate from the well-established T helper-1 (Th1) and T helper-2 (Th2) subsets[1,2]. Much work has been done since then to establish the characteristics of the Th17 cell lineage and to identify the many functional roles this subset plays in the immune system.

Th17 cells coordinate immune responses against extracellular bacteria and fungi. They have also been implicated in the development of various autoimmune diseases such as multiple sclerosis[3] (MS; a demyelinating disease of the central nervous system) and more recently, in the pathogenesis of asthma[4]. Asthma is a chronic airway disorder characterized by bronchospasm and airway hyperresponsiveness caused by inflammation[5]. Asthma has been classically thought of as a Th2 cell-driven allergic disease, but recent data suggests an additional role for Th17 cells in airway neutrophilia and acute airway hyperresponsiveness[4,6].

Th17 cells are defined by the transcription factor RORγt[7] (retinoic acid receptor-related orphan receptor-γt) and the ability to produce the cytokine IL-17 upon restimulation[8]. While IL-17A is the defining cytokine of the Th17 lineage, Th17 cells can also produce other effector cytokines, including IL-17F and IL-22[9,10]. The expression of CCR6 (C-C chemokine receptor, type 6), a homing receptor important for the function of Th17 cells, is commonly used to identify Th17 cells[11].

Transcriptional Regulation of Th17 Cells

The molecular mechanisms that control helper T cell differentiation can be studied using *in vitro* culture systems. Much of this work has identified the cytokines and transcription factors important for the regulation of Th17 cells and importantly, these factors operate to promote Th17 responses *in vivo.* Th17 cells are generated from naïve $CD4^+$ T cells when they are activated in the presence of the cytokines TGFβ and IL-6[8,12-14] (Fig. 1). TGFβ signals through multiple downstream mediators in the SMAD pathway to activate Th17 effector genes. IL-6 induces STAT3 activation via the JAK/STAT signaling pathway, which then induces the transcription of various Th17 genes[15]. Cytokines like IFNγ and IL-4, commonly produced by other T helper subsets, Th1 and Th2 respectively, have been shown to inhibit the development of Th17 cells and as such blocking antibodies are also commonly added to *in vitro* culture conditions[1,2]. TGFβ also helps induce the expression of IL-6Rα and thus, the responsiveness of Th17 cells to IL-6.

While TGFβ and IL-6 are most important for the initial induction of murine Th17 cells,

they also influence the T cells to become more responsive to other cytokines important for the maintenance of differentiated Th17 cells, including IL-23 and IL-21[16-19]. TGFβ also enhances Th17 development by inducing IL-23R expression so Th17 cells can respond to the IL-23 cytokine[20]. While IL-23 is not crucial to the induction of the Th17 subset, it is important in the survival and expansion of differentiated Th17 cells to maintain their fully differentiated state[21]. IL-6 driven activation of STAT3 is important for the expression of IL-21. IL-21 is not critical to Th17 development or immune function *in vivo*[22,23] but it can enhance Th17 cells through STAT3 by acting in an autocrine amplification loop[24,25]. IL-6 and IL-21 also both induce SOCS3, a negative regulator of STAT3, and TGFβ works in part by inhibiting this induction to sustain STAT3 activation[26].

Another important Th17 induction cytokine is IL-1β, which has been used in alternative Th17-polarizing culture conditions in place of or addition to TGFβ. The importance of IL-1β has been well defined as a necessary requirement for the differentiation of human Th17 cells, but the necessity for Th17 induction is less clear in murine T cells. IL-6 is known to induce the expression of the IL-1R1 on T cells allowing IL-1β to regulate the expression of Th17 genes such as RORγt[27]. One study showed that IL-1β could play a unique role in Th17 induction in coordination with IL-6, even in the absence of TGFβ[27]. IL-1β can also act in synergy with IL-23 to sustain Th17 differentiation. Culture conditions that include IL-1β often together with IL-23 may generate Th17 cells that better model the pathogenic Th17 cells found in autoimmune diseases[28]. Additionally, this subset of Th17 cells can have different cytokine expression profiles,

including IFNg^{+}IL-17A^{+} double-producers and/or the expression of GM-CSF *in vivo*[29-32]. Furthermore, compared to the classically-activated Th17 cell subset, this alternatively-activated subset of Th17 cells was shown to have greater pathogenic potential in a model of EAE causing more severe disease in mice[33].

Much work has been done to identify other important functional regulators for Th17 differentiation beyond RORγt. Th17-polarizing cytokines also induce the transcription factor, RORα, which is closely related to RORγt and has a well-established function in Th17 biology[34,35]. RORα acts together with RORγt to induce Th17 genes[35]. STAT3 activation is necessary but not sufficient to induce RORγt and its deficiency leads to lessened RORα and RORγt induction. *Hif1a*, previously found to activate Th17 genes such as RORγt[36], and components of TGFβ signaling like *Smad4*, which encodes a key transcription factor downstream of TGFβ signaling in differentiating Th17 cells, are important positive regulators of Th17 cells. There are also multiple factors that inhibit Th17 cell differentiation, including Foxp3, the master transcription factor of regulatory T cells, and Foxo1. Th17 induction cytokines such as IL-1β and IL-6 have been shown to inhibit the TGFβ-induced expression of Foxp3, and therefore promote the development of naive CD4^{+} T cells to become Th17 cells[37-39]. Other negative regulators include Ets1 and STAT5. While we are beginning to better understand the transcriptional and epigenetic regulatory networks that govern Th17 cell differentiation[40], there is still much to learn about the role of microRNAs (miRNAs) in the regulation of Th17 cell biology.

miRNA Programming of Th17 Cells

MicroRNAs are ~21 nucleotide RNAs that are endogenously expressed and regulate genes at the post-transcriptional level through direct mRNA interactions via complementary base pairing. miRNA genes are transcribed into primary miRNAs (pri-miRNAs) by RNA pol II. Pri-miRNAs are then bound by the microprocessor complex, which consists of the subunit DiGeorge syndrome critical region gene 8 (DGCR8) and the RNase III-type protein Drosha, and are cleaved at the stem region to produce a hairpin structure (pre-miRNA). These pre-miRNAs are shuttled into the cytoplasm where they are bound by the RNase III-type protein Dicer, which cleaves off the hairpin loop to leave an RNA duplex[41]. The mature single-stranded miRNA then associates with Argonaute proteins to form the functional miRNA-induced silencing complex (miRISC). MicroRNAs guide the Argonaute-containing miRISC to specific mRNAs to inhibit their translation, ultimately leading to mRNA degradation through deadenylation and decapping[42]. In contrast to short interfering RNAs (siRNAs), miRNAs do not have perfectly complementary base pairing with target mRNAs[43] and thus can target the miRISC to a diverse array of mRNA transcripts. Some of the many functions of miRNAs include roles in development[44,45], cell lineage specification[42], and oncogenesis[46,47]. Recent studies show that miRNAs mediate regulation of development and function in the immune system[48] and have an important role in helper T cell differentiation[42].

Several miRNAs have been identified to play important roles regulating Th17 cell differentiation (Fig. 2). miR-155, whose pri-miRNA is transcribed from the non-protein coding gene, *bic*, has several functions in the immune system, including regulation of $CD4^+$ helper T cell subsets like regulatory T (Treg) cells[49,50] and Th17 cells[51-54]. Overall,

work on miR-155 has shown it to be a powerful promoter of Th17 cells. One study found that $CD4^+$ T cells deficient in miR-155 had defective IL-17 expression. These authors identified that miR-155 binds to Jarid2, a DNA-binding protein, that affects chromatin accessibility and ultimately Th17 gene expression[51]. miR-155 expression levels were upregulated in patients with multiple sclerosis (MS) and in mice with experimental autoimmune encephalomyelitis (EAE), a mouse model for MS[52]. Similarly, miR-155 was shown to promote autoimmune pathogenesis while miR-155-deficient mice were resistant to EAE[53]. miR-155 has also been shown to regulate Th17 cell effector gene expression, including the IL-23R, by targeting Ets1, a transcription factor known to negatively regulate Th17 cells[55]. miR-326 was also found to target Ets1 to ultimately promote Th17 cell differentiation. Interestingly, the expression levels of miR-326 correlated with disease severity in patients with MS and mice with EAE[56].

miR-21 also promotes Th17 cells partially by mediating its effects through *Smad7*, an inhibitor of TGFβ signaling and a known suppressor of Th17 cells[57]. T cells deficient in miR-21 showed decreased Th17 differentiation. Expression of miR-21 was also upregulated in Th17-associated autoimmune conditions, including MS and psoriasis. In a passive transfer model of EAE, mice treated with a miR-21 inhibitor had suppressed EAE symptoms. Work from another group provided further evidence in support of miR-21 and miR-155 as positive regulators of Th17 cells. This work showed that miR-21 and miR-155, along with another miRNA, miR-301a, were all highly expressed in myelin antigen-specific $CD4^+$ Th17 cells that were responsive to the MOG peptide *in vitro* after immunization *in vivo*[58]. The MOG peptide is considered a myelin autoantigen and is

commonly used to induce the Th17-driven disease, EAE, in mice. miR-301a is part of the miR-130/301 family of miRNAs that includes miR-301a, -301b, -130a, and -130b. Studies additionally showed that inhibition of miR-301a led to decreased Th17 cell generation *in vitro* through its effects on *Pias3*, a component of the IL-6/STAT3 signaling pathway and also showed milder EAE symptoms *in vivo*. miR-301a has also been implicated in other Th17-associated diseases, including inflammatory bowel disease (IBD)[59]. This work showed that inhibition of miR-301a decreased the number of IL-17-expressing cells along with amounts of other pro-inflammatory mediators like TNFα in the gut in a mouse model of colitis. Taken together, miR-301a appears to be another important promoter of Th17 development.

Another recent publication showed that the miR-183 cluster (miR-183C) also regulated Th17 cells. This cluster produces 3 mature miRNAs, miR-183, miR-96, and miR-182, that have both overlapping and unique gene targets[60]. All three miRNAs from this cluster were induced by the IL-6-STAT3 signaling pathway and found to be upregulated in Th17 cells compared to other T helper subsets[61]. Ultimately, the miR-183C promotes the pathogenicity of Th17 cells in part by directly targeting Foxo1. Foxo1 was found to suppress the IL-1R1 in Th17 cells via its ability to interfere with the RORγt-mediated induction of the IL-1R1, highlighting at least one important mechanism for the regulation of 'more pathogenic' Th17 cells.

miR-210 has also been shown to be an important negative regulator of Th17 cells. This miRNA is known to be highly inducible during low oxygen (hypoxic) conditions and has

an important role in the regulation of B cell biology and cell proliferation[62-64]. miR-210 is regulated by several factors including the hypoxia inducible factor (HIF), HIF1α, and nuclear factor κB (NFκB). Surprisingly in other work, miR-210 was also found to directly target *Hif1a*, a known transcriptional regulator of Th17 cells[36], in a negative feedback loop[65]. This study also showed that miR-210 was specifically upregulated in Th17 cells. In hypoxic conditions, miR-210 deficiency promoted Th17 cell differentiation through its effects on *Hif1a*. In an IBD model of colitis, miR-210 also affected Th17 differentiation *in vivo* where mice deficient in miR-210 had symptoms that correlated with higher disease severity.

Recently, a member of the miR-30 family, miR-30a, was found to play an inhibitory role in Th17 cell differentiation[66]. The miR-30 family includes 5 mature miRNAs, including miR-30a, miR-30b, miR-30c, miR-30d, and miR-30e, some of which (miR-30a, -30d, and -30e) share much sequence similarity differing by only one nucleotide. A microarray analysis from this study showed that most members of the miR-30 family were downregulated in Th17 cells when compared to naïve T cells. miR-30a expression levels also declined throughout the *in vitro* Th17 differentiation process. Overexpression of miR-30a inhibited Th17 cell differentiation *in vitro* and alleviated the clinical symptoms and disease pathogenesis of EAE *in vivo* in mice. Additionally, the IL-21R was found to be a direct functional target of miR-30a mediating its inhibitory effect on Th17 cells.

miRNA members of the miR-17~92 cluster have also been implicated in Th17 cell biology. This cluster is transcribed as a polycistronic RNA that ultimately produces six

individual mature miRNAs, miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92a[67]. The miRNAs within this cluster can be further categorized into four families grouped by similar seed sequences (the first eight nucleotides at the 5' end that are the major determinant of target pairing)[43] (Fig. 3). The miR-17~92 cluster has been shown to play a role in oncogenesis[68]. In an Eμ-myc model of B cell lymphoma, miR-19 alone was found to have a dominant effect compared to other members of the miR-17~92 cluster in promoting *c-myc*-induced B cell oncogenesis[69,70]. While miR-17~92 has been extensively studied in cancer, earning the nickname Oncomir-1, it is also known to have several prominent roles in T cell biology[71-73].

Our group has shown that miRNAs, and particularly the miR-17~92 cluster, are important for robust T follicular helper (T_{FH}) cell differentiation[34]. This work also showed that miR-17~92 repressed a "Th17/Th22 signature" in T_{FH} cells. A microarray-based gene expression analysis of sorted T_{FH} cells found, surprisingly, that miR-17~92 knockout cells had upregulated genes reflecting a Th17/Th22 pattern including CCR6, IL-22, and RORα. *Ex vivo* cytokine production assays showed that wild-type (WT) cells hardly produced any IL-22 or IL-17A, but miR-17~92-deficient T_{FH} cells produced large amounts of these cytokines. Introducing a single defective allele of RORα, which is known to be important for Th17 cell differentiation, reduced expression of these aberrant Th17-like genes in the miR-17~92 knockout T_{FH} cells. This work suggested that miR-17~92 represses a Th17/Th22-associated gene expression program in T_{FH} cells at least in part through direct targeting of RORα.

Two recent studies further implicate this miRNA cluster and related family members in the regulation of Th17 biology[74,75]. miR-17 and miR-19 promote IL-17 production by targeting PTEN and IKZF4. Consistent with this work, we also found miR-17 and miR-19 family miRNAs to promote IL-17 production. Another miR-17 family member, miR-20b, was shown to inhibit Th17 differentiation through its regulation of STAT3. These previous studies of the miR-17~92 cluster differ on its overall effect on Th17 responses, exemplifying the necessity to further clarify the role of this cluster in Th17 cell biology. For the most part, many studies have focused on the importance of the miR-17 and miR-19 family miRNAs in a variety of roles in the immune system[73,75-78] while miR-18 has remained seemingly unimportant compared with its nearby counterparts. Our work attempts to clarify the role of the miR-17~92 cluster in Th17 biology and importantly uncovers a distinct and crucial inhibitory role for miR-18a in Th17 differentiation.

Thesis Work Overview

Since miR-17~92 was previously shown to promote T_{FH} cell differentiation and repress gene expression reminiscent of Th17 cells, these data have provided a foundation to further investigate the role of miR-17~92 in Th17 cells. Chapter 2 discusses our work aimed to define the role of the miR-17~92 cluster in Th17 cell differentiation. Using mouse genetics, we tested the importance of the entire miR-17~92 cluster in Th17 cell differentiation. We assessed the ability of control ($17\sim92^{+/+}$ = CD4-Cre^{-} miR-$17\sim92^{fl/fl}$) and miR-17~92-deficient ($17\sim92^{\Delta/\Delta}$ = CD4-Cre^{+} miR-$17\sim92^{fl/fl}$) T cells to become Th17 cells *in vitro* and found that $17\sim92^{\Delta/\Delta}$ mice had increased generation of $CCR6^{+}ROR\gamma t^{+}$ Th17 cells. These data suggested that the entire miR-17~92 cluster or a particular

member within this cluster functions to repress Th17 cell differentiation. To dissect the individual effects of miRNAs within this cluster on Th17 cell differentiation, we transfected miRNA mimics corresponding to each miRNA in the cluster using the Neon electroporation system into miR-17~92 knockout cells and analyzed changes in Th17 cell markers by flow cytometry. Interestingly, our data shows that multiple members of the miR-17~92 cluster differentially regulate multiple aspects of Th17 cell differentiation and effector function. These data revealed a distinct and unexpected inhibitory role for miR-18a showing that miR-18a alone can recapitulate the effects of the entire miR-17~92 cluster on the differentiation of $CCR6^{+}ROR\gamma t^{+}$ Th17 cells. Importantly, miRNA expression analyses also showed that among the 6 miRNAs in the miR-17~92 cluster, miR-18a was the most dynamically upregulated miRNA in activated T cells.

While miR-18a seemed to have a dominant effect inhibiting Th17 differentiation, studying T cells deficient in the entire miR-17~92 cluster meant that other miRNAs in the cluster will also contribute to the phenotype of miR-17~92-deficient T cells. To further dissect the specific role(s) of miR-18a, we wanted to compare cells lacking just miR-18a with those lacking the entire cluster to more clearly reveal the specific requirements for miR-18a *in vitro* and *in vivo*. To interrogate the specific role for miR-18a, we used a novel transgenic mouse deficient in miR-18a ($miR\text{-}18^{\Delta/\Delta}$), and analyzed changes in Th17 differentiation *in vitro*. These mice were made available to us through our collaboration with Dr. Andrea Ventura (Memorial Sloan-Kettering Cancer Center), whose lab has generated miR-18a-specific knockout mice that retain normal expression of the remaining miRNAs in the miR-17~92 cluster. This novel tool enabled us to directly

and fully characterize the requirements for miR-18a alone in Th17 cell differentiation and gene expression *in vitro* and in physiological settings. Additionally, we used transfectable miRNA family inhibitors in primary human T cells to test the conservation of miRNA activity in Th17 cells and its relevance to human biology. These experiments further confirmed that miR-18a limits Th17 differentiation.

To gain mechanistic understanding of the miR-18 mediated regulation of Th17 cell differentiation, it is important to identify the network of target genes involved in this process. To do this we generated a panel of potentially relevant target genes to further validate, guided by the extensive literature on miR-17~92 and by using miRTarBase[79], an online database of published miRNA targets. We identified known miR-18 target genes that are expressed in T cells and interrogated these candidates by a functional analysis with gene-specific siRNAs. We tested which of these genes were limiting factors for *in vitro* Th17 cell differentiation and cytokine production using siRNA knockdown of each target gene in miR-17~92 knockout T cells polarized under Th17 conditions. These studies identified candidate genes whose siRNA-mediated knockdown at least partially rescued the phenotype of these cells. Further target characterization was done to validate direct miRNA targeting of these candidate genes using 3'UTR dual luciferase reporter assays[80]. This work identified and confirmed several functionally relevant target genes, including *Smad4* and *Hif1a,* as well as *Rora,* a previously known miR-18a target. 3'UTRs of all candidates showed responsiveness to miR-18a, confirming they are all direct miR-18a targets. Additional experiments further supported these findings where we tested the importance of miR-18a fine-tuning of the

expression of SMAD4 and RORα, by assessing Th17 cell differentiation from 17~92$^{\Delta/\Delta}$ CD4^{+} T cells that were also heterozygous for either the *Rora*^{sg} mutation (which encodes a nonfunctional truncated RORα protein) or had a targeted conditional loss-of-function *Smad4* allele (*Smad4*$^{\Delta}$). By genetically limiting them to one allele, we could assess if this would partially rescue the miR-17~92 knockout phenotype. Indeed, we observed decreases in the elevated frequency of CCR6^{+}RORγt^{+} cells in miR-17~92-deficient mice that were also either *Rora*$^{sg/+}$ *or Smad4*$^{\Delta/+}$. These analyses provided mechanistic insight and enabled us to build a network model of the post-transcriptional regulation of Th17 cell differentiation by miRNAs.

We previously observed that miR-17~92 miRNAs are highly expressed in airway-infiltrating helper T cells in human subjects with asthma[76,81]. Additionally, there is evidence for the differential expression and regulation of this cluster in asthma as seen by the specific increase of miR-19a from the airways of patients with asthmatic disease. Many of these T cells express CCR6 and other markers of Th17 cells. Th17 cells play a role in the pathogenesis of mouse models of asthma[6,82] and they may also be pathogenic drivers of the most severe forms of this common inflammatory disease in humans[83]. As such, we used two mouse models of allergic airway inflammation to determine the functional significance of the role of miR-18 in airway inflammatory disease through its effects on Th17 cells.

First, we determined the *in vivo* function of miR-18 in a standard sensitization and challenge experimental airway inflammation mouse model of asthma. We also

examined the role of miR-18 in a longer inhaled LPS+OVA-driven model of airway inflammation. For both models of airway inflammation, we monitored the proportion and number of Th17 cells using the Th17 markers CCR6 and RORγt, and by their ability to produce IL-17 and other inflammatory cytokines upon activation *ex vivo*. We also enumerated myeloid cells including neutrophils, eosinophils, and macrophages. miR-18 deficiency in Th17 cells lead to a worsened airway inflammatory phenotype as seen by increased lung infiltration by Th17 cells. Additionally, we observed a shift towards a less eosinophilic phenotype in the airways, consistent with a Th17-mediated process[84]. Together, our work shows that miR-18 negatively regulates Th17 cell differentiation and immune function through a variety of factors important for Th17 cell biology.

These experiments have provided much insight into the unique role of an often-overlooked miRNA within a well-studied miRNA cluster. The differential regulation and distinct dynamic upregulation of miR-18a in comparison to other miRNAs within the same cluster is particularly interesting. Our work helps clarify how the role of this miRNA cluster fits into the network of genes regulating Th17 cell differentiation and immune function. We wanted to extend our findings by broadening the scope of the miRNA regulation of Th17 cells. Chapter 3 highlights this work using a robust technical method of screening to examine a wide list of miRNAs expressed in T cells and their regulation on Th17 cells. To do this, two functional screens were performed. The first functional screen makes use of genetic mice lacking mature miRNAs thus allowing us to individually put back each miRNA of interest using synthetic miRNA mimics to assess the effect on Th17 differentiation. The second loss-of-function screen takes an

alternative approach with human primary T cells using individual and family-wise miRNA inhibitors to assess their effects while expression of other miRNAs remains intact. Both approaches have provided a wide landscape view identifying multiple miRNAs that significantly have an effect on Th17 cell biology.

In order to perform a successful screen in human *in vitro* polarized Th17 cells, an optimized protocol had to be created since this had not been done before. Appendix 1 contains a first-author publication of the work I did optimizing conditions to successfully transfect primary human Th17 cells with small RNAs reagents. This work was vital to the experimental success of the functional screen performed with human Th17 cells presented in Chapter 3. Appendix 2 is my first publication with the Ansel lab. My contribution to this work was published in Supplementary Figure 2, which focuses on miRNA expression of miR-17~92 miRNAs by SYBR qPCR analysis in 17~92-deficient and control naïve $CD4^+$ T cells. This technique was later used in my own first-author manuscript. Both appendices show technical skills that were important to my own work in the Ansel lab.

Figure 1. Th17 cell differentiation. This graphic shows the process of Th17 cell differentiation. Th17 cells are generated from naïve $CD4^+$ T cells in the presence of the polarizing cytokines, TGFβ and IL-6. Other combinations of Th17-polarizing cytokines include IL-1β and IL-23, which are known to induce and/or maintain Th17 cells. Th17 cells are commonly identified by the chemokine receptor CCR6 and are characterized by the expression of RORα and RORγt as well as the cytokine IL-17A. Th17 cells are also capable of producing other effector cytokines including IL-22 and IL-17F.

Fig 1.

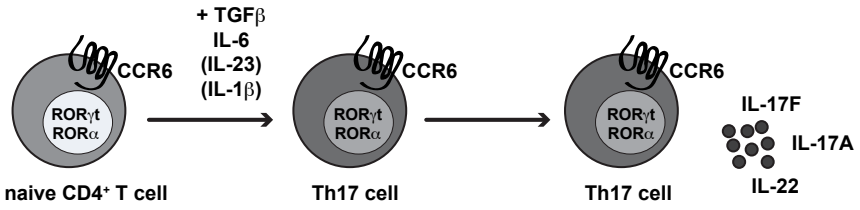


Figure 2. miRNAs known to regulate Th17 cell biology. A schematic of miRNAs known to negatively (red lines) or positively (green arrows) regulate Th17 cells. Various miRNAs target multiple genes involved in Th17 development. miRNAs in green identify miRNAs that promote Th17 cells while miRNAs listed in red inhibit Th17 cells. A shaded grey circle highlights work from this thesis, which shows the miR-18-mediated regulation of multiple transcription factors, including *Rora*, *Smad4*, and *Hif1a*, all of which are positive regulators of Th17 cells.

Fig 2.

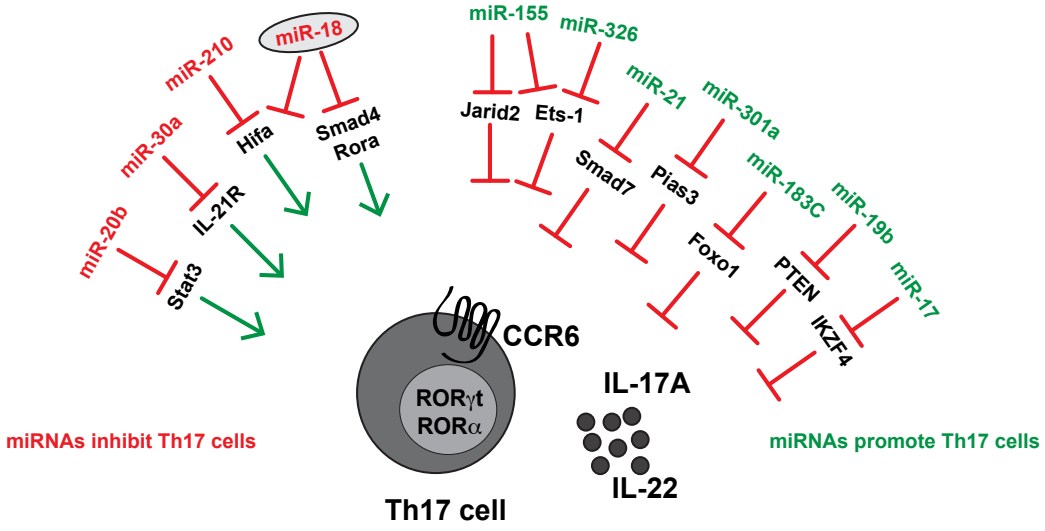
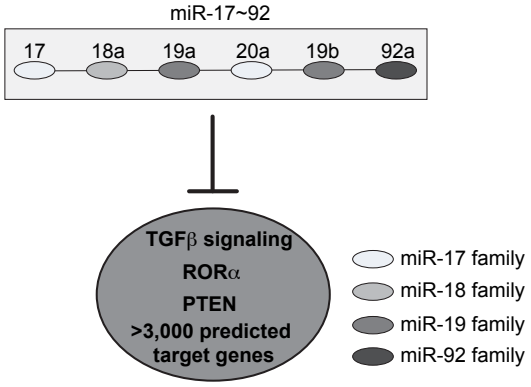


Figure 3. The miR-17~92 cluster miRNA families. Schematic of the miR-17~92 cluster. This cluster produces six mature miRNAs that can be categorized into four miRNA families (miR-17, miR-18, miR-19, and miR-92-families). miRNAs within this cluster have several known targets and many more predicted target genes.

Fig 3.



Chapter 2

A distinct inhibitory function for miR-18a in Th17 cell differentiation

Abstract

T helper (Th)17 cell responses orchestrate immunity against extracellular pathogens, but also underlie autoimmune disease pathogenesis. Here, we uncovered a distinct and critical role for miR-18a in limiting Th17 cell differentiation. miR-18a was the most dynamically upregulated miRNA of the miR-17~92 cluster in activated T cells. miR-18a deficiency enhanced $CCR6^{+}ROR\gamma t^{+}$ Th17 cell differentiation *in vitro* and increased the number of tissue Th17 cells expressing CCR6, RORγt and IL-17A in airway inflammation models *in vivo*. Sequence-specific miR-18 inhibitors increased CCR6 and RORγt expression in both mouse and human $CD4^{+}$ T cells, revealing functional conservation. miR-18a directly targeted *Smad4*, *Hif1a*, and *Rora,* all key transcription factors in the Th17 cell gene expression program. These findings indicate that activating signals influence the outcome of T helper cell differentiation via differential regulation of mature miRNAs within a common cluster.

Introduction

T helper (Th) cells orchestrate cellular and antibody-mediated immunity against a variety of pathogens. To accommodate this functional diversity, Th cells differentiate into distinct subsets classified by defined phenotypic characteristics and specialized functions in immunity[85]. Th17 cells combat extracellular bacteria and fungi, but they have also been implicated in the pathogenesis of several autoimmune and inflammatory diseases, including multiple sclerosis and psoriasis[86-88]. Th17 cells also mediate immune responses that are involved in maintaining epithelial barrier integrity, and it has been widely suggested that some cases of asthma may be caused by dysregulated Th17 responses[88,89]. Studies of both human asthma and mouse models indicate that lung inflammation can shift to a response marked by Th17 cytokines and neutrophil infiltration when Th2 cell-mediated eosinophilia is reduced[90,91]. Understanding how Th17 cells are programmed and contribute to tissue inflammation remains an important research frontier with high potential for therapeutic impact in a broad spectrum of inflammatory diseases.

Th17 cells can be generated *in vitro* by activating $CD4^+$ T cells in the presence of TGFβ and IL-6[12-14]. They are characterized by IL-17 secretion and expression of their lineage-defining transcription factor, RAR-related orphan receptor (ROR)γt. The closely related factor RORα is also an important transcriptional regulator of the Th17 gene expression program[35]. Th17 cells are commonly identified by expression of the RORγt/RORα target gene *Ccr6*, which encodes a chemokine receptor important for Th17 cell trafficking into the brain and mucosal tissues[11,92-94]. In addition to RORγt and RORα, Th17 cells utilize

multiple additional transcription factors in a robust transcriptional network that promotes their gene expression program[40,95]. Of note, HIF1α is a limiting factor that regulates Th17 cell biology[36,65,96], and SMAD4 cooperates with SMAD2/3 to transduce TGFβ signaling in differentiating Th17 cells.

MicroRNAs (miRNAs) have emerged as potent regulators of T helper cell differentiation, function and plasticity[42]. MicroRNAs are small endogenously expressed RNAs that regulate gene expression at the post-transcriptional level. Individual miRNAs can target hundreds of distinct mRNAs, and each mRNA can have several miRNA binding sites. Th17 cell differentiation is impaired in miRNA-deficient T cells[97], and important roles have been identified for several particular miRNAs[51,56,65]. Some of these miRNAs indirectly influence Th17 lineage commitment by acting on other cell types[53], while others act directly on cell-intrinsic signaling that induces Th17 cell programming, expansion and effector function *in vitro* and in mouse models of autoimmunity[53,58,65,98-100].

The *Mirc1* locus, better known as the miR-17~92 cluster, encodes six miRNAs in four families (miR-17, miR-18, miR-19, and miR-92 families), each defined by a common 'seed sequence' and predicted targets[67]. The miR-17~92 cluster and miRNAs in these four families are important for T cell proliferation and survival, and for the proper differentiation and immunological functions of Treg, Tfh, Th1, Th2 and Th17 cells[34,42,71-78,101,102]. In Tfh cells, miR-17~92 deficiency also induced inappropriate expression of Th17-associated genes[34]. Studies that dissected the functionally relevant miRNAs

within the miR-17~92 cluster in T cells have focused almost entirely on the miR-17 and miR-19 families, and uncovered similar roles in promoting clonal expansion and cytokine production in a variety of Th subsets[73,75-78]. In contrast, miR-18a has drawn little attention. No unique function has been ascribed to this miRNA in immune cells, and recently characterized miR-18a-deficient mice did not show any overt immunopathological features[103].

Here, we uncovered a unique role for miR-18a as a highly inducible inhibitor of Th17 differentiation. Accordingly, miR-18a-deficient mice exhibited increased Th17 responses in airway inflammation models *in vivo*. We identified *Smad4*, *Hif1a*, and *Rora* as important target genes mediating miR-18a regulation of Th17 cell differentiation.

Results

miR-18a is the most dynamically regulated miRNA of the miR-17~92 cluster during Th17 cell differentiation

The miR-17~92 cluster comprises six different miRNAs that can be grouped into four distinct families based on their seed sequence (Fig. 1A). To gain insight into the dynamics of miR-17~92 expression during Th17 cell differentiation, we first cultured purified control (17~92$^{+/+}$ = CD4-Cre^{-} miR-17~92$^{fl/fl}$) and miR-17~92-deficient (17~92$^{\Delta/\Delta}$ = CD4-Cre^{+} miR-17~92$^{fl/fl}$) CD4^{+} T cells *in vitro* for 4 days under classical Th17-polarizing conditions (TGFβ+IL-6). We assessed the expression of each miRNA in the miR-17~92 cluster during the course of Th17 differentiation by quantitative PCR (Fig. 1B). All of the miR-17~92 cluster miRNAs were expressed in 17~92$^{+/+}$ CD4^{+} T cells under these conditions and, as expected, all six miRNAs were absent in 17~92$^{\Delta/\Delta}$ CD4^{+} T cells. While most miRNAs from this cluster were induced upon activation, miR-18a was the most dynamically regulated, showing a greater than ten-fold induction that was sustained over three days in culture. Mature miR-92a was not induced together with the other cluster miRNAs. Importantly, representative miRNAs from the paralogous miRNA gene clusters miR-106b~25 and miR-106a~363 (Fig. 1A) were expressed at comparable levels in 17~92$^{+/+}$ and 17~92$^{\Delta/\Delta}$ CD4^{+} T cells throughout the time course (Fig. 1C). Consistent with previous reports[104], miR-25 was abundant in developing Th17 cells, whereas miR-363 was barely detectable (Fig. 1C).

miR-18a is active in differentiating CD4^{+} T cells

To assess the inhibitory activity of each miRNA family represented within the miR-17~92 cluster, we used previously developed retroviral miRNA activity sensors in developing Th17 cells[76,78]. Each reporter encodes EGFP with four binding sites for the miRNA of interest within an artificial 3'UTR, resulting in EGFP expression that is inhibited by miRNAs of the corresponding family in a sequence-specific fashion (Supplementary Fig. 1). We assessed the activity of the miR-17, miR-18, miR-19, and miR-92 families in 17~92$^{+/+}$ control and 17~92$^{\Delta/\Delta}$ Th17 cells (Fig. 1D). Sensor EGFP expression was increased in 17~92$^{\Delta/\Delta}$ Th17 cells as compared to 17~92$^{+/+}$ control cells, indicating that endogenous miR-17~92 cluster miRNAs from all four families actively inhibit target mRNAs. However, the increase in EGFP expression was greatest for miR-18 and miR-19 sensors. 17~92$^{\Delta/\Delta}$ Th17 cells displayed only intermediate EGFP expression from miR-17 and miR-92 sensors, suggesting that other family members encoded outside of the miR-17~92 cluster make substantial contributions to miR-17 and miR-92 activity in Th17 cells. These findings were further corroborated by experiments with locked nucleic acid miRNA “family inhibitors”, designed to simultaneously inhibit all members of each family (Supplementary Fig. 1). The miR-17 family inhibitor further increased the already elevated miR-17 sensor EGFP expression in 17~92$^{\Delta/\Delta}$ Th17 cells. Similar results were obtained for miR-19 and miR-92 family inhibitors and matched sensors, suggesting residual activity beyond what is accounted for by miR-17~92 cluster miRNAs. In contrast, the miR-18 sensor EGFP expression was unaffected by the miR-18 family inhibitor in 17~92$^{\Delta/\Delta}$ Th17 cells. Taken together, the expression and activity data show that deletion of miR-17~92 was sufficient to nearly abolish miR-18

and strongly reduce miR-19 activity, but that other members of the miR-17 and miR-92 families exhibit substantial activity in 17~92$^{\Delta/\Delta}$ Th17 cells.

miR-18a inhibits Th17 cell differentiation

To determine the individual contribution of miR-18a and the other miR-17~92 cluster miRNAs to Th17 cell differentiation, we first cultured purified control (17~92$^{+/+}$) and miR-17~92-deficient (17~92$^{\Delta/\Delta}$) CD4$^+$ T cells *in vitro* for 4 days under classical Th17-polarizing conditions (TGFβ+IL-6). 17~92$^{\Delta/\Delta}$ T cells showed increased expression of CCR6 and RORγt (Fig. 2A) with variable IL-17A production (Fig. 2B). To isolate individual effects of each member of the miR-17~92 cluster on Th17 cell differentiation, miRNA mimics of the cluster were transfected into activated 17~92$^{\Delta/\Delta}$ CD4$^+$ T cells cultured under Th17-polarizing conditions, and the effect on CCR6, RORγt, and IL-17A expression was assessed by flow cytometry. Strikingly, only miR-18a could significantly rescue the increased frequency of CCR6$^+$RORγt$^+$ cells in 17~92$^{\Delta/\Delta}$ Th17 cells, restoring it to the frequency observed in cultures of control 17~92$^{+/+}$ cells (Fig. 2C). In contrast, several other miRNAs of the cluster further increased the already enhanced frequency of CCR6$^+$RORγt$^+$ cells in 17~92$^{\Delta/\Delta}$ Th17 cell cultures (Fig. 2C). Consistent with a previous report[75], miR-17 family members also enhanced IL-17 production (Supplementary Fig. 2). These data demonstrate that miR-18a exhibits a distinct inhibitory function on CCR6$^+$RORγt$^+$ Th17 cells.

Genetic ablation of miR-18a alone increases the frequency of CCR6$^+$RORγt$^+$ Th17 cells

To directly assess the requirement for endogenous miR-18a, we tested Th17 differentiation in miR-18$^{\Delta/\Delta}$ CD4^{+} T cells from recently developed mice that harbor a targeted deletion of miR-18 while retaining normal expression of all the remaining miRNAs of the miR-17~92 cluster[103]. Similar to what we observed for miR-17~92 cluster deletion, loss of miR-18a alone increased the frequency of CCR6^{+} cells and CCR6^{+}RORγt^{+} cells among cultured Th17 cells as compared to wildtype controls, with no consistent effect on IL-17 production (Fig. 3A). These defects could be rescued by transfecting a miR-18a mimic into miR-18$^{\Delta/\Delta}$ CD4^{+} T cells (Fig. 3B). Taken together, these data demonstrate that miR-18a plays a critical role in limiting Th17 cell differentiation.

miR-18a deficiency increases lung Th17 cell frequencies in airway inflammation models

Next, to test the requirement for endogenous miR-18a for Th17 responses *in vivo*, we induced asthma-like inflammation in the lungs of control and miR-18$^{\Delta/\Delta}$ mice by sensitizing/challenging these mice with ovalbumin (OVA) (Fig. 4A). While we observed no differences in the numbers of CD4^{+} T cells or overall CD45^{+} leukocytes, frequencies of CCR6^{+} and CCR6^{+}RORγt^{+} CD4^{+} T cells were significantly increased in miR-18$^{\Delta/\Delta}$ mice (Fig. 4B). This was also reflected in elevated RORγt gMFI of miR-18$^{\Delta/\Delta}$ CD4^{+} T cells compared to miR-18$^{+/+}$ CD4^{+} T cells. Importantly, frequencies of IL-17-producing cells were also significantly increased among re-stimulated miR-18-deficient lung CD4^{+} T cells, while frequencies of IL-13-producing Th2 cells and IFNγ-producing Th1 cells were not affected (Fig. 4C). The number of lung eosinophils, but not neutrophils, was

decreased (Fig. 4D), consistent with a shift from Th2-mediated to Th17-mediated inflammation[84] in miR-18$^{\Delta/\Delta}$ airways. miR-18 deficiency also increased Th17 cells in the lung and draining mediastinal lymph nodes in an inhaled LPS+OVA model of airway inflammation (Supplementary Fig. 3).

Inhibition of the miR-18 family increases mouse and human Th17 cell differentiation

The ability to specifically inhibit miRNAs from the miR-17~92 cluster in a family-wise manner using the transfectable inhibitors gave us the opportunity to test whether endogenous miR-18 is important for Th17 differentiation. In line with the observed increase in CCR6$^+$RORγt$^+$ Th17 cell differentiation of miR-18a-deficient cells *in vitro* (Fig. 3) and *in vivo* (Fig. 4), inhibition of the miR-18 family significantly increased the expression of both CCR6 and RORγt in both mouse and human CD4$^+$ T cells cultured under Th17 differentiation conditions (Fig. 5A and B, respectively). These data indicate that miR-18 acts as an evolutionarily conserved inhibitor of Th17 cell differentiation. Our miRNA family inhibitor experiments also confirmed the miR-17 family's role in promoting cytokine production[75], as the miR-17 family inhibitor reduced IL-17 production in transfected Th17 cells (Supplementary Fig. 2).

***Smad4*, *Hif1a,* and *Rora* are functionally relevant direct miR-18a target genes**

To identify relevant miR-18a target genes in Th17 cell differentiation, we compiled a list of 18 previously reported miR-18 family target genes from miRTarBase[79] and performed siRNA-mediated inhibition of these genes in 17~92$^{\Delta/\Delta}$ CD4$^+$ T cells to determine whether

any of them are limiting factors for *in vitro* Th17 cell differentiation and effector cytokine production. siRNA SmartPools against several of these genes altered the expression of CCR6, RORγt and/or IL-17 (Supplementary Fig. 4). Following retesting with siRNA SmartPools to confirm effects observed in the primary screen (data not shown), the top five target genes whose pooled siRNAs at least partially rescued the increased CCR6 and/or RORγt expression of 17~92$^{\Delta/\Delta}$ CD4^{+} T cells were examined further by separately transfecting 17~92$^{\Delta/\Delta}$ CD4^{+} T cells with three individual unique siRNAs against each gene (Fig. 6A). Multiple siRNAs against *Smad4*, a transcriptional mediator of TGFβ signaling, reversed the exaggerated increase in CCR6^{+}RORγt^{+} 17~92$^{\Delta/\Delta}$ T cells. The siRNA SmartPool against *Hif1a*, another important transcriptional regulator of Th17 cell differentiation, rescued the increased generation of CCR6^{+} Th17 cells (Supplementary Fig. 4). Individual siRNAs against *Hif1a* also partially rescued the increased frequency of CCR6^{+}RORγt^{+} Th17 cells (Fig. 6A). Targeting either of these genes also reduced IL-17 production, as did siRNAs against the miR-17 family target gene *Stat3* (Supplementary Fig. 4). As previously reported[75,76], siRNAs targeting *Pten* promoted IL-17A production, but they did so without consistently affecting the generation of CCR6^{+}RORγt^{+} Th17 cells (Fig. 6A and Supplementary Fig. 4).

To further validate direct miRNA targeting of candidate target genes from the siRNA screen, we used 3'UTR dual luciferase reporter assays in primary mouse T cells. Although *Smad4* lacks a predicted miR-18 binding site, it had previously been suggested to be a miR-18 target[105]. While we did detect de-repression of the annotated *Smad4* 3'UTR reporter in 17~92$^{\Delta/\Delta}$ CD4^{+} T cells (Fig. 6B), we could not significantly

reverse that effect with any individual miRNA mimic (Fig. 6C). Based on RNA sequencing results[106] (and data not shown), we suspected that a longer *Smad4* transcript with an extended 3'UTR might be the functionally relevant target in this system. Quantitative RT-PCR confirmed that such a transcript is indeed expressed in naïve $CD4^+$ T cells (Supplementary Fig. 5), and a *Smad4* 3'UTR extension segment luciferase reporter was inhibited by both miR-18a and miR-17 in transfected *Dgcr8*$^{\Delta/\Delta}$ $CD4^+$ T cells (Fig. 6D). The *Hif1a* 3'UTR reporter was also de-repressed in 17~92$^{\Delta/\Delta}$ $CD4^+$ T cells compared to 17~92$^{+/+}$ $CD4^+$ T cells (Fig. 6E). Importantly, the *Hif1a* 3'UTR was responsive to miR-18a as well as miR-17, establishing *Hif1a* as a novel miR-18 target (Fig. 6F). Finally, miR-18a repressed *Rora* 3'UTR reporter expression in 17~92$^{\Delta/\Delta}$ $CD4^+$ T cells, providing further evidence (Baumjohann et al., 2013) that *Rora* is a direct target of miR-18a, even in the presence of residual miR-17 family activity (Fig. 6G).

These data indicate that miR-18a directly targets the mRNAs encoding three transcription factors that are important inducers of the Th17 cell gene expression program. siRNA experiments indicated that all three of these mRNAs could be limiting factors for Th17 cell differentiation, suggesting that regulation by miR-17~92 cluster miRNAs could impact their function. To further test this possibility, we generated 17~92$^{\Delta/\Delta}$ $CD4^+$ T cells that were also heterozygous for either the *Rora*^{sg} mutation (which encodes a nonfunctional truncated RORα protein) or a targeted conditional loss-of-function *Smad4* allele (*Smad4*$^{\Delta}$*)*. $CD4^+$ T cells from both 17~92$^{\Delta/\Delta}$*Rora*$^{sg/+}$ (Fig. 6H) and 17~92$^{\Delta/\Delta}$*Smad4*$^{\Delta/+}$ (Fig. 6I) mice exhibited a decrease in the characteristic elevated frequency of 17~92$^{\Delta/\Delta}$ $CCR6^+ROR\gamma t^+$ cells to near miR-17~92$^{+/+}$ control levels,

especially when cultured in Th17 conditions with higher doses of TGFβ. Thus, genetically limiting *Rora* or *Smad4* to one allele partially rescued the 17~92$^{\Delta/\Delta}$ phenotype. In summary, these data strongly suggest that *Smad4, Hif1a,* and *Rora* are all functionally relevant target genes of miR-18a.

Discussion

Here, we showed that miR-18a is the key miRNA of the miR-17~92 cluster that negatively regulates Th17 cell differentiation. Our experiments revealed that miR-18a is the most dynamically regulated miRNA of the miR-17~92 cluster in developing Th17 cells. Individually restoring each miRNA in the miR-17~92 cluster by transfection with miRNA mimics revealed miR-18a's ability to inhibit Th17 cell differentiation, while other cluster members actually further enhanced Th17 cell differentiation in 17~92$^{\Delta/\Delta}$ CD4^{+} T cells. Importantly, these observations were further corroborated by *in vitro* and *in vivo* experiments utilizing T cells from miR-18a-deficient mice. CCR6 and RORγt expression was also increased in mouse and human CD4^{+} T cells transfected with miR-18 family specific inhibitors. Mechanistically, we identified and validated three miR-18a target genes that encode transcription factors important for Th17 cell differentiation, including *Smad4*, a component of the TGFβ signaling pathway, *Hif1a*, an important target of miR-210 in its regulation of Th17 cell differentiation[65], and *Rora*, which we had previously shown to prevent subset-inappropriate gene expression in Tfh cells[34]. The finding that miR-18a inhibits Th17 differentiation contrasts strikingly with previous work that described the miR-17~92 cluster's many roles as a positive regulator of CD4^{+} T cell differentiation, including Th1 cells[73], Tfh cells[34,72,101] and Th2 cells[76]. Overall, the present study illustrates how redundancy within families and clusters coupled with signal-regulated expression enables miRNAs to confer robustness to T cell differentiation by targeting multiple genes in convergent pathways.

While Th1 and Th2 cell subsets were initially regarded as stable 'lineages', more recent work, especially on Th17 and Treg cells, has drawn attention to the plasticity and flexibility of Th cell subsets[85,107]. Th cell differentiation is driven by the balanced expression of key transcription factors that form a regulatory network in which small changes in gene expression determine cell fate decisions[107]. One important layer of gene regulation that contributes to the control of these processes consists of evolutionary highly conserved miRNAs[42]. For example, we and others have previously shown the importance of the miR-17~92 cluster for promoting robust Tfh cell differentiation[42,72,101]. In addition, miR-17~92-deficient Tfh cells inappropriately upregulated a set of genes that are normally associated with Th17 cells, including *Ccr6*, *Il1r1*, *Il1r2*, the cytokine *Il22*, and the transcription factor *Rora,* the latter being a direct target of all four miRNA families represented in the miR-17~92 cluster[34]. Thus, miR-17~92 emerges as a central regulator of Th cell plasticity, e.g. by preventing a Th17 program in differentiating Tfh cells[108].

Two previous reports implicated miR-17~92 miRNAs or related family members in the regulation of Th17 cell biology[74,75]. In one study, miR-17 and miR-19b were identified as responsible miR-17~92 miRNAs that promoted Th17-mediated inflammation[75]. This is consistent with our finding that miR-17, miR-20a, and miR-19b mimics further enhanced CCR6 and IL-17A expression in 17~92$^{\Delta/\Delta}$ Th17 cells. We extended these findings by showing that inhibition of the entire miR-17 family decreased IL-17A production. Multiple lines of evidence, including new siRNA experiments presented herein, indicate that *Pten* is an important miR-17~92 target gene that regulates IL-17 production[75,76].

Nevertheless, we did not observe a consistent decrease in IL-17 production in 17~92$^{\Delta/\Delta}$ CD4^{+} T cells, indicating the presence of counter-regulation of Th17 differentiation through other miR-17~92 target genes. This possibility was raised in a previous study in which decreased IL-17 production was observed in miR-17~92-deficient CD4^{+} T cells using different Th17 culture conditions with additional polarizing cytokines (IL-1β and IL-23) and a longer culture period prior to analysis[75]. Interestingly, the same study also showed an increase in *Rora* expression in miR-17~92-deficient CD4^{+} T cells, which is consistent with our earlier work on Tfh cells[42] and data presented in the current study that showed that *Rora* is a direct miR-18a target gene involved in Th17 cell differentiation. A second study showed that miR-20b can directly target *Stat3* and *Rorc*, further suggesting a complex balancing role for the miR-17 family in the regulation of IL-17 production[74]. Our results extend these previous studies and others that focused on the varying roles of the miR-17~92 cluster in T cell biology and, importantly, identify a novel and distinct role for miR-18a as an inhibitor of Th17 cell differentiation.

The large impact of miR-18a in determining the net effect of miR-17~92 deficiency may be a product of the differential expression of individual miR-17~92 miRNAs and their related family members in differentiating Th17 cells. T cells strongly express the paralogous miR-106b~25 cluster, which contains two miR-17 family members and one miR-92 family member, and also retain weak but detectable expression of the miR-106a~363 cluster. Activity sensors revealed that 17~92$^{\Delta/\Delta}$ CD4^{+} T cells almost completely lack miR-18 family activity, but retain a large fraction of the miR-17 and miR-92 family activity observed in wildtype CD4^{+} T cells, and a small residual amount of

miR-19 family activity. These findings may explain why so many of the requirements for miR-17~92 in T cell biology have been mapped to miR-19 family function.

The importance of miR-18a function in Th17 cells may also relate to its particularly dynamic regulation during Th17 cell differentiation. *Mirc1* transcription is induced in activated T cells[109], but expression of the mature miR-17~92 cluster miRNAs is subject to differential regulation. Our data suggest that mature miR-92a induction may be limited by pre-processing of the primary miR-17~92 transcript in developing Th17 cells similarly as in differentiating ES cells[110]. They also indicate further independent regulation of miR-18a processing or stability that maintains it at very low abundance in naïve T cells and allows it to be sharply upregulated during T cell activation (Fig. 1B and ([104,111]). miR-18a is also the most strongly upregulated miRNA in response to Myc-induced miR-17~92 transcription[112]. Previous studies have indicated that miR-17, miR-19a, and miR-20a are also specifically regulated in lymphocytes during disease processes that they promote, including lymphomagenesis and allergic inflammation in asthma[67,76]. Further studies are needed to dissect the intricate regulation of miR-17~92 and its paralogs, and the relationships between regulated expression and the biological functions of each mature miRNA. Complete deficiency in miR-17, miR-18, and miR-19 could be studied in mice lacking all three paralogous clusters[72], and each miRNA in the miR-17~92 cluster could be further interrogated using recently reported miR-17~92 allelic series mutant mice[103].

The distinct regulation and functions of miR-18a and miR-17 illustrate how a cluster of miRNAs can evolve to exert nuanced regulation of gene expression and cell behavior. Phylogenetic comparison suggests that miR-18a was created by a duplication of miR-17 that shifted the miRNA seed sequence by a single nucleotide. As such, miR-18a and miR-17 share many target binding sites, such as in the *Smad4* 3'UTR extension. Yet these two miRNAs have diverged and acquired independent targets[103], differential regulation of processing and/or stability during T cell activation, and different degrees of redundancy with miRNAs in other clusters. Together, these features allow T cells to translate environmental signals into complex regulation of immune responses through closely related miRNAs within a single miRNA cluster. In future studies, it will be interesting to investigate the upstream mechanisms that regulate the induction of miR-17~92 expression and the differential expression and processing of the individual cluster miRNAs. Given their clear functional differences in regards to regulating Th17 differentiation, this is particularly the case for the closely related miRNAs miR-18a and miR-17. In summary, our detailed analyses of the function of miR-18a in activated $CD4^+$ T cells *in vitro* and *in vivo* highlight the distinct negative impact of this miRNA on Th17 cell differentiation, which is in clear contrast to the function of the other miR-17~92 cluster members. These insights might provide the basis for the development of therapeutic approaches that strengthen the expression of this miRNA or inhibit its target genes.

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Author contributions

M.M. performed and analyzed most of the experiments under the supervision of K.M.A. and D.B.; J.M., P.S., and D.B. performed and analyzed some of the experiments. M.M., K.M.A., and D.B. designed the experiments, interpreted the data, and wrote the manuscript.

Materials and methods

Mice

Mice with *lox*P sites flanking the miR-17~92 cluster (*Mirc1*$^{tm1.1Tyj}$; The Jackson Laboratory, 008458) were crossed to CD4-Cre mice (*Tg(CD4-cre)1Cwi;* Taconic, 4196) to generate T cell-specific miR-17~92-deficient mice. For some experiments, these mice were further crossed with *Smad4*$^{tm2.1Cxd}$ mice containing loxP sites flanking exon 8 of the *Smad4* gene (The Jackson Laboratory, 017462) or with mice heterozygous for the spontaneous *Rora*^{sg} mutation (The Jackson Laboratory, 002651) to generate miR-17~92-deficient mice with heterozygous deletion of *Smad4* or with one defective *Rora* allele and appropriate littermate controls. Mice with a targeted deletion of miR-18a (*Mir18*$^{tm1.1Aven}$) were recently described[103]. Mice with *lox*P-flanked *Dgcr8* alleles (*Dgcr8*$^{tm1.1Biel}$) have been described before[113] and were bred to CD4-Cre and R26-stop-EYFP mutant mice (*Gt(ROSA)26Sor*$^{tm3(CAG-EYFP)Hze}$*;* The Jackson Laboratory, 006148). All mice were housed and bred in the specific pathogen-free barrier facilities at the University of California San Francisco or the Ludwig-Maximilians-Universität München. All experiments were performed according to the Institutional Animal Care and Use Committee (IACUC) guidelines of the University of California, San Francisco, or in accordance with the regulations of the Regierung von Oberbayern.

In vitro mouse primary T cell polarization

Single-cell suspensions from spleen and lymph nodes were prepared by mincing the tissues between the frosted ends of glass slides. Cells were filtered through fine mesh and counted. $CD4^+$ T cells were enriched with the Easy Sep Mouse $CD4^+$ T Cell

Isolation Kit (Stemcell Technologies). Purified $CD4^+$ T cells were plated at $4x10^6$ cells per well in complete medium (RPMI-1640 supplemented with 10% fetal bovine serum, pyruvate, nonessential amino acids, l-arginine, l-asparagine, l-glutamine, folic acid, beta mercaptoethanol, penicillin and streptomycin) in 6-well plates (Corning Costar) or $1x10^5$ cells per well in 96-well, flat-bottom plates (Corning Costar) pre-coated with 2μg/ml anti-CD3 (clone 17A2; Bio X Cell) and anti-CD28 (clone 37.51; Bio X Cell). For Th17 polarizing conditions, media were supplemented with anti-IFNγ (10μg/ml, clone XMG1.2; Bio X Cell), anti-IL-4 (10μg/ml, clone 11B11; Bio X Cell), human TGFβ (5ng/ml; Peprotech), and murine IL-6 (25ng/ml; Peprotech), unless otherwise stated. On day 2 of culture, cells were collected, counted, suspended in transfection buffer together with miRNA mimics, siRNAs or inhibitors, and transfected with the Neon transfection system (Invitrogen). Cells were immediately transferred into fresh culture medium containing Th17-polarizing cytokines plus murine IL-23 (20ng/ml; R&D Systems) at $4x10^5$ cells per well in 96-well flat-bottom plates pre-coated with anti-CD3 and anti-CD28. Cultured cells were usually analyzed on day 3.5 of initial culture unless otherwise stated.

In vitro human cord blood T cell polarization

Cord blood mononuclear cells (CBMCs) from anonymous human cord blood donors were isolated by Lymphoprep gradient (1114545; Accurate Chemical & Scientific). $CD4^+$ T cells were isolated from CBMCs using the Dynabeads Untouched Human $CD4^+$ T Cell Isolation Kit (Invitrogen). Cells were stimulated for ~48 h on plates coated with 2μg/ml anti-CD3 (clone OKT-3; UCSF Monoclonal Antibody Core) and 4μg/ml anti-CD28 (clone

15E8; Miltenyi Biotec) at an initial density of 4-5 × 10^6 cells per well in complete medium (RPMI-1640 media with 10% FCS, pyruvate, nonessential amino acids, l-arginine, l-asparagine, l-glutamine, folic acid, beta mercaptoethanol, penicillin and streptomycin) in 6-well plates (Corning Costar). After 2 days of stimulation, cells were collected, counted, suspended in transfection buffer together with miRNA inhibitors, and transfected with the Neon transfection system (Invitrogen). Cells were immediately transferred into 48-well plates at a density of 4x10^5 per well pre-coated with anti-CD3 and anti-CD28 in fresh culture medium containing Th17-polarizing cytokines. For Th17-polarizing conditions, media were supplemented with anti-human IFNγ (10µg/ml, clone NIB42; eBioscience), anti-human IL-4 (10µg/ml, clone MP4-25D2; Biolegend), human TGFβ (5ng/ml; Peprotech), human IL-6 (25ng/ml; Peprotech), human IL-1β (20ng/ml; Peprotech), and human IL-23 (20ng/ml; Peprotech).

miRNA mimics, miRNA inhibitors, siRNAs, and miRNA sensors

Th17-polarized human or mouse primary $CD4^+$ T cells were transfected with miRNA mimics, siRNAs or inhibitors at 48 h of cell culture with the Neon Transfection System (Invitrogen) as previously described (Steiner et al., 2011). miRIDIAN miRNA mimics (Dharmacon) or siGENOME SmartPools (Dharmacon) were used at 500nM and miRCURY LNA microRNA Power family inhibitors (Exiqon) were used at 5µM or 20µM with appropriate controls. MSCV-PGK-hCD25 miRNA sensors for miR-17, miR-18a, miR-92a, and miR-19b[76] were constructed to express EGFP with four perfectly complementary binding sites for the miRNA in the 3'UTR as previously described[78]. Cells were transduced by spin infection early on day 2 of Th17 cultures and transfected

with miRNA mimics or inhibitors later on day 2 of Th17 cultures. hCD25$^+$ CD4$^+$ T cells were analyzed on day 3.5 for EGFP expression.

3'UTR cloning and Luciferase assays

3'UTR dual luciferase plasmids containing near full length 3'UTRs of *Smad4*, *Hif1a* or an extension 3'UTR for *Smad4* were cloned into the psiCHECK-2 luciferase reporter construct (Promega). Primer sequences were: *Smad4* F: TAGTAGCTCGAGCTCTGCAGCTCTTGGATGAA, *Smad4* R: TAGTAGGCGGCCGCCATGGGAAAGTCCTGGTAGAG, *Hif1a* F: TAGTAGCTCGAGGGCAGCAGAAACCTACTGCAGG, *Hif1a* R: TAGTAGGCGGCCGCTAAACGTAAGCGCTGACCCAGG, *Smad4* extension F: TAGTAGCTCGAGACTGAGTCACTATACGAAGTGG, *Smad4* extension R: TAGTAGGCGGCCGCTTGGCTCTGAAGAGATACTTCC. psiCHECK-2 *Rora* 3'UTR P1 was described previously[34]. CD4$^+$ T cells were transfected on day 2 of ThN (non-polarizing, no exogenous cytokines or blocking antibodies) culture with luciferase reporter constructs and/or miRNA mimics using the Neon Transfection System. Media were additionally supplemented with 20 units/ml recombinant human IL-2 (NCI) on day 2 of culture. Luciferase activity was measured 24 h after transfection with the Dual Luciferase Reporter Assay System (Promega) and a FLUOstar Optima plate-reader (BMG Labtech).

Flow cytometry

Cultured cells were collected, washed, and stained with antibodies against cell surface

proteins and transcription factors as described before[114]. Nonspecific binding was blocked with anti-CD16/CD32 (clone 2.4G2; UCSF Monoclonal Antibody Core), 2% normal rat/mouse serum for mouse T cell staining or with human FcR binding inhibitor (eBioscience) for human T cells. Dead cells were excluded with Fixable Viability Dye eFluor780 (eBioscience). For intracellular staining of transcription factors, the Foxp3 staining set (eBioscience) was used. Intracellular cytokine detection was performed after stimulation with 10nM PMA and 1μM ionomycin for 2 h, followed by the addition of 5μg/ml Brefeldin A for another 2 h. Cells were fixed with 4% PFA for 8 min at room temperature, washed with ice-cold PBS, and permeabilized with 0.5% saponin. The following fluorochrome-conjugated antibodies were used in the study for mouse T cells: anti-CD4 (clones RM4-5 or GK1.5), anti-IL-17A (eBio17B7), anti-IFNγ (XMG1.2), and anti-IL-13 (eBio13A; all from eBioscience); anti-CD11b (M1/70; Biolegend); anti-CCR6 (140706), anti-RORγt (Q31-378), anti-Siglec F (E50-2440), anti-CD45 (30-F11), anti-Ly6G (1A8; all from BD Biosciences); and for human T cells: anti-human CD4 (OKT4; Biolegend), anti-human CCR6 (R6H1; eBioscience); and anti-human RORγt (Q21-559; BD Biosciences).

In vivo airway hypersensitivity models

Acute 10-day OVA model: Sex and age matched wildtype and miR-18$^{\Delta/\Delta}$ mice were sensitized by i.p. injection with 50μg ovalbumin (OVA, Sigma-Aldrich) in 100μl of PBS plus 100μl of Imject Alum Adjuvant (Thermo Scientific). After seven days, the mice were challenged oropharyngeally with 50μg OVA in 20μl of PBS daily for 3 consecutive days. Lungs were harvested on day 10 of the experiment. 27-day OVA+LPS model: Sex and

age matched wildtype and miR-18$^{\Delta/\Delta}$ mice were sensitized with 100μg of EndoFit Ovalbumin (OVA, InvivoGen) and 10μg of LPS (Sigma-Aldrich) in 30μl of PBS delivered by oropharyngeal instillation on days 0, 2, 4, and 11. The mice were then challenged oropharyngeally with 40μg of OVA in 30μl of PBS on days 18, 20, 25, and 26. Lungs and and lung-draining mediastinal lymph nodes were harvested on day 27. For both airway hypersensitivity models, lungs were digested following the lung dissociation kit protocol and gentleMACS dissociator (Miltenyi Biotec). Liberase TM (Roche) was used at 50μg/ml and DNase I (Roche) at 25μg/ml. Cells collected from the lung and lung-draining lymph nodes were analyzed by flow cytometry for surface markers, transcription factors and cytokines as described above. Prior to euthanasia, mice were injected retro-orbitally with 2μg of fluorochrome-conjugated anti-CD45 antibody (clone 30-F11; BD Biosciences) in 200μl of PBS to distinguish vascular and non-vascular cells in the lung.

qPCR

CD4$^+$ T cells from spleen and lymph nodes of mice were enriched with the EasySep Mouse CD4$^+$ T Cell Isolation Kit (Stemcell Technologies). Cells were lysed in Trizol LS (Life Technologies), total RNA was isolated, and RNA was then quantified with a ND-1000 spectrophotometer (NanoDrop). Reverse transcription of miRNA was performed with the NCode miRNA First-Strand cDNA Synthesis Kit (Life Technologies). Forward primers were the mature miRNA sequence[115] and a universal reverse primer was used from the kit. Expression values were normalized to 5.8S ribosomal RNA (F: ATCGTAGGCACCGCTACGCCTGTCTG). Reverse transcription of mRNA was

performed with SuperScript III First-Strand Synthesis for RT-PCR (Invitrogen). Primers for *Smad4* total transcript were CACAATGAGCTTGCATTCCAG (F) and ACCTTAAACGTCTCTCCTACCT (R). Primers for *Smad4* extended transcript were CTGAGTCACTATACGAAGTGGAAT (F) and GTCATTTAGCAGAAGGTGTCTTG (R). Expression values were normalized to *Gapdh* (F: GTGTTCCTACCCCCAATGTGT; R:ATTGTCATACCAGGAAATGAGCTT). qPCR was performed in technical duplicates using FastStart Universal SYBR Green Master mix (Roche) on a Realplex^2 instrument (Eppendorf).

Statistics

Excel (Microsoft) and Prism (GraphPad) were used for data analysis. For all figures, bar graphs display mean+s.e.m., unless otherwise stated. Z-scores were calculated from mean and s.d.. *P<0.05, **P<0.01, and ***P<0.001 for significance. Appropriate statistical analyses were performed for all data and are specified in each corresponding figure legend.

Figure 1. miR-18a is active and dynamically regulated during Th17 cell differentiation. (**A**) Schematic of miRNAs within the miR-17~92 cluster and its paralogous miRNA gene clusters miR-106a~363 and miR-106b~25. Individual miRNA families represented in the three clusters are color-coded. (**B, C**) Naive control ($17\sim92^{+/+}$; circles) and miR-17~92-deficient ($17\sim92^{\Delta/\Delta}$; squares) $CD4^+$ T cells were cultured *in vitro* under Th17-polarizing conditions. Cells were harvested at the indicated time points and quantitative real-time PCR was performed to determine expression levels of individual miRNAs of the miR-17~92 cluster (**B**) or representative miRNAs from the paralogous miRNA gene clusters miR-106b~25 and miR-106a~363 (**C**); expression was normalized to 5.8S rRNA in each sample. (**D**) $17\sim92^{+/+}$ and $17\sim92^{\Delta/\Delta}$ $CD4^+$ T cells were transduced with retroviral sensors expressing EGFP together with 4 perfectly complementary binding sites for miR-17 (psens17), miR-18a (psens18a), miR-19b (psens19b) or miR-92a (psens92a) in the 3'UTR and analyzed by flow cytometry. Data are pooled from three independent experiments, each with one mouse per genotype (**B, C**; error bars represent s.e.m.) or are representative of four independent experiments, each with one mouse per genotype (**D**).

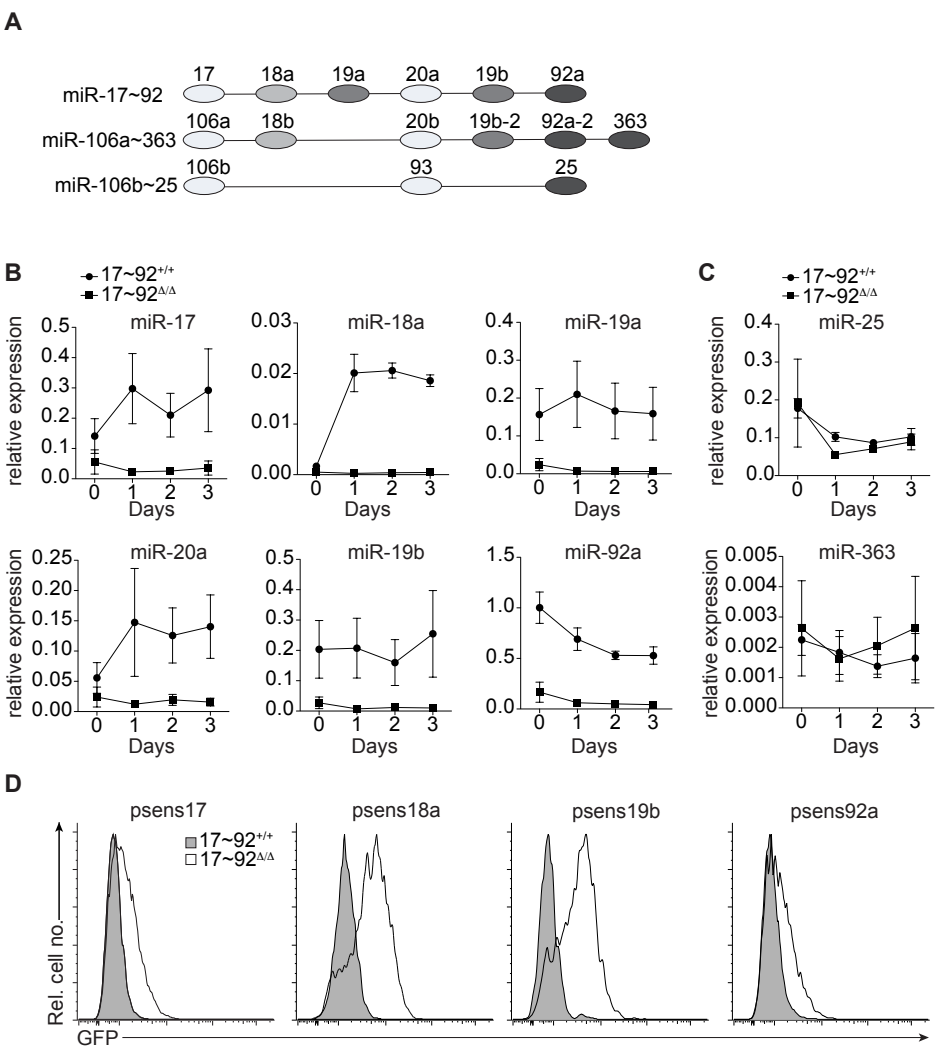

A
miR-17~92
17
18a
19a
20a
19b
92a
miR-106a~363
106a
18b
20b
19b-2
92a-2
363
miR-106b~25
106b
93
25
B
17~92+/+
17~92Δ/Δ
miR-17
miR-18a
miR-19a
miR-20a
miR-19b
miR-92a
relative expression
Days
C
miR-25
miR-363
D
psens17
psens18a
psens19b
psens92a
Rel. cell no.
GFP

Figure 2. miR-18a inhibits Th17 cell differentiation. Naive 17~92$^{+/+}$ and 17~92$^{\Delta/\Delta}$ CD4^{+} T cells were cultured *in vitro* under Th17 conditions (TGFβ+IL-6) and analyzed on day 3.5 for Th17 marker expression by flow cytometry: (**A**) Surface CCR6 and intracellular RORγt staining of CD4^{+} T cells. Numbers in quadrants indicate percent CCR6 and/or RORγt-positive live singlet cells (left) and the frequencies of CCR6^{+}RORγt^{+} cells are quantified in the bar graph (right). (**B**) IL-17A production after restimulation with PMA/ionomycin is shown in a representative histogram (left) and the frequency of IL-17A^{+} cells is quantified in the bar graph (right). (**C**) Naive 17~92$^{+/+}$ and 17~92$^{\Delta/\Delta}$ CD4^{+} T cells were cultured under Th17-polarizing conditions and transfected on day 2 with control miRNA mimics (Ctl mimic) or miRNA mimics of the individual miR-17~92 cluster members. Cells were analyzed on day 3.5 for Th17 marker expression by flow cytometry. The frequencies of CCR6^{+}RORγt^{+} cells among CD4^{+} T cells are shown in the bar graph (left); representative contour plots display surface CCR6 expression and intracellular RORγt co-staining of CD4^{+} T cells (right). Numbers in quadrants indicate percent CCR6 and/or RORγt-positive live singlet cells. **P<0.01 and ***P<0.001 (two-tailed paired *t*-test with pre-assigned littermate pairs (**A, B**) or one-way ANOVA with Dunnett's multiple comparison post-test comparing all columns to control miRNA mimic-transfected 17~92$^{\Delta/\Delta}$ CD4^{+} T cells (**C**)). Data are pooled from five (**C**), eight (**B**), or six (**A**) independent experiments, each with one to two mice per genotype (mean and s.e.m.; **A-C**).

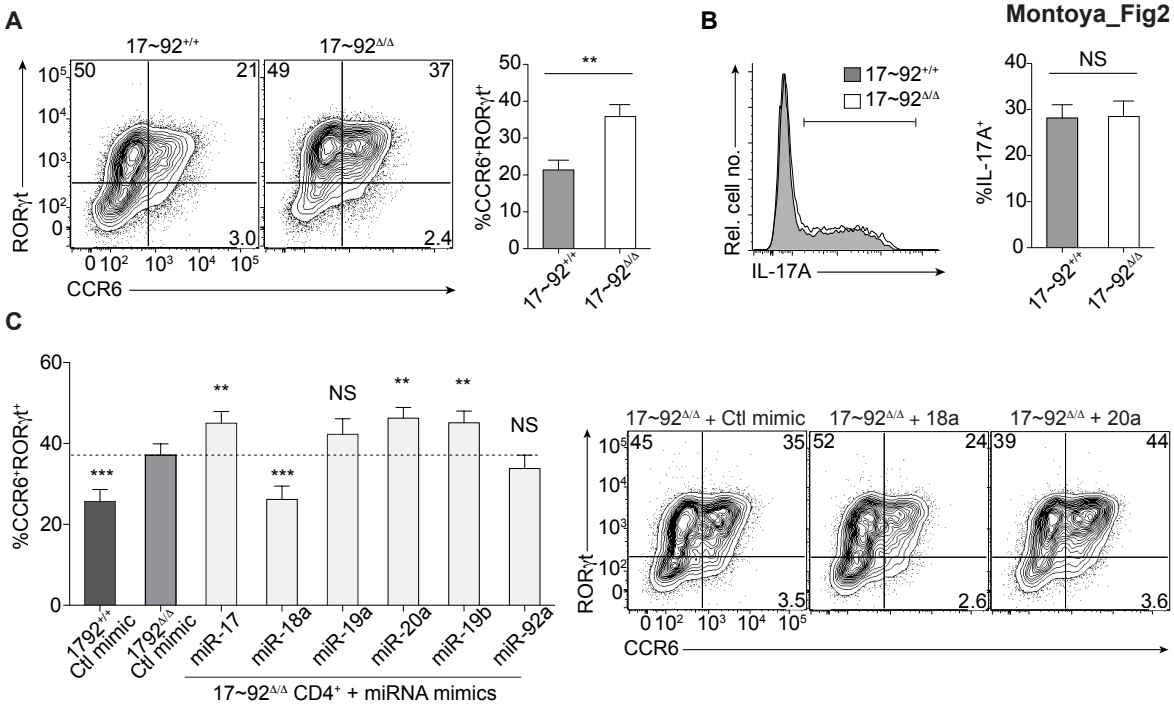

Montoya_Fig2
A
17~92+/+
17~92Δ/Δ
50
21
49
37
3.0
2.4
RORγt
CCR6
%CCR6+RORγt+
**
B
17~92+/+
17~92Δ/Δ
Rel. cell no.
IL-17A
%IL-17A+
NS
C
%CCR6+RORγt+
***
**
NS
**
**
NS
1792+/+ Ctl mimic
1792Δ/Δ Ctl mimic
miR-17
miR-18a
miR-19a
miR-20a
miR-19b
miR-92a
17~92Δ/Δ CD4+ + miRNA mimics
17~92Δ/Δ + Ctl mimic
17~92Δ/Δ + 18a
17~92Δ/Δ + 20a
45
35
52
24
39
44
3.5
2.6
3.6
RORγt
CCR6

Figure 3. Genetic ablation of miR-18a alone increases the frequency of CCR6$^+$ and CCR6$^+$RORγt$^+$ CD4$^+$ T cells. (A) Naive wildtype (WT) and miR-18$^{\Delta/\Delta}$ CD4$^+$ T cells were cultured *in vitro* under Th17 conditions (TGFβ+IL-6) and analyzed on day 3.5 for Th17 marker expression by flow cytometry: Surface CCR6 expression is shown in a representative histogram and the frequency of CCR6$^+$ cells is quantified in the bar graph (top left). IL-17A production after restimulation with PMA/ionomycin is shown in a representative histogram and the frequency of IL-17A$^+$ cells is quantified in the bar graph (top right). Surface CCR6 and intracellular RORγt co-staining of CD4$^+$ T cells with numbers in quadrants indicating percent CCR6 and/or RORγt-positive live singlet cells and the frequency of CCR6$^+$RORγt$^+$ cells is quantified in the bar graph (bottom left). (**B**) Naive WT and miR-18$^{\Delta/\Delta}$ CD4$^+$ T cells were cultured under Th17-polarizing conditions and transfected on day 2 with control miRNA mimics (Ctl mimic) or miR-18a mimics. Cells were analyzed by flow cytometry on day 3.5. The frequency of CCR6$^+$ (left) and CCR6$^+$RORγt$^+$ (right) cells is quantified in the bar graphs. **P<0.01 and ***P<0.001 (two-tailed paired *t*-test (**A**) or one-way ANOVA with Dunnett's multiple comparison post-test comparing all columns to control miRNA mimic-transfected miR-18$^{\Delta/\Delta}$ CD4$^+$ T cells (**B**)). Data are pooled from two independent experiments, each with three mice per genotype, and error bars represent s.e.m. (**A**, **B**).

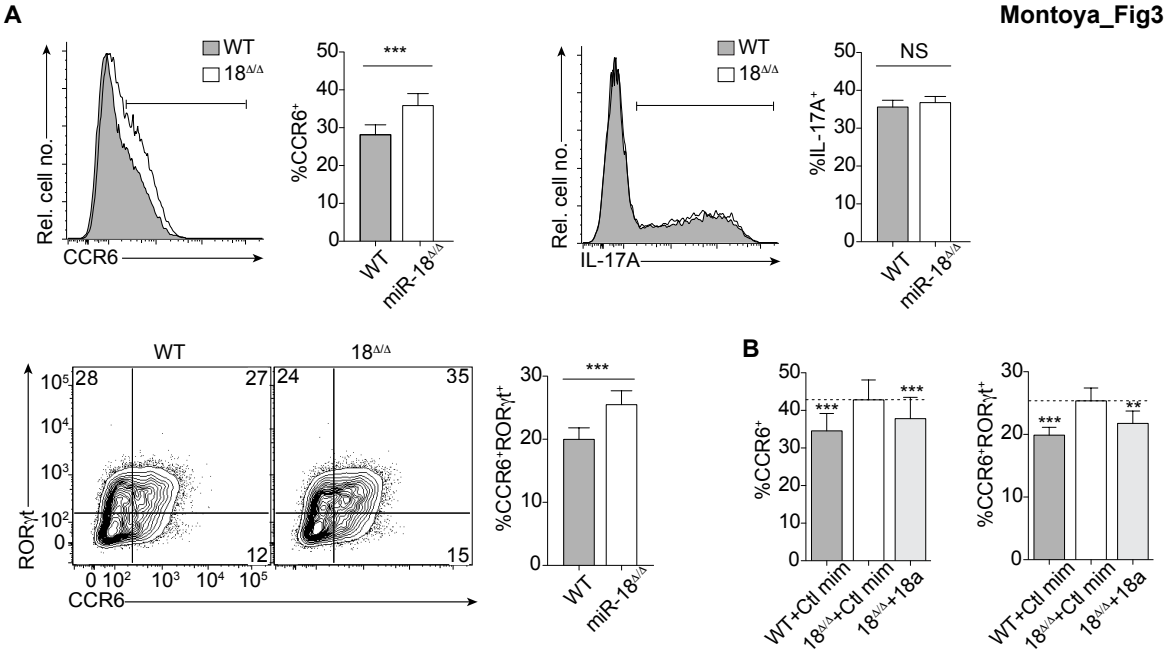

A
WT
18Δ/Δ
Rel. cell no.
CCR6
%CCR6+
***
WT
miR-18Δ/Δ
IL-17A
%IL-17A+
NS
WT
18Δ/Δ
28
27
12
24
35
15
RORγt
CCR6
%CCR6+RORγt+
B
%CCR6+
%CCR6+RORγt+
WT+Ctl mim
18Δ/Δ+Ctl mim
18Δ/Δ+18a

Figure 4. miR-18a deficiency increases lung Th17 cell frequencies in an airway inflammation model. (A) Schematic of the 10-day *in vivo* airway hypersensitivity model with intraperitoneal (i.p.) OVA+alum sensitization on day 0, followed by three consecutive daily challenges with OVA oropharyngeally (o.p.) on days 7, 8, and 9. On day 10, cells from the lungs of WT and miR-18$^{\Delta/\Delta}$ mice were harvested and analyzed by flow cytometry for: (**B**) Total CD4$^+$ T cell and total CD45$^+$ leukocyte numbers per lung; frequencies of CCR6$^+$ and CCR6$^+$RORγt$^+$ cells; intracellular RORγt expression levels as determined by gMFI. (**C**) Frequencies of IL-17A, IL-13 or IFNγ-producing cells among CD4$^+$ T cells after restimulation with PMA/ionomycin. (**D**) Frequencies of inflammatory cells in the lungs, including eosinophils (CD11b$^+$SiglecF$^+$) and neutrophils (CD11b$^+$Ly6G$^+$). **P<0.01 and ***P<0.001 (two-tailed unpaired *t*-test). Data are pooled from three independent experiments, each with three to nine mice per genotype (n=21 mice total per genotype).

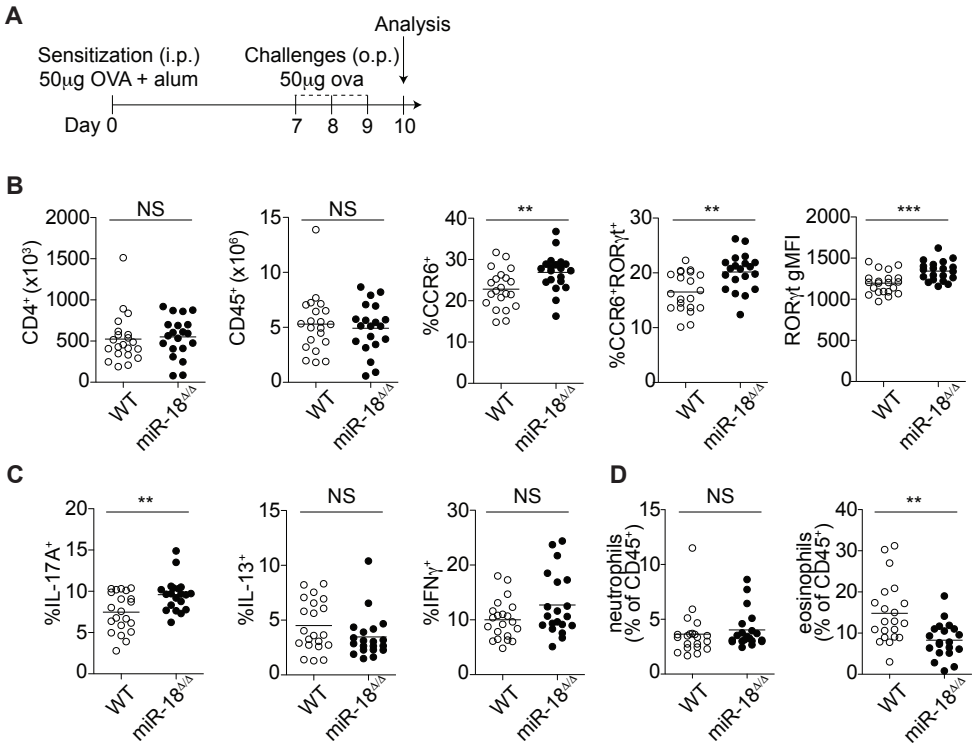

A
Analysis
Sensitization (i.p.)
50μg OVA + alum
Challenges (o.p.)
50μg ova
Day 0
7
8
9
10
B
NS
NS
**
**
***
CD4+ (x10³)
CD45+ (x10⁶)
%CCR6+
%CCR6+RORγt+
RORγt gMFI
WT
miR-18Δ/Δ
C
D
**
NS
NS
NS
**
%IL-17A+
%IL-13+
%IFNγ+
neutrophils (% of CD45+)
eosinophils (% of CD45+)

Figure 5. Inhibition of the miR-18 family increases mouse and human Th17 cell differentiation. (**A**) Flow cytometric analyses of murine WT $CD4^+$ T cells polarized under Th17 conditions and transfected with either control inhibitor (Ctl inh.) or miR-18 family inhibitor (18 fam. inh.) on day 2. Cells were analyzed on day 3.5 of culture. Histograms show representative stainings of surface CCR6 and intracellular RORγt. Frequencies of $CCR6^+$ cells as well as RORγt gMFI are quantified in the dot plots. (**B**) Flow cytometric analyses of CCR6 expression and RORγt expression of $CD4^+$ T cells isolated from human cord blood and transfected with control inhibitor (Ctl inh.) or miR-18 family inhibitor (18 fam. inh.) on day 2 of culture under Th17-polarizing conditions. Cells were analyzed on day 3.5 of culture. Histograms show representative stainings of surface CCR6 and intracellular RORγt. Frequencies of $CCR6^+$ cells as well as RORγt gMFI are quantified in the dot plots. Dashed lines represent background staining in the CCR6 or RORγt-detecting channels (fluorescence-minus-one (FMO) controls). Circles (Ctl inh.) and squares (18 fam. inh.) in the dot plots represent the mean of two to three individual transfections of each inhibitor (**A, B**); lines connect individual WT mice (**A**) or individual cord blood donors (**B**) that received respective inhibitors. *P<0.05, **P<0.01 and ***P<0.001 (two-tailed paired *t*-test). Data are pooled from three independent experiments with two to three mice per experiment (n=7) (**A**) or from one experiment with three different donor samples (n=3) (**B**).

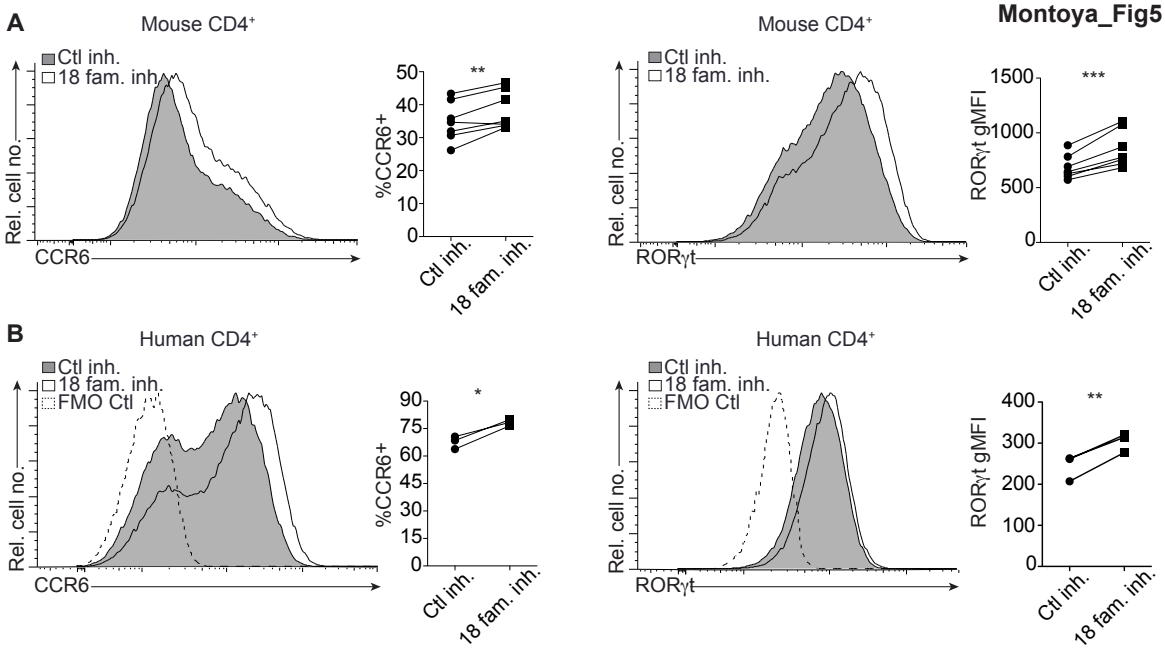
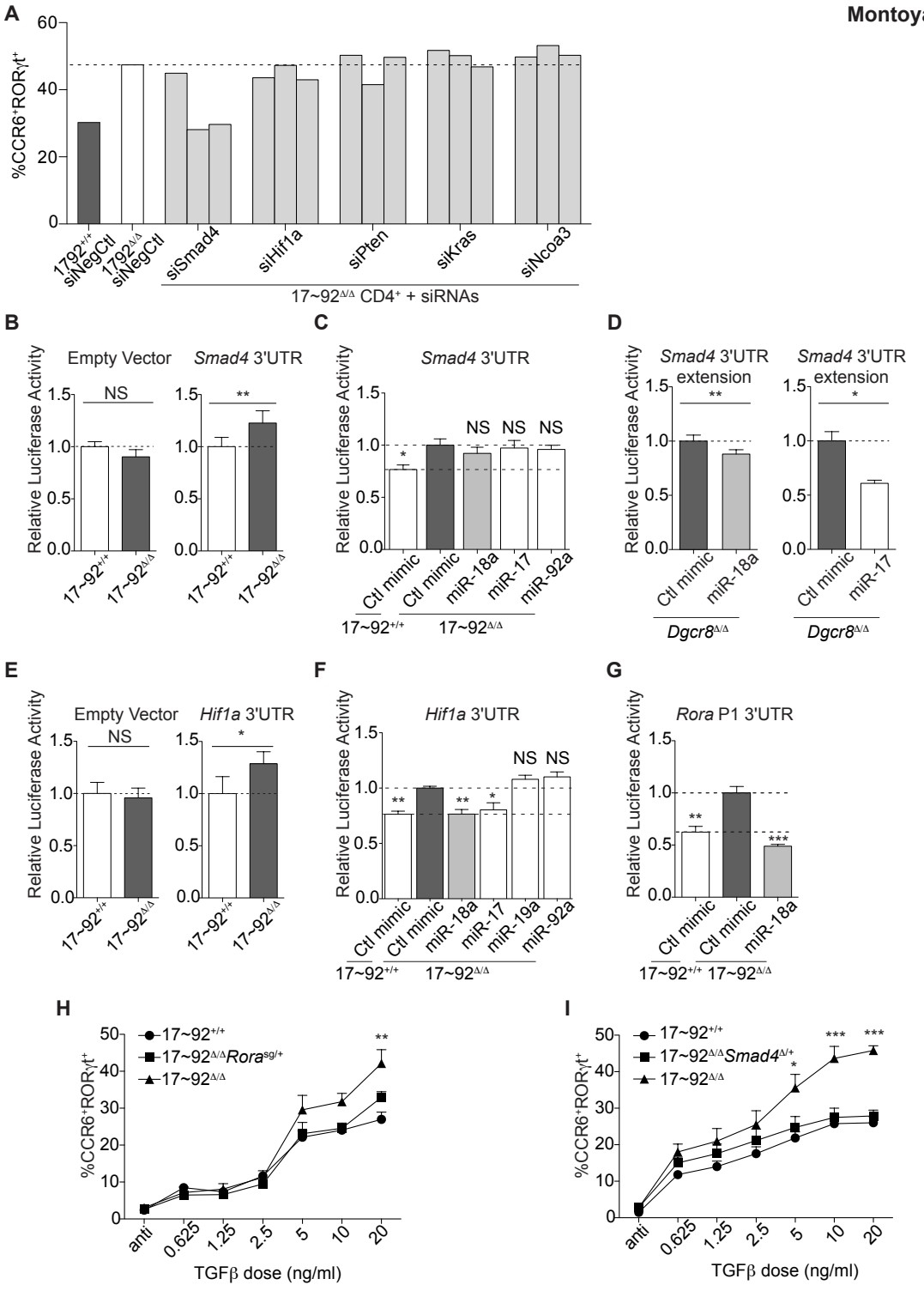
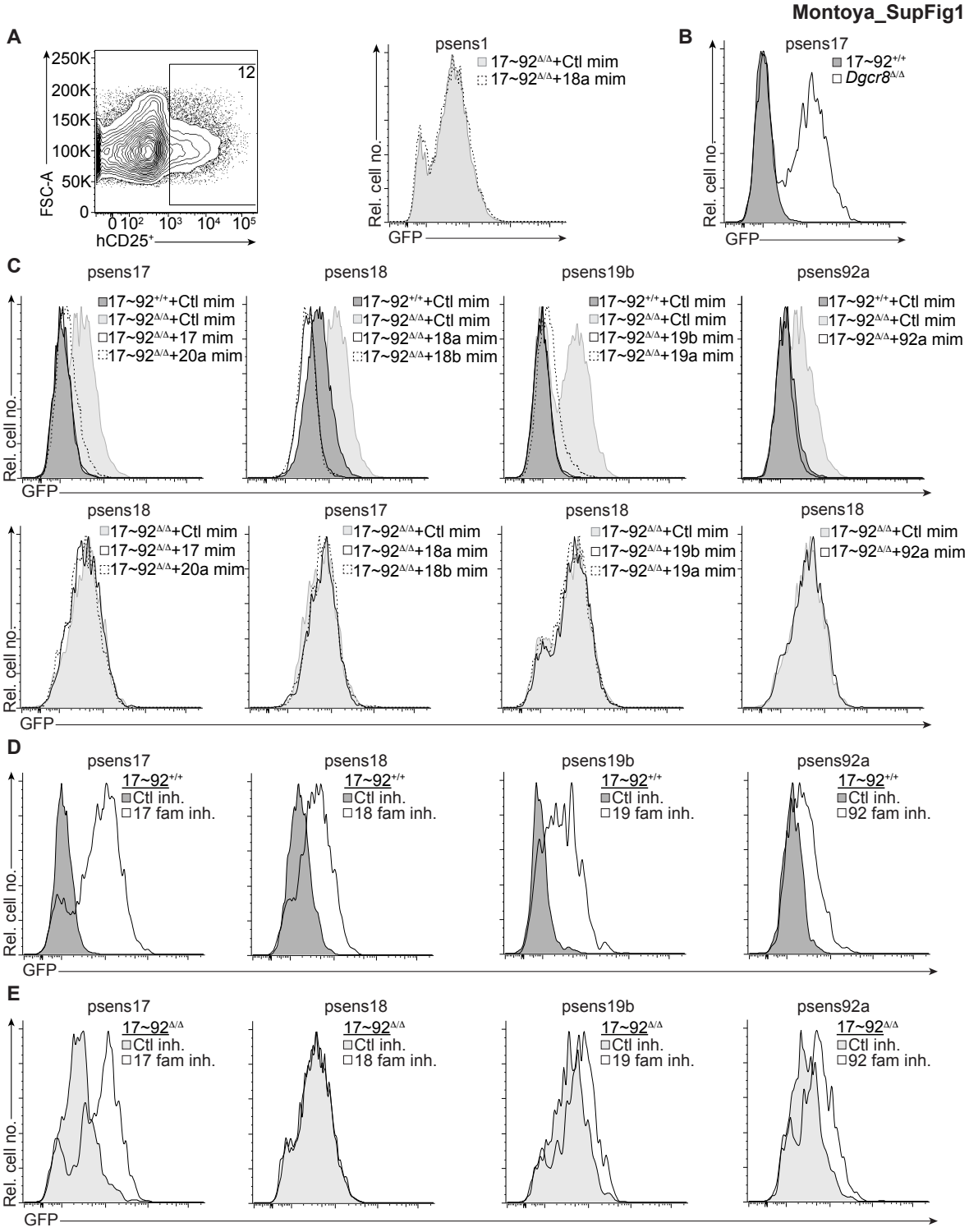

Montoya_Fig5
A
Mouse CD4+
Ctl inh.
18 fam. inh.
Rel. cell no.
CCR6
%CCR6+
**
Ctl inh.
18 fam. inh.
Mouse CD4+
Ctl inh.
18 fam. inh.
Rel. cell no.
RORγt
RORγt gMFI
***
Ctl inh.
18 fam. inh.
B
Human CD4+
Ctl inh.
18 fam. inh.
FMO Ctl
Rel. cell no.
CCR6
%CCR6+
*
Ctl inh.
18 fam. inh.
Human CD4+
Ctl inh.
18 fam. inh.
FMO Ctl
Rel. cell no.
RORγt
RORγt gMFI
**
Ctl inh.
18 fam. inh.

Figure 6. *Smad4*, *Hif1a,* and *Rora* are functionally relevant target genes of miR-18a. (**A**) Naive 17~92$^{+/+}$ and 17~92$^{\Delta/\Delta}$ CD4^{+} T cells were cultured under Th17-polarizing conditions and transfected with an siRNA non-targeting control (siNegCtl) or three individual siRNAs against each of five different candidate genes. Bars quantify the frequency of CCR6^{+}RORγt^{+} cells among live CD4^{+} singlet cells. Dashed line indicates the level of negative-control siRNA-transfected 17~92$^{\Delta/\Delta}$ CD4^{+} cells. (**B**) Renilla luciferase activity in 17~92$^{+/+}$ control and 17~92$^{\Delta/\Delta}$ CD4^{+} T cells transfected with empty vector or with dual luciferase reporters for *Smad4* 3' untranslated (3'UTR) regions assessed 24 h after transfection; results were normalized to firefly luciferase activity and are presented relative to those of transfected 17~92$^{+/+}$ control CD4^{+} T cells. (**C**) Renilla luciferase activity in primary 17~92$^{+/+}$ control and 17~92$^{\Delta/\Delta}$ CD4^{+} T cells co-transfected with *Smad4* 3'UTR luciferase reporters and individual miR-17~92 miRNA mimics or Ctl mimic, assessed 24 h after transfection; results were normalized to firefly luciferase activity and are presented relative to those of control miRNA mimic-transfected 17~92$^{\Delta/\Delta}$ CD4^{+} T cells. (**D**) Renilla luciferase activity in global miRNA-deficient *Dgcr8*$^{\Delta/\Delta}$ CD4^{+} T cells co-transfected with the *Smad4* 3'UTR extension luciferase reporter and either control miRNA mimic (Ctl mimic) or miR-18a mimic (left) or with miR-17 mimic (right), assessed 24 h after transfection; results were normalized to firefly luciferase activity and are presented relative to those of control miRNA mimic-transfected *Dgcr8*$^{\Delta/\Delta}$ CD4^{+} T cells. (**E**) Renilla luciferase activity of 17~92$^{+/+}$ control and 17~92$^{\Delta/\Delta}$ CD4^{+} T cells transfected with empty vector or with dual luciferase reporters for *Hif1a* 3'UTR regions as described in B. (**F**) Renilla luciferase activity in primary 17~92$^{+/+}$ control and 17~92$^{\Delta/\Delta}$ CD4^{+} T cells transfected with *Hif1a* 3'UTR luciferase reporters together with miR-17~92

miRNA mimics or Ctl mimic as described in C. (**G**) Renilla luciferase activity in primary 17~92$^{+/+}$ control and miR-17~92-deficient CD4^{+} T cells transfected with position 1 (P1) of *Rora* 3'UTR luciferase reporters (see methods) together with miR-18a mimic or control miRNA mimics (Ctl mimic), as described in C. (**H, I**) Frequencies of CCR6^{+}RORγt^{+} cells from 17~92$^{+/+}$ control (circles), 17~92$^{\Delta/\Delta}$ (triangles), and 17~92$^{\Delta/\Delta}$*Rora*$^{sg/+}$ (squares) (**H**) or 17~92$^{\Delta/\Delta}$*Smad4*$^{\Delta/+}$ (squares) (**I**) cells after 3.5 days of *in vitro culture* under Th17-polarizing conditions with varying doses of TGFβ, as assessed by flow cytometry. *P<0.05, **P<0.01, ***P<0.001 (one-tailed paired *t*-test; (**B, D, E**) or one-way ANOVA with Dunnett's post-test (comparing each column to 17~92$^{\Delta/\Delta}$ + Ctl mimic mean; (**C, F, G**)) or two-way ANOVA with Bonferroni post-test (comparing 17~92$^{\Delta/\Delta}$ and 17~92$^{\Delta/\Delta}$*Rora*$^{sg/+}$ (**H**) or 17~92$^{\Delta/\Delta}$ and 17~92$^{\Delta/\Delta}$*Smad4*$^{\Delta/+}$ (**I**); (**H, I**)). Data are pooled from six independent experiments (**B, D** (left)) or two to four independent experiments (**C, D** (right), **E-I**) with one to two mice per genotype (mean and s.e.m; **A-I**).


A
%CCR6+RORγt+
1792+/+ siNegCtl
1792Δ/Δ siNegCtl
siSmad4
siHif1a
siPten
siKras
siNcoa3
17~92Δ/Δ CD4+ + siRNAs
B
Empty Vector
Smad4 3'UTR
Relative Luciferase Activity
NS
**
17~92+/+
17~92Δ/Δ
C
Smad4 3'UTR
NS NS NS
Ctl mimic
miR-18a
miR-17
miR-92a
D
Smad4 3'UTR extension
Dgcr8Δ/Δ
E
Hif1a 3'UTR
F
Hif1a 3'UTR
NS NS
miR-19a
G
Rora P1 3'UTR
H
17~92+/+
17~92Δ/ΔRorasg/+
17~92Δ/Δ
TGFβ dose (ng/ml)
I
17~92Δ/ΔSmad4Δ/+

Supplementary Figure 1. MicroRNA sensors are sensitive and specific and show residual miR-17 and miR-92 activity in miR-17~92-deficient mice. (**A**) Pre-gating strategies for murine $CD4^+$ T cells either retrovirally transduced (resulting in positive staining for human CD25) (left) or murine $17\sim92^{\Delta/\Delta}$ $CD4^+$ T cells transduced with a retroviral sensor expressing EGFP with 4 perfectly complementary binding sites for miR-1 (psens1) and transfected with control mimic (Ctl mim) or miR-18a mimic (18a mim). miR-1 is not expressed in $CD4^+$ T cells, thus representing maximum GFP expression using retroviral sensors (right). (**B**) $17\sim92^{+/+}$ and *Dgcr8*$^{\Delta/\Delta}$ $CD4^+$ T cells transduced with retroviral sensors expressing EGFP with 4 perfectly complementary binding sites for miR-17 (psens17) in the 3'UTR and analyzed by flow cytometry. (**C**) Histograms show EGFP fluorescence in $17\sim92^{+/+}$ and $17\sim92^{\Delta/\Delta}$ $CD4^+$ T cells transduced with the indicated retroviral miRNA sensors (psens) and transfected with the indicated control (Ctl) and miR-17~92 cluster miRNA mimics. Results demonstrate the sensitivity of each sensor for its corresponding miRNA family (top row), and the specificity of each miRNA mimic (bottom row). (**D, E**) $17\sim92^{+/+}$ $CD4^+$ T cells (**D**) or $17\sim92^{\Delta/\Delta}$ $CD4^+$ T cells (**E**) were transduced with miR-17 (psens17), miR-18a (psens18a), miR-19b (psens19b) or miR-92a (psens92a) sensors and transfected with control inhibitor (Ctl inh.) and miR-17 family inhibitor (17 fam inh.), miR-18 family inhibitor (18 fam inh.), miR-19 family inhibitor (19 fam. inh.), or miR-92 family inhibitor (92 fam. inh.). Data are representative of four (**A-C**, **E**) or seven (**D**) independent experiments each with one mouse per genotype.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Rel. cell no.
GFP
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Dgcr8Δ/Δ
C
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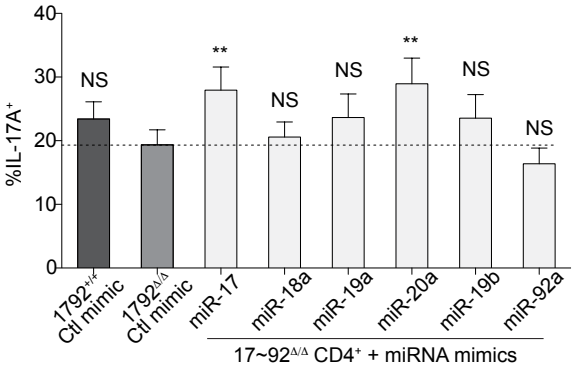
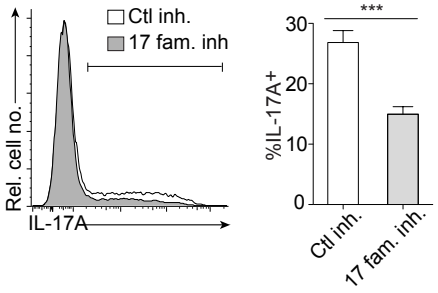
Supplementary Figure 2. miRNAs of the miR-17 family promote IL-17 production. **(A)** Naive 17~92$^{+/+}$ and 17~92$^{\Delta/\Delta}$ CD4^{+} T cells were cultured under Th17-polarizing conditions and transfected on day 2 with control miRNA mimics (Ctl mimic) or miRNA mimics of the individual miR-17~92 cluster members. Cells were analyzed by flow cytometry on day 3.5 for IL-17A production after restimulation with PMA/ionomycin. **(B)** Flow cytometric analysis of IL-17A expression of 17~92$^{+/+}$ control CD4^{+} T cells polarized under Th17-conditions and transfected with control inhibitor (Ctl inh.) or miR-17 family inhibitor (17 fam. inh) on day 2, analyzed on day 3.5. Representative histograms show IL-17 production after restimulation with PMA/ionomycin and the frequency of IL-17A^{+} cells is quantified in the respective bar graph. **P<0.01 and ***P<0.001 (one-way ANOVA with Dunnett's multiple comparison post-test comparing all columns to control miRNA mimic-transfected 17~92$^{\Delta/\Delta}$ CD4^{+} T cells (**A)** or two-tailed paired *t*-test (**B**)). Data are pooled from five (**A**) or three (**B**) independent experiments, each with one (**A**) or two to three (**B**) mice per experiment (mean and s.e.m.).

Montoya_SupFig2

A

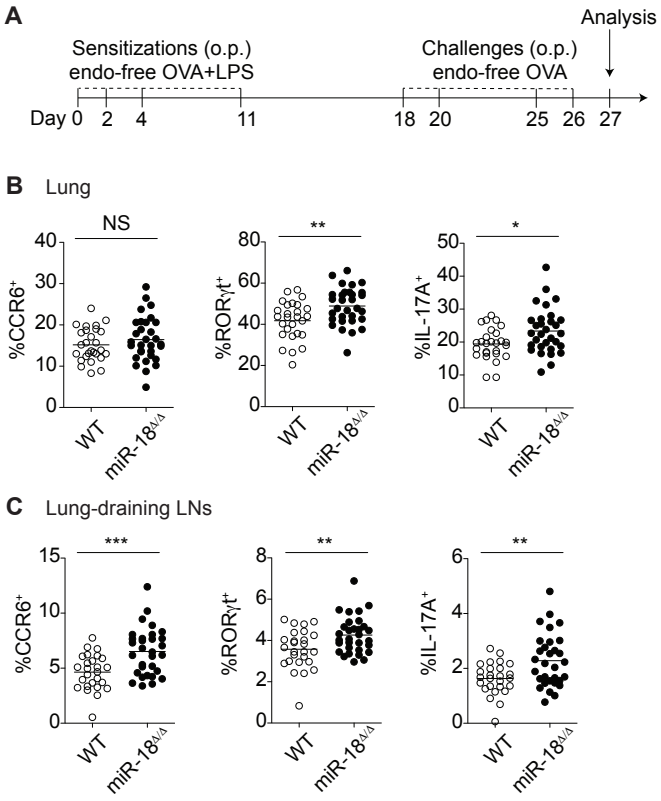


B



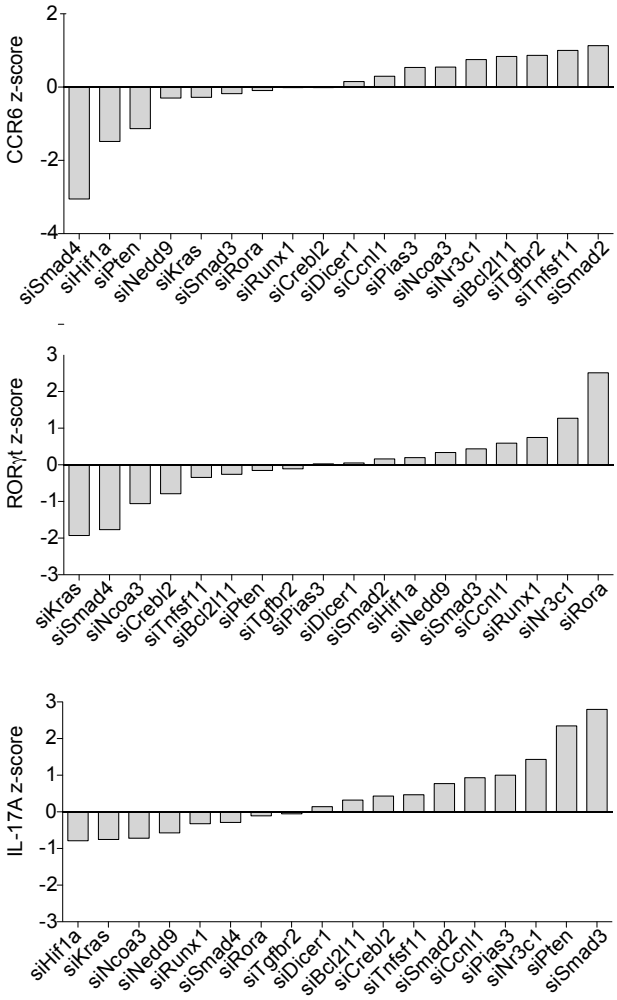
Supplementary Figure 3. miR-18a deficiency increases lung and draining lymph node Th17 cell frequencies in an OVA+LPS airway inflammation model. (A) Schematic of the 27-day *in vivo* airway hypersensitivity model with OVA+LPS sensitizations on days 0, 2, 4, and 11 by oropharyngeal instillation, followed by oropharyngeal (o.p.) OVA challenge on days 18, 20, 25, and 26. On day 27, cells from the lungs (**B**) and lung-draining lymph nodes (**C**) of WT and miR-18$^{\Delta/\Delta}$ mice were harvested and analyzed by flow cytometry for the frequency of CCR6^{+} and RORγt^{+} cells as well as the frequency of IL-17A-producing cells among CD4^{+} T cells after restimulation with PMA/ionomycin. *P<0.05, **P<0.01 and ***P<0.001 (two-tailed unpaired *t*-test). Data are pooled from three independent experiments, each with seven to twelve mice per genotype (n=26 to 31 mice total per genotype).

Montoya_SupFig3



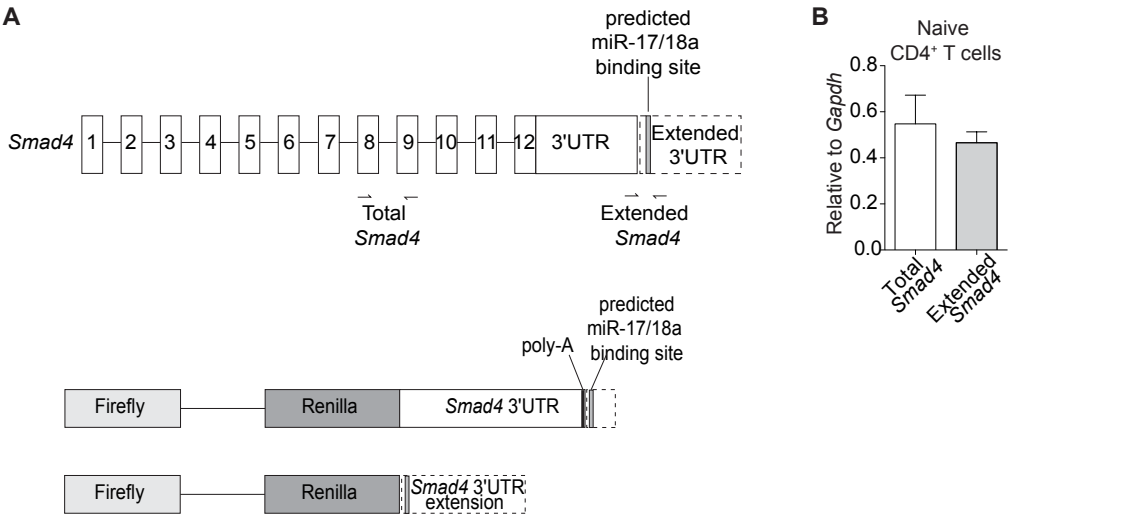
Supplementary Figure 4. siRNA screen identifies functionally relevant miR-18a target genes. Primary screen of miR-18a target genes**,** presented as analysis of 17~92$^{\Delta/\Delta}$ CD4^{+} T cells cultured under Th17-polarizing conditions and transfected with an siRNA nontargeting control or 18 targeting pools each comprising 4 individual siRNAs. Cells were analyzed by flow cytometry on day 3.5 for surface CCR6 expression (top), intracellular RORγt expression (middle), and IL-17A production after restimulation with PMA/ionomycin (bottom); Results were normalized to negative control siRNA. *z*-score= *x*- mean/s.d., where *x* represents the RORγt gMFI or percentage of CCR6^{+} or IL-17A^{+} cells for each siRNA treatment and the mean is across all treatments.

Montoya_SupFig4



Supplementary Figure 5. *Smad4* extended transcript is expressed in CD4$^+$ T cells. (**A**) Positions of qPCR primers used for total *Smad4* and extended *Smad4* transcript quantification (top). Dual luciferase constructs for the annotated *Smad4* 3'UTR and the *Smad4* 3'UTR extension (bottom). Bolded line indicates polyA signal sequence at the end of the annotated 3'UTR. Dashed lines represent the *Smad4* extended transcript supported by human and mouse EST (expressed sequence tag) data. (**B**) mRNA expression of *Smad4* and the *Smad4* extended transcript in naive CD4$^+$ T cells by qPCR; expression was normalized to *Gapdh* in each sample. Data are pooled from eight independent experiments with one to three mice per experiment (**B;** mean and s.e.m).

Montoya_SupFig5



Chapter 3

Defining the scope of miRNA regulation in mouse and human Th17 cells

Introduction

Th17 cells are a subset of $CD4^+$ helper T cells that have been implicated in autoimmune disease pathogenesis and are important for the control of extracellular bacterial and fungal infections. Both murine and human Th17 cells characteristically produce IL-17, are defined by the transcription factor RORγt, and are commonly identified by the chemokine receptor, CCR6[92,116-118]. Several miRNAs (small non-coding RNAs) have been identified to have specific roles in Th17 cell biology, including our work described here in Chapter 2 showing that miR-18a acts as a dynamically inducible inhibitor of Th17 cell differentiation. However, the scope of miRNA control of Th17 cell differentiation remains unexplored.

To expand our work in this area, we performed functional screens in both mouse and human T cells to determine the scope of regulation of all miRNAs expressed in T cells on Th17 cell differentiation. Functional screens like this can define the spectrum of effects that miRNAs can have on Th17 cells and identify novel powerful regulators of Th17 biology. Ultimately, these detailed studies can help define how miRNAs should be integrated into gene expression networks that govern Th17 cell differentiation.

For the murine Th17 screen, miRNA-deficient (*Dgcr8*$^{\Delta/\Delta}$ = CD4-Cre^+ *Dgcr8*$^{fl/fl}$) T cells were used. Since *Dgcr8* is a critical component of miRNA processing in the miRNA biogenesis pathway, *Dgcr8*$^{\Delta/\Delta}$ T cells essentially lack all endogenous miRNAs. This enhances the sensitivity of the assay to the effects of each re-introduced miRNA.

Using *Dgcr8*-floxed mice that have been crossed to a mouse line that expresses Cre recombinase under the control of the CD4 promoter allows for conditional deletion of *Dgcr8* only in cells that have expressed CD4. We further crossed *Dgcr8* CD4-Cre mice to *Rosa26*-YFP mice to introduce a fluorescent reporter of Cre expression. This YFP reporter can then be used during flow cytometry to gate only on cells that have deleted the *Dgcr8* alleles.

After isolating T cells from these mice, our first challenge was identifying the optimal culture conditions to generate robust Th17 cultures. One study showed that this was possible in T cells depleted of endogenous miRNAs where they cultured Th17 cells from $CD4^+$ T cells deficient in *Dicer,* another essential factor for miRNA processing[97]. They showed that IL-17 expression was induced in $Dicer^{\Delta/\Delta}$ T cells in Th17-polarizing conditions (TGFβ+IL-6) although much less than control $CD4^+$ T cells in the same conditions. We found similar results after optimizing conditions for transfection in $Dgcr8^{\Delta/\Delta}$ Th17 cells.

Using the miRNA-deficient $Dgcr8^{\Delta/\Delta}$ polarized Th17 cells, we tested the regulatory effects of individual miRNAs during the Th17 differentiation process by transfecting in every miRNA expressed in T cells one at a time using synthetic miRNA mimics. This strategy was first used by others whose work was done in embryonic stem cells[119,120]. Our lab has also routinely performed functional screens like these with much success for Th1 differentiation[78] and Th2 differentiation[121]. Further characterization of top 'hits' from these screens confirmed phenotypes as predicted from these screening

experiments in follow-up studies. In a functional screen of miRNAs that regulate miRNA-deficient $CD4^+$ T cells, miR-29 was found to rescue the abnormal IFNγ production from *Dgcr8*$^{\Delta/\Delta}$ T cells. Additional experiments showed that miR-29 regulates Th1 differentiation by limiting IFNγ production through direct targeting of the transcription factors Eomes and T-bet. Top hits from a screen for miRNA function in regulating Th2 differentiation uncovered distinct roles for miR-24 and miR-27 inhibiting IL-4 production. Mice deficient in two clusters which co-express these miRNAs showed increased Th2 responses *in vitro* and *in vivo* in an allergic airway inflammation model through several unique and shared target genes. The success of these screens provides confidence in our strategy to assess the miRNA function in helper T cell differentiation and suggests a similar strategy could be used to interrogate miRNA function in Th17 cell differentiation.

To test the conservation of miRNA activity in Th17 cells and its relevance to human biology, we also performed a loss of function screen in human Th17 cells. To do this, we used transfectable individual miRNA inhibitors and miRNA family inhibitors in primary human T cells. Similar to the murine Th17 screen with miRNA-deficient $CD4^+$ T cells, for the human Th17 screen our first task was to develop strong Th17-polarizing culture conditions to generate human Th17 cells. Much work has been done to ascertain the factors necessary for human Th17 cell differentiation[122]. Based on this work, our strategy was to use full-polarizing conditions utilizing all the known Th17-inducing cytokines (TGFβ, IL-1β, IL-6, and IL-23) to optimally generate robust Th17 cultures as measured by induction of CCR6, RORγt, and IL-17 expression. Our work described in Appendix 1 details the optimized protocol. After we established a strong

human Th17 culturing system, we performed the loss of function screen by transfecting in locked nucleic acid (LNA) inhibitors ("miRCURY" products commercially available from Exiqon) using the Neon electroporation system. Importantly, experiments in mouse T cells had confirmed the efficacy and specificity of inhibitors targeted against individual miRNAs (e.g. blocking miR-18a only) and others with family-wise specificity (e.g. blocking both miR-18a and miR-18b) as shown in Chapter 2 of this work.

For both functional screens, we monitored changes in Th17 cell differentiation by flow cytometry. Since individual miRNAs may alter this process in a variety of ways, we assessed the effects of each re-introduced miRNA (murine screen) or miRNA inhibition (human screen) on multiple Th17 cell markers, including CCR6, RORγt, and IL-17, as a readout of changes in Th17 cell differentiation. Together, these experiments define the miRNA requirements for Th17 differentiation.

Results

Th17 cell differentiation in miRNA-deficient CD4$^+$ T cells

To perform a screen in *Dgcr8*$^{\Delta/\Delta}$ T cells, we first optimized culture conditions for polarizing naïve *Dgcr8*$^{\Delta/\Delta}$ CD4$^+$ T cells into Th17 cells. CD4$^+$ T cells were isolated from spleens and lymph nodes of *Dgcr8*$^{\Delta/\Delta}$ mice and cultured *in vitro* for 4 days with TCR stimulation and exposure to the Th17-induction cytokines, TGFβ, IL-1β, IL-6, and IL-23. On day 4 of culture, we did flow cytometric analysis of several Th17 markers. *Dgcr8*$^{\Delta/\Delta}$ Th17 cells that were YFP$^+$ (Fig. 1A) expressed both CCR6 and RORγt and also produced IL-17 upon re-stimulation as expected (Fig. 1B). Expression of all three

markers was lower in *Dgcr8*$^{\Delta/\Delta}$ Th17 cells when compared to control Th17 cells, consistent with published literature in *Dicer*$^{\Delta/\Delta}$ Th17 cells, which are also deficient in endogenous miRNAs. Importantly, expression levels were all high enough to show upregulation or downregulation of these markers for screening purposes. However, the RORγt staining protocol to observe RORγt expression by flow cytometry technically interferes with the ability to gate on the YFP$^+$ population of cells, which have confirmed deletion of *Dgcr8* alleles and are therefore truly miRNA-deficient. Although younger mice tend to have mostly YFP$^+$ cells with very minimal YFP$^-$ population, for the purposes of the screen, we decided it was best to only stain for CCR6 and IL-17A so that we could previously gate on only the YFP$^+$ population of cells.

Next, conditions were optimized for introducing miRNA mimics into differentiating Th17 cells during primary activation in Th17-polarizing conditions *in vitro*. We delivered synthetic miRNA mimics during the differentiation process on day 2 of culture (Fig. 1C). miR-18a miRNA mimic was used as a 'positive control' for CCR6 expression since our work in Chapter 2 shows that miR-18 inhibits the expression of CCR6 in Th17 cells (Fig. 1D). The miR-17 miRNA mimic was used as a 'negative control' for CCR6 expression (Fig. 1D) and as a 'positive control' for IL-17 production since we expected miR-17 to promote IL-17 expression based on our work and that of others (Fig. 1E). Both of these mimic transfections successfully phenocopied the response we expected giving us confidence in our ability to screen more miRNAs on a larger scale. The same *in vitro* culture and transfections conditions were used for the screen.

miRNA functional screen in miRNA-deficient mouse Th17 cells

From a list of miRNAs known to be expressed at considerable levels in T cells, we created a panel of miRNA mimics to transfect into miRNA-deficient *Dgcr8*$^{\Delta/\Delta}$ Th17 cells. On day two of culture, we individually reintroduced each miRNA expressed in T cells into *Dgcr8*$^{\Delta/\Delta}$ polarized Th17 cells. We transfected the miRNA-deficient cells with 115 synthetic miRNA mimics or non-specific controls and 48 hours later, assessed several Th17 markers including surface markers and cytokines using flow cytometry. This strategy uncovered several miRNAs, in addition to miR-18, that inhibited or enhanced Th17 cell differentiation (Fig. 2).

The rescue screen successfully identified known miRNAs that play a role in Th17 cell differentiation and revealed new miRNAs that may be important regulators of Th17 biology. This primary screen was repeated on a slightly smaller scale in a confirmatory screen, which showed high reliability and reproducibility of the primary screen (Fig. 3). As expected, miR-17 and miR-19 miRNA family members were found to be top hits promoting Th17 cell differentiation while miR-18a was observed to be one of the top inhibitory miRNAs. In line with previously published literature, miR-155 was again identified as a top hit promoting Th17 differentiation, which provides further confidence of these results. Importantly, this functional screen identified multiple individual miRNAs and miRNA families that can regulate Th17 cell differentiation and cytokine production that have not been previously studied in Th17 cells. In particular, multiple miRNAs from the miR-30 family, the miR-181 family, and the miR-130 family, all inhibited Th17 cell differentiation.

miR-181-deficient T cells show increased Th17 cell frequencies

We further characterized the role of miR-181, one of the top hits from the screen, in Th17 cells. The miR-181 family consists of four mature miRNAs: miR-181a, miR-181b, miR-181c, and miR-181d. Mature miR-181a and miR-181b are transcribed from two separate genetic loci, the *Mirc14* cluster (*miR-181a-1* and *miR-181b-1*) and the *Mirc15* cluster (*miR-181a-2* and *miR-181b-2*) while miR-181c and miR-181d are transcribed from their own locus, the *Mirc16* cluster. In T cells, miR-181a and miR-181b are highly expressed. Previous studies showed that expression of mature miR-181a and miR-181b is mostly due to the *Mirc14* cluster[123] so we used a conditional mutant mouse strain with floxed *miR-181ab1* alleles. We crossed this line to mice with Cre recombinase under the control of the CD4 promoter, which allowed for conditional deletion of *Mirc14* only in cells that have expressed CD4.

To interrogate the role of miR-181 in Th17 cells, we used these conditional knockout mice that lack miR-181 only in T cells and studied their phenotype in Th17 differentiation. We isolated $CD4^+$ T cells from the spleen and lymph nodes of miR-181 knockout mice (miR-$181^{\Delta/\Delta}$ = CD4-Cre^+ *miR181ab1*$^{fl/fl}$), mice heterozygous for the *miR-181ab1* floxed allele (miR-$181^{+/\Delta}$ = CD4-Cre^+ miR-$181^{+/fl}$), and control mice (miR-$181^{+/+}$= CD4-Cre^- miR-$181^{fl/fl}$) and cultured them *in vitro* in Th17-polarizing conditions for 4 days. To assess the Th17 phenotype, we used two different culture conditions including the classical Th17 culture (TGFβ and IL-6) and a full-polarizing culture, which included all Th17-inducing cytokines (TGFβ, IL-1β, IL-23, and IL-6). In classical Th17-polarizing conditions, compared to control miR-$181^{+/+}$ $CD4^+$ T cells, miR-$181^{+/\Delta}$ and miR-$181^{\Delta/\Delta}$

both showed increased expression of RORγt and IL-17A production (Fig. 4A). In the full Th17-polarizing condition, miR-181$^{+/\Delta}$ and miR-181$^{\Delta/\Delta}$ showed overall significantly higher levels of CCR6 and RORγt expression as well as IL-17A production upon re-stimulation when compared to miR-181$^{+/+}$ CD4^{+} T cells (Fig. 4B). These *in vitro* data suggest an inhibitory role for miR-181a and/or miR-181b in Th17 cell differentiation as predicted from the primary functional screen.

miRNA inhibitor screen in human Th17 cells

To test the potential functional relevance of the miRNA contribution to human biology, we performed a similar functional screen in human Th17 cells. To do this, we isolated CD4^{+} T helper lymphocytes from de-identified human cord blood samples. Importantly, we used optimized cell culture conditions, 4 days of TCR stimulation and exposure to TGFβ, IL-1β, IL-23, and IL-6 in serum-free media, to polarize these cells into Th17 cells while introducing transfectable miRNA inhibitors (Fig. 5A). Before performing the screen, we conducted experiments transfecting in inhibitors that should have predicted effects on Th17 differentiation (Fig. 5B,C). Based on the literature, miR-155 promotes Th17 cells and therefore, inhibition of miR-155 should decrease expression of Th17 cell markers. Here we show that inhibition of miR-155 in human Th17 cells showed decreased frequencies of CCR6-, RORγt-, and IL-17A-producing T cells. Similarly, work on miR-210 suggests that inhibition of miR-210 should promote Th17 cells. In line with this, we see increased frequencies of CCR6-, RORγt-, and IL-17A-expressing human Th17 cells after miR-210 inhibition. These same culture and transfection conditions were used to perform the human Th17 screen.

During the human Th17 differentiation process, 77 LNA inhibitors were transfected in one at a time (Fig. 6). These LNA inhibitors allowed us to effectively and specifically inhibit miRNAs expressed in T cells by targeting individual miRNAs and other miRNA families, which are grouped together by similar seed sequences. We then assessed the effects of these miRNAs on Th17 cell differentiation by examining the levels of CCR6, RORγt, and IL-17 expression post-transfection. This functional screen performed in primary human T cells identified many endogenous miRNAs that differentially regulated various markers associated with Th17 cells. miRNAs were shown to have differential effects on CCR6, RORγt, and IL-17A expression. Of note, multiple miRNAs that were identified as top hits in the functional screen in mouse Th17 cells were also observed as top hits in the human Th17 cell screen. Importantly, a slightly smaller confirmatory screen was also performed for the human Th17 cells, which showed strong reproducibility and reliability when compared to the original primary human Th17 screen (Fig. 7). Inhibition of several miRNAs were top hits in both screens, providing confidence as potential promising candidates to follow-up on in future studies.

Discussion

While my data in Chapter 2 suggest an essential limiting role for miR-18 in Th17 cell differentiation, the scope of regulation of all miRNAs expressed in T helper cells remains to be determined. To build a systematic view of miRNA regulation of Th17 biology, we performed two screens to examine miRNA function in murine and human Th17 differentiation. First we performed a rescue screen where we re-introduced a

panel of 115 synthetic miRNA mimics into mutant miRNA-deficient murine Th17 cells. Second, we did a loss-of-function screen using 77 miRNA inhibitors to examine the effects of limiting their function in human *in vitro* polarized Th17 cells. Using this approach, we identified powerful regulators of Th17 cells and uncovered new miRNAs not previously known to play a role in Th17 biology.

The Th17 cell rescue screen uncovered a complex role for members of the miR-17~92 cluster. It is clear from our work in Chapter 2 that miRNA members of the miR-17~92 cluster have differential effects on Th17 differentiation. Data from this screen highlighted the complexity of this cluster showing miRNAs from the miR-17 family (miR-17, -20a, -20b, -106a, -106b, and -93) and miRNAs from the miR-19 family (miR-19a, -19b) as top hits that promote Th17 cells while the miR-18 family miRNAs (miR-18a, -18b) were top hits as inhibitory miRNAs. In fact, miR-18a had one of the largest effects inhibiting CCR6 expression in *Dgcr8*$^{\Delta/\Delta}$ T cells. Both of these findings were as expected based on our work and others and provided confidence in the validity and power of our screen.

Notably, several miRNAs that belong to the same miRNA families were identified in the primary and confirmatory screens as miRNAs that can regulate CCR6 and/or IL-17A expression. All members of the miR-30 family included in the screen, miR-30b, miR-30c, miR-30d and miR-30e, inhibited CCR6 and IL-17A expression. miRNAs from the miR-130 family had more complex effects since the miRNAs miR-301a and miR-130b regulated CCR6 expression and IL-17A production differently. These miRNAs seem to have either no effect or slightly decreased IL-17A expression but were strong promoters

of CCR6 expression suggesting a potential role for this miRNA family early on in the induction of CCR6-expressing T cells without effects later in the differentiation process on effector cytokine production. Because miRNA family members share common seed sequences, it is not surprising to find them cluster together to similarly regulate these Th17 markers and even provides more confidence in their candidacy as true Th17 regulators.

Recent work has been done by others that confirmed our findings on the importance of the miR-30 and miR-130 miRNA families in Th17 cell differentiation. Qu et al. showed that miR-30a directly targets the IL-21R to inhibit Th17 cell differentiation and alleviate EAE symptoms in mice[66]. Another study showed that $CD4^+$ T cells transfected with a miR-301a mimic showed increased CCR6 expression[58].

While it is important to confirm expected hits, we hoped to uncover novel miRNAs that have yet to be identified as key regulators of Th17 cells. Several members of the miR-181 family, miR-181a, miR-181b, and miR-181c miRNAs all decreased the frequencies of both CCR6 and IL-17-producing cells. miR-181 has mostly been studied as a tuner of TCR signaling[124,125]. One study showed that deletion of *miR-181a-1/b-1* did cause an increase in Th17 cell differentiation as shown by increased IL-17A production in the spleens of mice with EAE, however due to migration defects into the CNS overall had delayed onset of EAE symptoms[123]. This study used a full *miR-181a-1/b-1* knockout so it is possible that conditional deletion of *miR-181a-1/b-1* only in $CD4^+$ expressing T cells might not exhibit the same migration defects and could lead to increased *in vivo* Th17

responses in the CNS of mice with EAE. This remains to be determined. Another study looked at the role of miR-181 in human T cells[126]. Their work showed that at a late time point in Th17 cell activation, miR-181 can induce a self-regulatory mechanism to dampen inflammation by modulating ERK phosphorylation, which when sustained induces ID3, a negative regulator of *Rorc*. This suggests that over time miR-181 can inhibit Th17 cells through this regulatory network.

Based on this evidence, we wanted to further examine the role of miR-181 in Th17 cell differentiation. Our preliminary studies using *in vitro* assays showed that miR-181 deficiency specifically in $CD4^+$ T cells led to increased Th17 cell frequencies. More work should be done to further interrogate the role of this miRNA in Th17 cell biology. Future experiments should focus on identifying target genes of miR-181 and determining its functional significance *in vivo.* Based on the preliminary data, I expect miR-181 to directly target multiple genes important for Th17 cells and mice lacking miR-181 to have worsened Th17-driven disease phenotypes.

While top candidates identified in the murine rescue screen and discussed further here demonstrate the importance of this technique in identifying known and novel Th17 regulators, this screening method does have caveats. One such consideration when using miRNA mimics to interrogate individual miRNAs in miRNA-deficient T cells is that miRNAs that must act in combination with other cluster or family members can be missed. To identify such miRNAs, one could perform the screen using miRNA inhibitors for each individual miRNA and for whole families in WT mouse Th17 cell cultures. This

allows for the examination of effects of inhibition of only one miRNA (or one miRNA family) at a time while other miRNAs remain active at normal endogenous levels. Importantly, this approach also enables us to test the requirement for each miRNA in human Th17 cells.

Our technical expertise in performing these screening methods and in studying T cell differentiation allowed us to use this approach with primary human T cells. To assess miRNA regulation of human Th17 cells, we performed a loss-of-function screen in human T cells using a catalog of miRNA inhibitors. Again, this technique allowed us to identify both known and novel regulators of human Th17 cell differentiation.

In particular, miR-155 and miR-210, two well studied miRNA regulators of murine Th17 cells also regulated human Th17 cells in our primary screen. Inhibition of miRNA families (the miR-181, miR-30, and miR-130 families) that were identified as top hits in our murine rescue screen also proved to have similar effects in human Th17 cells. Since work on these miRNAs was mostly done in murine T cells, this is an important observation that the effects of these miRNAs in mice are translatable to primary human T cells as well.

A follow-up confirmatory screen showed many miRNA inhibitors had similar regulatory effects as in the primary screen. We noted several miRNAs, including miR-574, miR-532, and the miR-10 and miR-15 families, that could be interesting candidates to negatively regulate Th17 cells since their inhibition increased Th17 cells. We also

highlighted multiple miRNAs that might be positive regulators of Th17 cells since their inhibition greatly decreased Th17 differentiation, including miR-31, miR-598, miR-590, miR-126, miR-451, miR-142, and miR-361. All of these miRNAs were identified as top hits in both the primary and confirmatory screens and whose regulatory role in Th17 cells is unknown. Future work should aim to investigate the role of these top miRNA candidates in both human and mouse Th17 cells.

These two functional screens in mouse and human Th17 cells have proven to be a powerful way to find functionally relevant miRNAs that regulate Th17 differentiation. More detailed studies should be done with top candidates to verify and further characterize their precise role in Th17 biology. miRNA biology is not a linear pathway since miRNAs are known to target multiple genes and multiple miRNAs can target the same gene. As such, we believe that a network of post-transcriptionally regulated genes determines the mechanism by which miRNAs control Th17 cell differentiation. Future studies of miR-181 and any other interesting hits from either functional screen should work to characterize these mechanistic interactions with target genes as well as their physiological relevance by studying their immune effector functions *in vivo*.

Materials and Methods

Mice

Mice with *lox*P sites flanking microRNA miR-181ab1 (*Mirc14*$^{tm1.1Czc}$; The Jackson Laboratory, 025872) were crossed to CD4-Cre mice (*Tg(CD4-cre)1Cwi;* Taconic, 4196) to generate T cell-specific miR-181-deficient mice. Mice with *lox*P-flanked *Dgcr8* alleles (*Dgcr8*$^{tm1.1Blel}$) have been described before[113] and were bred to CD4-Cre and R26-stop-EYFP mutant mice (*Gt(ROSA)26Sor*$^{tm3(CAG-EYFP)Hze}$*;* The Jackson Laboratory, 006148). All mice were housed and bred in the specific pathogen-free barrier facilities at the University of California San Francisco. All experiments were performed according to the Institutional Animal Care and Use Committee (IACUC) guidelines of the University of California, San Francisco.

In vitro mouse primary T cell polarization and miRNA screening

Single-cell suspensions from spleen and lymph nodes were prepared by mincing, filtered through fine mesh and counted. $CD4^+$ T cells were enriched with the Easy Sep Mouse $CD4^+$ T Cell Isolation Kit (Stemcell Technologies). Purified $CD4^+$ T cells were plated at $4x10^6$ cells per well in complete medium (RPMI-1640 supplemented with 10% fetal bovine serum, pyruvate, nonessential amino acids, l-arginine, l-asparagine, l-glutamine, folic acid, beta mercaptoethanol, penicillin and streptomycin) in 6-well plates (Corning Costar) pre-coated with 2μg/ml anti-CD3 (clone 17A2; Bio X Cell) and anti-CD28 (clone 37.51; Bio X Cell) for ~48 hr. For Th17 polarizing conditions, media were supplemented with anti-IFNγ (10μg/ml, clone XMG1.2; Bio X Cell), anti-IL-4 (10μg/ml, clone 11B11; Bio X Cell), human TGFβ (5ng/ml; Peprotech), murine IL-23 (20ng/ml;

R&D Systems), murine IL-1β (20ng/ml; R&D Systems) and murine IL-6 (25ng/ml; Peprotech), unless otherwise stated. For miRNA screening, on day 2 of culture, cells were collected, counted, suspended in transfection buffer together with miRIDIAN miRNA mimics (Dharmacon) used at 500nM and transfected with the Neon transfection system (Invitrogen). A total of 115 miRNAs with appropriate controls were screened. Cells were immediately transferred into fresh culture medium containing Th17-polarizing cytokines plus at $4x10^5$ cells per well in 96-well flat-bottom plates pre-coated with anti-CD3 and anti-CD28. All cultured cells were analyzed on day 4 of initial culture by flow cytometry. Z-scores were generated for the entire set of miRNAs screened (Z=x-median/SD, where x is the value for each individual miRNA).

***In vitro* human cord blood T cell polarization and miRNA screening**

Cord blood mononuclear cells (CBMCs) from anonymous human cord blood donors were isolated by Lymphoprep gradient (1114545; Accurate Chemical & Scientific). $CD4^+$ T cells were isolated from CBMCs using the Dynabeads Untouched Human $CD4^+$ T Cell Isolation Kit (Invitrogen). Cells were stimulated for ~48 h on plates coated with 2μg/ml anti-CD3 (clone OKT-3; UCSF Monoclonal Antibody Core) and 4μg/ml anti-CD28 (clone 15E8; Miltenyi Biotec) at an initial density of 3-4 × 10^6 cells per well in serum-free base medium (Stemcell Technologies Immunocult™ XF T Cell Expansion Medium) supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 10mM HEPES, 1 mM sodium pyruvate and 100 μM 2-mercaptoethanol in 6-well plates (Corning Costar). After 2 days of stimulation, cells were collected, counted, suspended in transfection buffer together with miRNA inhibitors, and transfected with

the Neon transfection system (Invitrogen). miRNA inhibitors included exiqon miRCURY LNA microRNA power inhibitors or miRCURY LNA microRNA power family inhibitors and were used at 20μM with appropriate controls. For miRNA screening, 77 miRNA individual or family inhibitors were screened. Cells were immediately transferred into 48-well plates at a density of $4x10^5$ per well pre-coated with anti-CD3 and anti-CD28 in fresh culture medium containing Th17-polarizing cytokines. For Th17-polarizing conditions, media were supplemented with anti-human IFNγ (10μg/ml, clone NIB42; eBioscience), anti-human IL-4 (10μg/ml, clone MP4-25D2; Biolegend), human TGFβ (5ng/ml; Peprotech), human IL-6 (25ng/ml; Peprotech), human IL-1β (20ng/ml; Peprotech), and human IL-23 (20ng/ml; Peprotech). Cultured cells were analyzed on day 4 of initial culture by flow cytometry. Z-scores were generated for the entire set of miRNAs screened (Z=x-median/SD, where x is the value for each individual miRNA).

Flow cytometry

Cultured cells were collected, washed, and stained with antibodies against cell surface proteins and transcription factors as described before[114]. Nonspecific binding was blocked with anti-CD16/CD32 (clone 2.4G2; UCSF Monoclonal Antibody Core), 2% normal rat/mouse serum for mouse T cell staining or with human FcR binding inhibitor (eBioscience) for human T cells. Dead cells were excluded with Fixable Viability Dye eFluor780 (eBioscience). For intracellular staining of transcription factors, the Foxp3 staining set (eBioscience) was used. Intracellular cytokine detection was performed after stimulation with 10nM PMA and 1μM ionomycin for 2 h, followed by the addition of 5μg/ml Brefeldin A for another 2 h. Cells were fixed with 4% PFA for 8 min at room

temperature, washed with ice-cold PBS, and permeabilized with 0.5% saponin. The following fluorochrome-conjugated antibodies were used in the study for mouse T cells: anti-CD4 (clones RM4-5 or GK1.5), anti-IL-17A (eBio17B7), anti-IFNγ (XMG1.2; all from eBioscience); anti-CCR6 (140706), anti-RORγt (Q31-378; both from BD Biosciences); and for human T cells: anti-human CD4 (OKT4; Biolegend); anti-human IL-17A (eBio64DEC17), and anti-human CCR6 (R6H1; both from eBioscience); and anti-human RORγt (Q21-559; BD Biosciences).

Figure 1. Th17 cell differentiation pre- and post-transfection in miRNA-deficient T cells. (A) Representative histogram displays expression of the fluorescent reporter gene, YFP. Gating of YFP^+ population selects only for cells that have deleted the *Dgcr8* alleles. (B) Control (*Dgcr8*$^{+/\Delta}$ = CD4-Cre^+ *Dgcr8*$^{+/fl}$) and miRNA-deficient (*Dgcr8*$^{\Delta/\Delta}$ = CD4-Cre^+ *Dgcr8*$^{fl/fl}$) $CD4^+$ T cells were cultured *in vitro* under Th17-polarizing conditions and analyzed on day 4 for Th17 marker expression by flow cytometry. Representative contour plots display surface CCR6 expression and intracellular RORγt staining of $CD4^+$ T cells (left) and IL-17A production after restimulation with PMA/ionomycin (right). Numbers in quadrants indicate percent CCR6 and/or RORγt-positive or IL-17A-positive live singlet cells. (C) Schematic of workflow for miRNA screen in mouse T cells in Th17-polarizing conditions. *Dgcr8*$^{\Delta/\Delta}$ $CD4^+$ T cells were cultured *in vitro* in Th17-polarizing conditions and transfected with miR-18a (blue line) or miR-17 (red line) miRNA mimics or appropriate control mimic (Ctl mim) on day 2 and analyzed 48 hours later for Th17 marker expression by flow cytometry: (D) Representative contour plots display surface CCR6 expression of $CD4^+$ T cells (left). Numbers in quadrants indicate percent CCR6-positive live singlet cells. Surface CCR6 expression is shown in a representative histogram (right). (E) Representative contour plots display IL-17A production after restimulation with PMA/ionomycin. Numbers in quadrants indicate percent IL-17A-positive live singlet cells (left). IL-17A expression is shown in a representative histogram (right).

Fig 1.

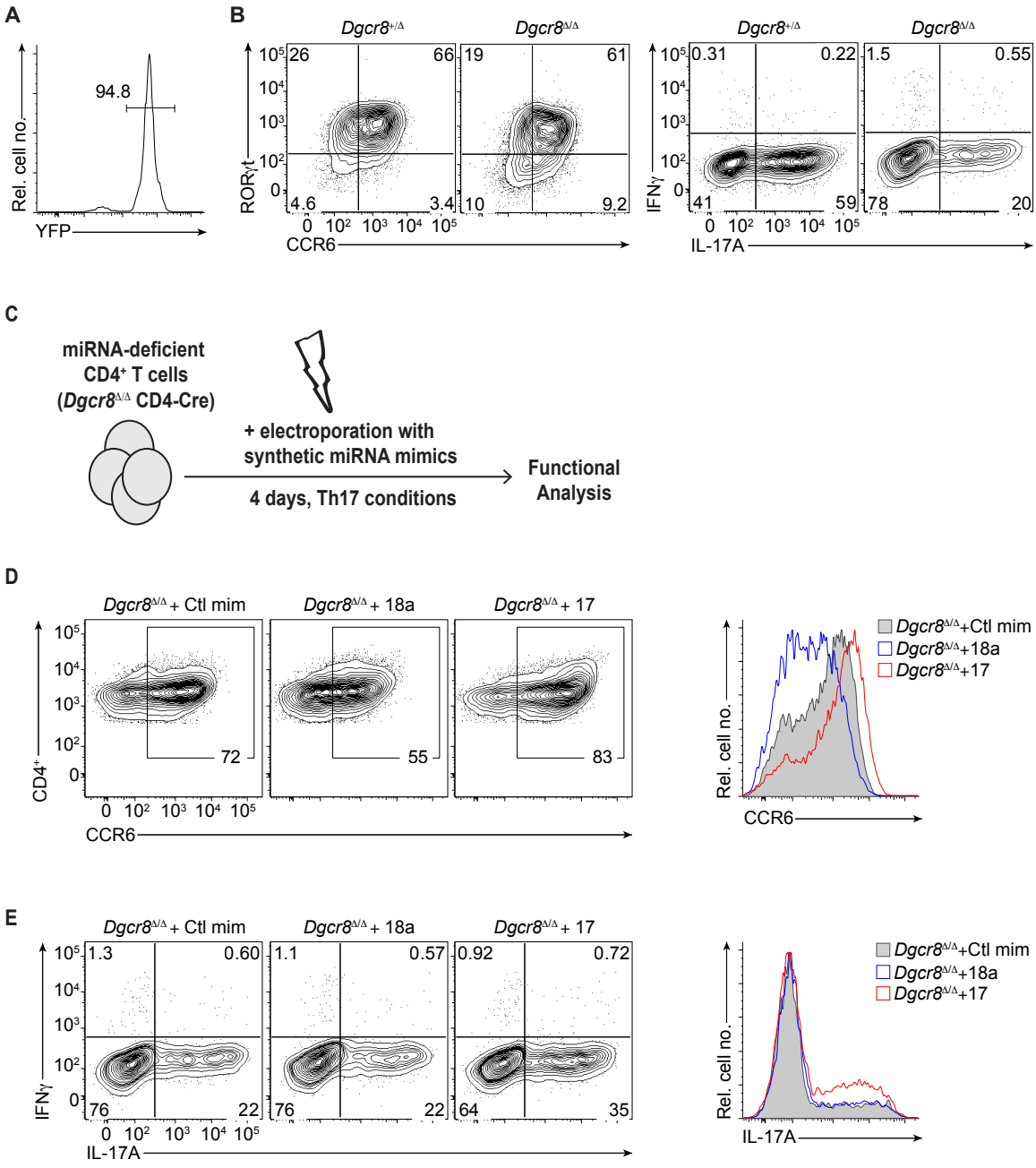

A
94.8
Rel. cell no.
YFP
B
Dgcr8+/Δ
DgcrΔ/Δ
26
66
19
61
4.6
3.4
10
9.2
RORγt
CCR6
0.31
0.22
1.5
0.55
41
59
78
20
IFNγ
IL-17A
C
miRNA-deficient
CD4+ T cells
(Dgcr8Δ/Δ CD4-Cre)
+ electroporation with
synthetic miRNA mimics
4 days, Th17 conditions
Functional
Analysis
D
Dgcr8Δ/Δ + Ctl mim
Dgcr8Δ/Δ + 18a
Dgcr8Δ/Δ + 17
72
55
83
CD4+
CCR6
Dgcr8Δ/Δ+Ctl mim
Dgcr8Δ/Δ+18a
Dgcr8Δ/Δ+17
Rel. cell no.
E
Dgcr8Δ/Δ + Ctl mim
Dgcr8Δ/Δ + 18a
Dgcr8Δ/Δ + 17
1.3
0.60
1.1
0.57
0.92
0.72
76
22
76
22
64
35
IFNγ
IL-17A
Dgcr8Δ/Δ+Ctl mim
Dgcr8Δ/Δ+18a
Dgcr8Δ/Δ+17
Rel. cell no.

Figure 2. miRNA functional screen in miRNA-deficient mouse Th17 cells. *Dgcr8*$^{\Delta/\Delta}$ CD4^{+} T cells were cultured *in vitro* in Th17-polarizing conditions transfected with 115 miRNA mimics or appropriate control mimic (Ctl mim) on day 2 and analyzed 48 hours later for Th17 marker expression by flow cytometry. Frequencies of CCR6^{+} and IL-17A^{+} cells were determined for each miRNA and used to calculate Z scores. miRNAs of interest have been highlighted in color.

Fig 2.

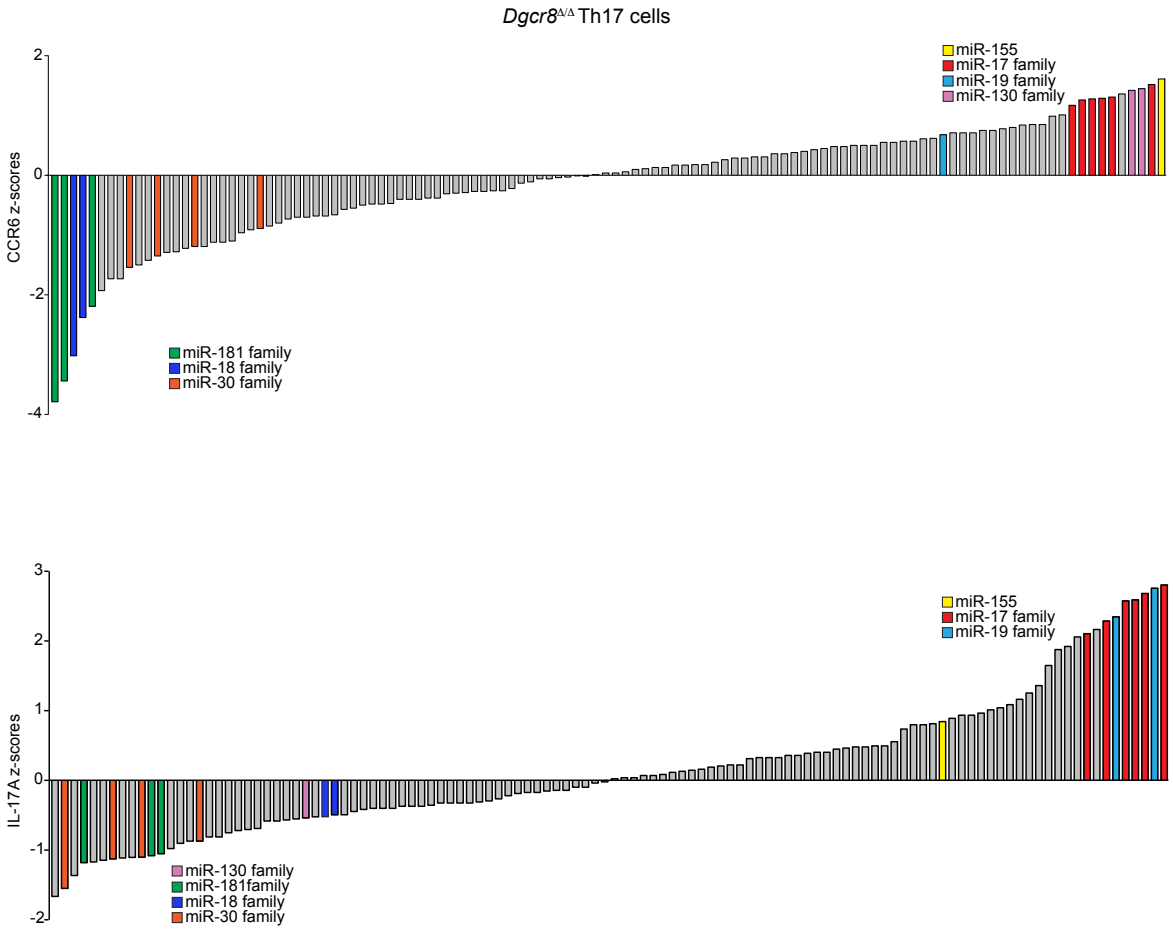

Dgcr8$^{\Delta/\Delta}$ Th17 cells
CCR6 z-scores
2
0
-2
-4
miR-155
miR-17 family
miR-19 family
miR-130 family
miR-181 family
miR-18 family
miR-30 family
IL-17A z-scores
3
2
1
0
-1
-2
miR-155
miR-17 family
miR-19 family
miR-130 family
miR-181family
miR-18 family
miR-30 family

Figure 3. miRNA confirmatory screen in miRNA-deficient mouse Th17 cells.

Dgcr8$^{\Delta/\Delta}$ CD4^{+} T cells were cultured *in vitro* under Th17-polarizing conditions transfected with 77 miRNA mimics or appropriate control mimic (Ctl mimic) on day 2 and analyzed 48 hours later for Th17 marker expression by flow cytometry. Frequencies of CCR6^{+} and IL-17A^{+} cells were determined for each miRNA. miRNAs of interest have been highlighted in color.

Fig 3.

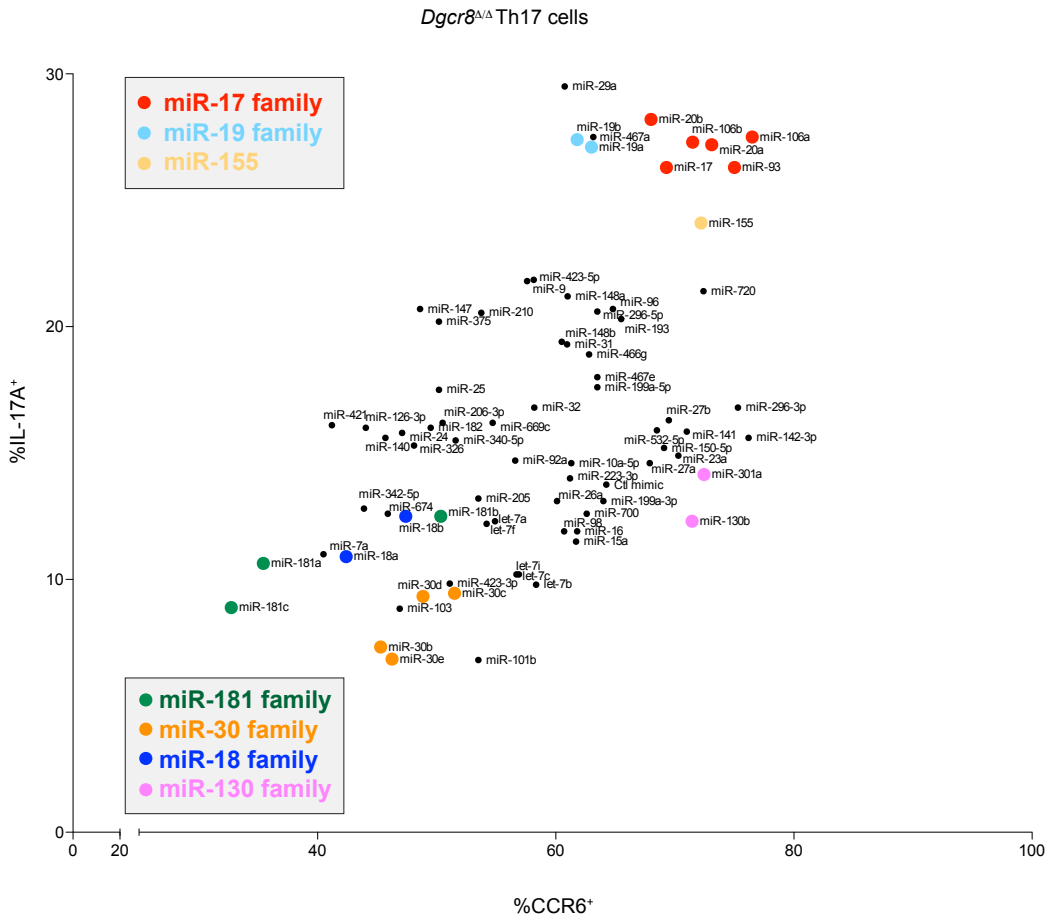

Dgcr8Δ/Δ Th17 cells
miR-17 family
miR-19 family
miR-155
miR-181 family
miR-30 family
miR-18 family
miR-130 family
%IL-17A+
%CCR6+
0
10
20
30
0
20
40
60
80
100
miR-29a
miR-20b
miR-106b
miR-106a
miR-19b
miR-467a
miR-19a
miR-20a
miR-17
miR-93
miR-155
miR-423-5p
miR-9
miR-148a
miR-96
miR-720
miR-147
miR-210
miR-296-5p
miR-375
miR-193
miR-148b
miR-31
miR-466g
miR-467e
miR-199a-5p
miR-25
miR-421
miR-126-3p
miR-206-3p
miR-32
miR-27b
miR-296-3p
miR-182
miR-669c
miR-24
miR-340-5p
miR-532-5p
miR-141
miR-142-3p
miR-140
miR-326
miR-150-5p
miR-92a
miR-10a-5p
miR-23a
miR-27a
miR-301a
miR-223-3p
Ctl mimic
miR-342-5p
miR-205
miR-26a
miR-199a-3p
miR-674
miR-181b
miR-700
miR-18b
let-7a
let-7f
miR-98
miR-16
miR-130b
miR-15a
miR-7a
miR-18a
miR-181a
let-7i
let-7c
miR-30d
miR-423-3p
let-7b
miR-30c
miR-181c
miR-103
miR-30b
miR-30e
miR-101b

Figure 4. miR-181-deficient T cells show increased Th17 cell frequencies. miR-181$^{+/+}$, miR-181$^{+/\Delta}$, and miR-181$^{\Delta/\Delta}$ CD4^{+} T cells were cultured *in vitro* for 4 days in classical Th17-polarizing conditions (TGFβ and IL-6) (A) or full-polarizing conditions (TGFβ, IL-1β, IL-6, and IL-23) (B) and analyzed on day 4 for Th17 marker expression by flow cytometry: (A) Representative contour plots display surface CCR6 expression and intracellular RORγt staining of CD4^{+} T cells. Numbers in quadrants indicate percent CCR6 and/or RORγt-positive live singlet cells (top, left). RORγt geometric mean fluorescence intensity (gMFI) and frequency of CCR6^{+} cells are quantified in the bar graphs (top, right). Representative contour plots display IL-17A production after restimulation with PMA/ionomycin. Numbers in quadrants indicate percent IL-17A-positive live singlet cells (bottom, left). Frequency of IL-17A^{+} cells is quantified in the bar graph (bottom, right). (B) Representative contour plots display surface CCR6 expression and intracellular RORγt staining of CD4^{+} T cells. Numbers in quadrants indicate percent CCR6 and/or RORγt-positive live singlet cells (top, left). RORγt geometric mean fluorescence intensity (gMFI) and frequency of CCR6^{+} cells are quantified in the bar graphs (top, right). Representative contour plots display IL-17A production after restimulation with PMA/ionomycin. Numbers in quadrants indicate percent IL-17A-positive live singlet cells (bottom, left). Frequency of IL-17A^{+} cells is quantified in the bar graph (bottom, right). *P<0.05 and **P<0.01 (one-way ANOVA with Dunnett's post-test (comparing each column to miR-181$^{+/+}$). Data are pooled from three independent experiments with two to three mice per experiment (n=7).

Fig 4.

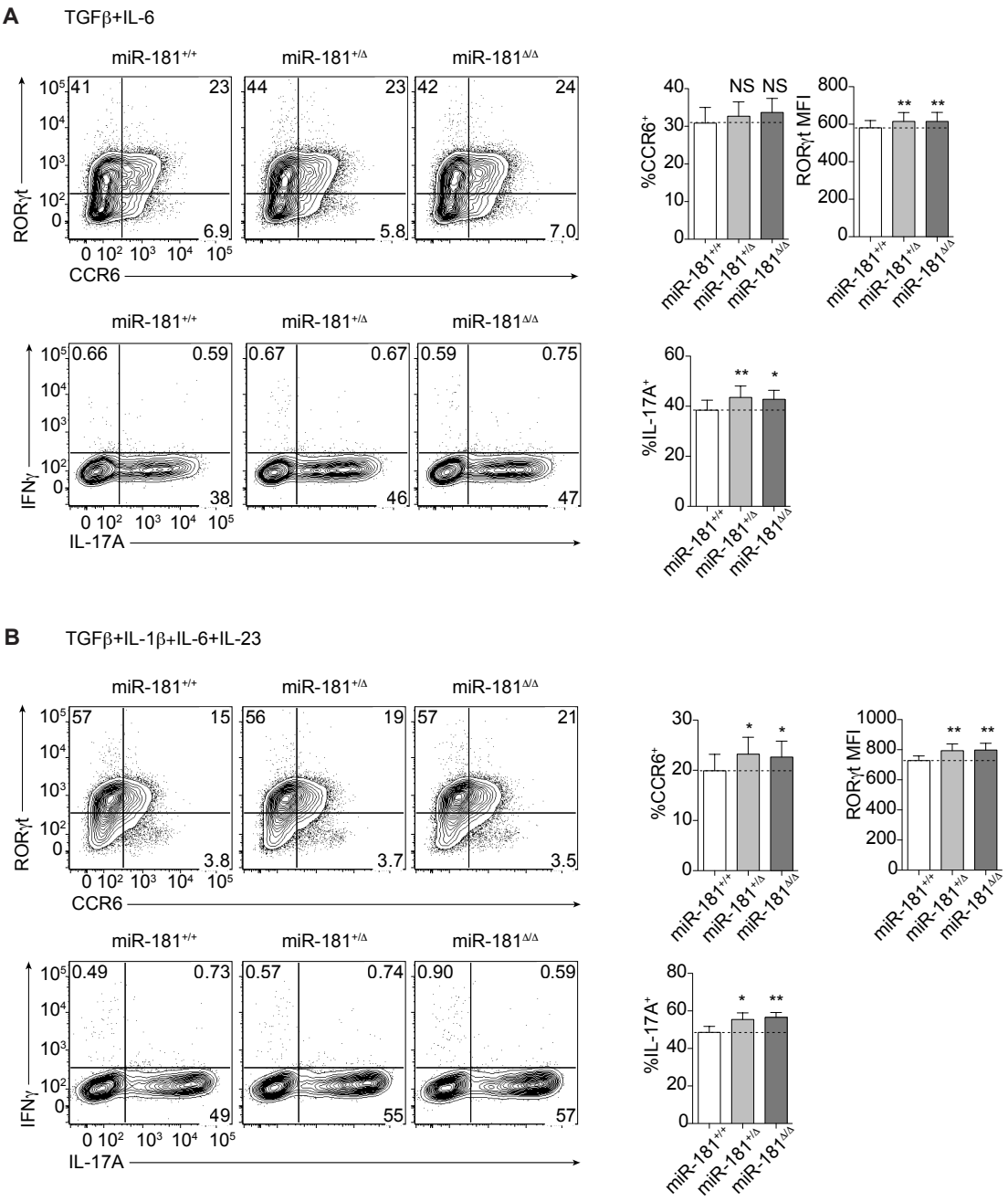

A
TGFβ+IL-6
miR-181+/+
miR-181+/Δ
miR-181Δ/Δ
RORγt
CCR6
41
23
44
23
42
24
6.9
5.8
7.0
IFNγ
IL-17A
0.66
0.59
0.67
0.67
0.59
0.75
38
46
47
%CCR6+
NS NS
RORγt MFI
%IL-17A+
B
TGFβ+IL-1β+IL-6+IL-23
57
15
56
19
57
21
3.8
3.7
3.5
0.49
0.73
0.57
0.74
0.90
0.59
49
55
57

Figure 5. Inhibition of miRNAs alters human Th17 cell differentiation. (A) Schematic of workflow for miRNA screen in human T cells in Th17-polarizing conditions. Human $CD4^+$ T cells were cultured *in vitro* in Th17-polarizing conditions transfected with miR-155 (155i) or miR-210 (210i) miRNA inhibitors or appropriate control inhibitor (Ctl inh) on day 2 and analyzed 48 hours later for Th17 marker expression by flow cytometry: (B) Representative contour plots display surface CCR6 expression and intracellular RORγt staining of $CD4^+$ T cells. Numbers in quadrants indicate percent CCR6 and/or RORγt-positive live singlet cells. (C) Representative contour plots display IL-17A production after restimulation with PMA/ionomycin. Numbers in quadrants indicate percent IL-17A-positive live singlet cells.

Fig 5.

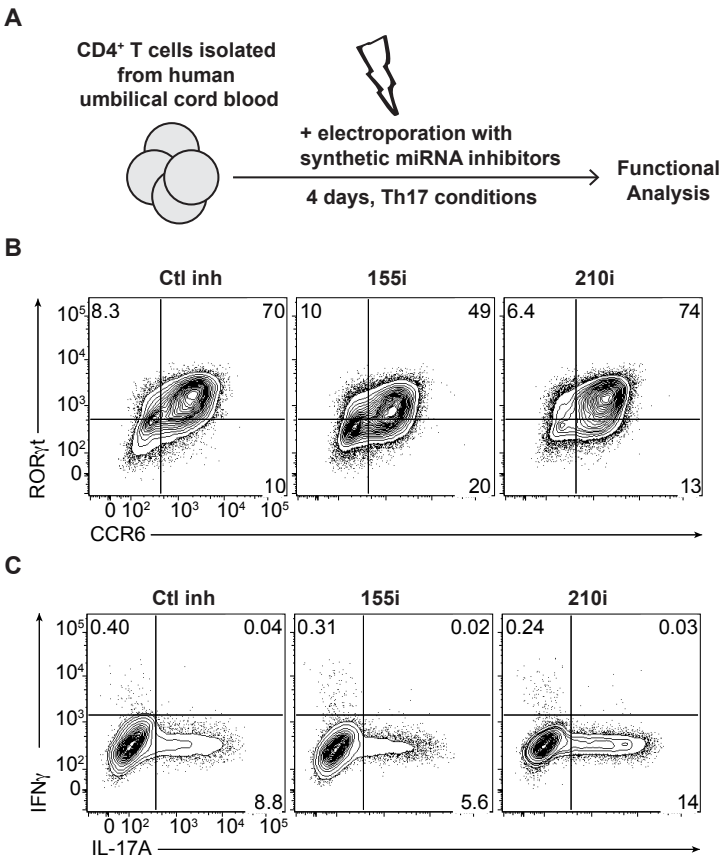

A
CD4+ T cells isolated from human umbilical cord blood
+ electroporation with synthetic miRNA inhibitors
4 days, Th17 conditions
Functional Analysis
B
Ctl inh
155i
210i
8.3
70
10
10
49
20
6.4
74
13
RORγt
CCR6
C
Ctl inh
155i
210i
0.40
0.04
8.8
0.31
0.02
5.6
0.24
0.03
14
IFNγ
IL-17A

Figure 6. miRNA inhibitor screen in human Th17 cells. Human $CD4^+$ T cells were cultured *in vitro* in Th17-polarizing conditions transfected with 77 miRNA inhibitors on day 2 and analyzed 48 hours later for Th17 marker expression by flow cytometry. RORγt geometric mean fluorescence intensity (gMFI) and frequencies of $CCR6^+$ and IL-$17A^+$ cells were determined for each miRNA and used to calculate Z scores. miRNAs of interest have been highlighted in color.

Fig 6.

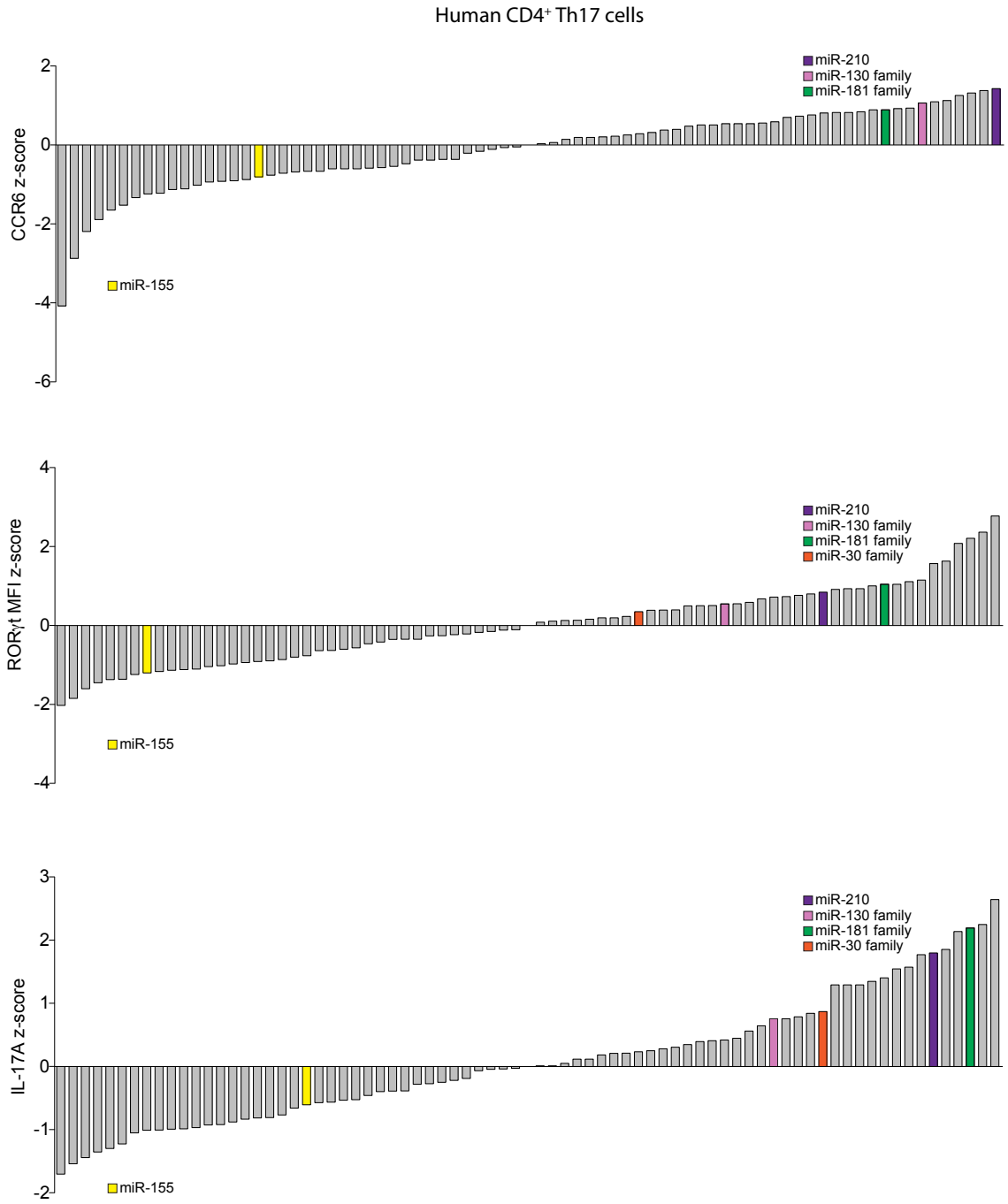

Human CD4+ Th17 cells
CCR6 z-score
2
0
-2
-4
-6
miR-155
miR-210
miR-130 family
miR-181 family
RORγt MFI z-score
4
2
0
-2
-4
miR-155
miR-210
miR-130 family
miR-181 family
miR-30 family
IL-17A z-score
3
2
1
0
-1
-2
miR-155
miR-210
miR-130 family
miR-181 family
miR-30 family

Figure 7. miRNA inhibitor confirmatory screen in human Th17 cells. Human $CD4^+$ T cells were cultured *in vitro* in Th17-polarizing conditions transfected with 77 miRNA inhibitors (primary screen) or 43 miRNA inhibitors and appropriate synthetic small RNA control inhibitor (Ctl) (confirmatory screen) on day 2 and analyzed 48 hours later for Th17 marker expression by flow cytometry: (A) RORγt geometric mean fluorescence intensity (gMFI) and frequencies of $CCR6^+$ cells were determined for each miRNA and shown in the 2D scatter dot plots for the primary miRNA inhibitor screen (left) and confirmatory inhibitor screen (right). (B) RORγt geometric mean fluorescence intensity (gMFI) and frequencies of $IL\text{-}17A^+$ cells were determined for each miRNA and shown in the 2D scatter dot plots for the primary miRNA inhibitor screen (left) and confirmatory inhibitor screen (right). miRNAs of interest have been highlighted in color.

Fig 7. Human CD4⁺ Th17 cells

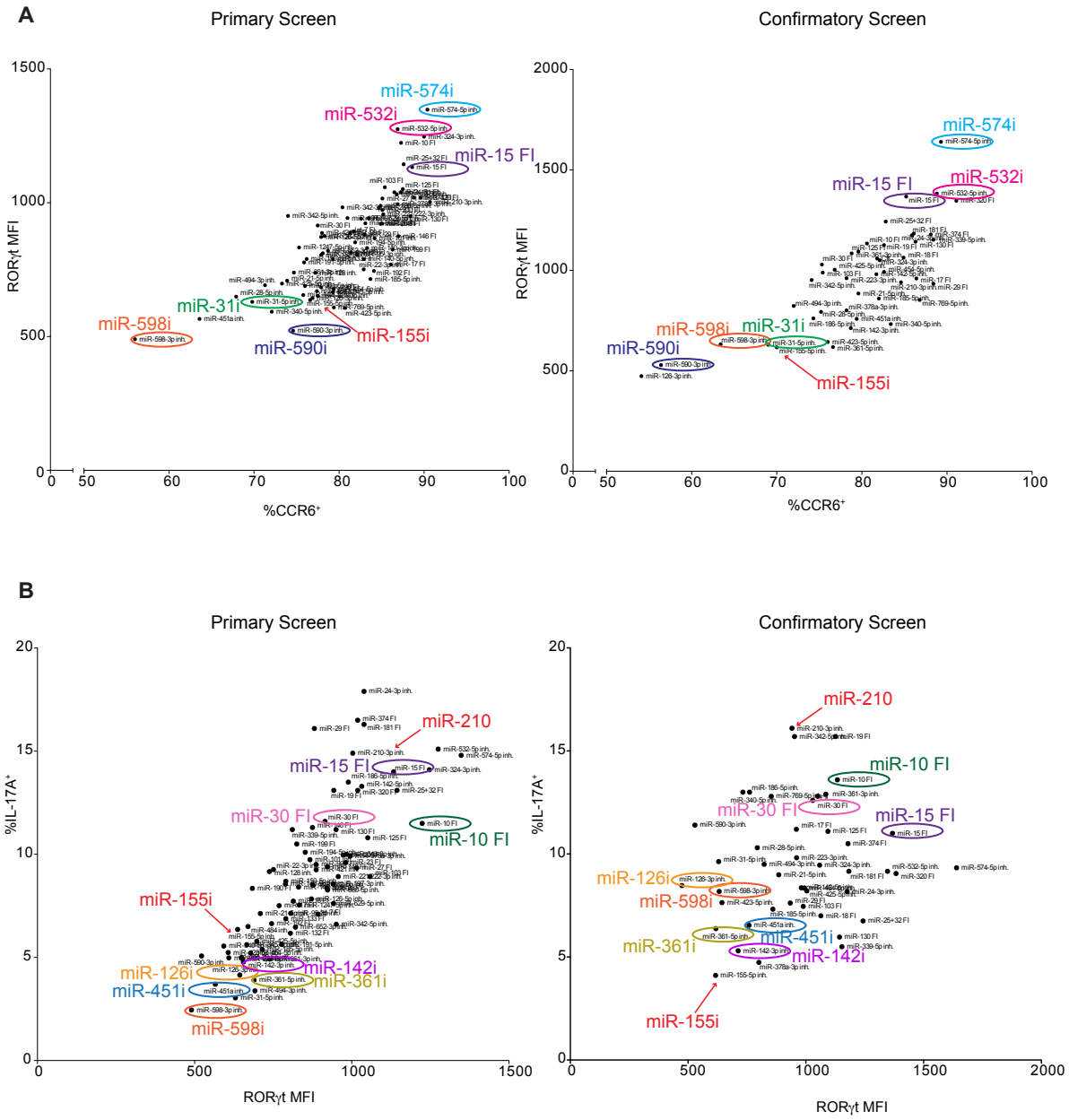


Table 1. Summary of Z scores for CCR6 and IL-17A following miRNA transfection in primary mouse T cell screen

miRNA	CCR6 Z score	miRNA	IL-17A Z score
mmu-miR-181c	-3.79	mmu-miR-101b	-1.67
mmu-miR-181a	-3.44	mmu-miR-30e	-1.55
mmu-miR-18a	-3.02	mmu-miR-700	-1.37
mmu-miR-18b	-2.38	mmu-miR-181b	-1.18
mmu-miR-181b	-2.19	mmu-let-7b	-1.17
mmu-miR-326	-1.93	mmu-let-7a	-1.15
mmu-let-7b	-1.73	mmu-miR-30b	-1.13
mmu-miR-342-5p	-1.73	mmu-miR-7a	-1.11
mmu-miR-30d	-1.54	mmu-let-7i	-1.11
mmu-miR-140	-1.50	mmu-miR-30d	-1.10
mmu-miR-674	-1.42	mmu-miR-181c	-1.08
mmu-miR-30e	-1.35	mmu-miR-181a	-1.05
mmu-miR-126-3p	-1.29	mmu-miR-16	-0.98
mmu-miR-206-3p	-1.28	mmu-let-7f	-0.90
mmu-miR-340-5p	-1.22	mmu-let-7c	-0.87
mmu-miR-30b	-1.19	mmu-miR-30c	-0.87
mmu-miR-7a	-1.19	mmu-miR-103	-0.81
mmu-miR-24	-1.12	mmu-miR-223-3p	-0.81
mmu-miR-467e	-1.12	mmu-miR-205	-0.75
mmu-miR-147	-1.10	mmu-miR-423-3p	-0.72
mmu-miR-669c	-0.96	mmu-miR-322	-0.71
mmu-miR-375	-0.91	mmu-miR-15a	-0.69
mmu-miR-30c	-0.89	mmu-miR-674	-0.58
mmu-miR-466g	-0.85	mmu-miR-98	-0.58
mmu-miR-421	-0.80	mmu-miR-669c	-0.57
mmu-let-7i	-0.73	mmu-miR-374	-0.55
mmu-miR-182	-0.70	mmu-miR-301a	-0.54
mmu-miR-101b	-0.70	mmu-miR-15b	-0.52
mmu-let-7c	-0.68	mmu-miR-18a	-0.52
mmu-let-7a	-0.68	mmu-let-7g	-0.49
mmu-miR-223-3p	-0.66	mmu-miR-18b	-0.49
mmu-miR-10a-5p	-0.57	mmu-miR-191	-0.45
mmu-miR-423-3p	-0.55	mmu-let-7d	-0.42
mmu-miR-378	-0.50	mmu-miR-140	-0.40
mmu-let-7f	-0.48	mmu-miR-151-3p	-0.40
mmu-miR-98	-0.48	mmu-miR-744	-0.40
mmu-miR-103	-0.47	mmu-miR-185	-0.37
mmu-miR-25	-0.40	mmu-miR-199a-3p	-0.37
mmu-miR-425	-0.40	mmu-miR-33	-0.37
mmu-miR-361	-0.40	mmu-miR-467e	-0.36
mmu-miR-374	-0.38	mmu-miR-28	-0.33

miRNA	CCR6 Z score	miRNA	IL-17A Z score
mmu-miR-872	-0.38	mmu-miR-425	-0.33
mmu-miR-9	-0.31	mmu-miR-466l	-0.33
Ctl mim	-0.30	mmu-miR-872	-0.33
mmu-let-7d	-0.29	mmu-miR-146a	-0.31
mmu-miR-423-5p	-0.27	mmu-miR-150-5p	-0.30
mmu-miR-185	-0.27	mmu-miR-326	-0.27
mmu-miR-297a	-0.26	mmu-miR-342-5p	-0.22
mmu-miR-652	-0.26	mmu-miR-128	-0.19
mmu-miR-205	-0.22	mmu-miR-212	-0.17
mmu-miR-363	-0.13	mmu-miR-26a	-0.17
mmu-miR-186	-0.11	Ctl mimic	-0.15
mmu-miR-210	-0.06	mmu-miR-10a-5p	-0.14
mmu-miR-19b	-0.06	mmu-miR-130b	-0.14
mmu-miR-191	-0.04	mmu-miR-186	-0.10
mmu-miR-500	-0.03	mmu-miR-92a	-0.10
mmu-miR-199a-5p	-0.01	mmu-miR-132	-0.04
mmu-miR-29a	-0.01	mmu-miR-361	-0.02
mmu-miR-15b	0.01	mmu-miR-126-3p	0.02
mmu-miR-194	0.04	mmu-miR-421	0.04
mmu-miR-15a	0.04	mmu-miR-532-5p	0.04
mmu-miR-322	0.06	mmu-miR-21	0.07
mmu-miR-21	0.10	mmu-miR-297a	0.07
mmu-miR-16	0.11	mmu-miR-200c	0.08
mmu-miR-33	0.13	mmu-miR-183	0.11
mmu-miR-92a	0.13	mmu-miR-500	0.13
mmu-let-7g	0.17	mmu-miR-142-5p	0.14
mmu-miR-328	0.17	mmu-miR-467c	0.16
mmu-miR-183	0.18	mmu-miR-328	0.19
mmu-miR-142-5p	0.18	mmu-miR-22	0.21
mmu-miR-296-5p	0.22	mmu-miR-144	0.22
mmu-miR-212	0.26	mmu-miR-342-3p	0.22
mmu-miR-744	0.29	mmu-miR-340-5p	0.31
mmu-miR-199a-3p	0.29	mmu-miR-194	0.33
mmu-miR-700	0.31	mmu-miR-206-3p	0.33
mmu-miR-146a	0.31	mmu-miR-23a	0.33
mmu-miR-22	0.36	mmu-miR-141	0.36
mmu-miR-150-5p	0.36	mmu-miR-378	0.36
mmu-miR-467a	0.38	mmu-miR-24	0.39
mmu-miR-148a	0.40	mmu-miR-26b	0.40
mmu-miR-125a-5p	0.43	mmu-miR-363	0.40
mmu-miR-128	0.45	mmu-miR-298	0.45
mmu-miR-132	0.48	mmu-miR-182	0.46

miRNA	CCR6 Z score	miRNA	IL-17A Z score
mmu-miR-148b	0.48	mmu-miR-125a-5p	0.48
mmu-miR-32	0.50	mmu-miR-142-3p	0.48
mmu-miR-26b	0.50	mmu-miR-466g	0.49
mmu-miR-466l	0.50	mmu-miR-652	0.49
mmu-miR-151-3p	0.55	mmu-miR-199a-5p	0.55
mmu-miR-342-3p	0.55	mmu-miR-148b	0.74
mmu-miR-31	0.57	mmu-miR-147	0.80
mmu-miR-298	0.57	mmu-miR-720	0.80
mmu-miR-28	0.61	mmu-miR-27a	0.81
mmu-miR-467c	0.62	mmu-miR-155	0.84
mmu-miR-19a	0.68	mmu-miR-148a	0.89
mmu-miR-720	0.71	mmu-miR-25	0.93
mmu-miR-96	0.71	mmu-miR-27b	0.93
mmu-miR-200c	0.71	mmu-miR-296-5p	0.96
mmu-miR-193	0.75	mmu-miR-193	1.01
mmu-miR-144	0.75	mmu-miR-375	1.04
mmu-miR-532-5p	0.78	mmu-miR-296-3p	1.09
mmu-miR-27a	0.80	mmu-miR-9	1.16
mmu-miR-27b	0.84	mmu-miR-96	1.25
mmu-miR-142-3p	0.85	mmu-miR-32	1.36
mmu-miR-26a	0.85	mmu-miR-210	1.65
mmu-miR-296-3p	0.99	mmu-miR-423-5p	1.88
mmu-miR-23a	1.01	mmu-miR-31	1.92
mmu-miR-17	1.17	mmu-miR-467a	2.06
mmu-miR-20b	1.26	mmu-miR-93	2.10
mmu-miR-93	1.28	mmu-miR-29a	2.16
mmu-miR-106b	1.29	mmu-miR-20a	2.29
mmu-miR-20a	1.31	mmu-miR-19b	2.35
mmu-miR-141	1.36	mmu-miR-20b	2.57
mmu-miR-130b	1.42	mmu-miR-17	2.59
mmu-miR-301a	1.45	mmu-miR-106a	2.68
mmu-miR-106a	1.52	mmu-miR-19a	2.76
mmu-miR-155	1.61	mmu-miR-106b	2.80

Table 2. Summary of Z scores for CCR6, RORγt, and IL-17A in primary human T cell screen with individual miRNA inhibitors (inh.) or miRNA family inhibitors (FI)

CCR6 Z Scores		RORγt Z scores		IL-17A Z score	
miRNA	**Z score**	**miRNA**	**Z score**	**miRNA**	**Z score**
hsa-miR-598-3p inh.	-4.08	hsa-miR-598-3p inh.	-2.02	hsa-miR-598-3p inh.	-1.71
hsa-miR-451a inh.	-2.87	hsa-miR-590-3p inh.	-1.85	hsa-miR-31-5p inh.	-1.54
hsa-miR-28-5p inh.	-2.19	hsa-miR-451a inh.	-1.60	hsa-miR-494-3p inh.	-1.44
hsa-miR-31-5p inh.	-1.89	hsa-miR-340-5p inh.	-1.45	hsa-miR-451a inh.	-1.35
hsa-miR-494-3p inh.	-1.65	hsa-miR-423-5p inh.	-1.37	hsa-miR-361-5p inh.	-1.30
hsa-miR-340-5p inh.	-1.52	hsa-miR-769-5p inh.	-1.36	hsa-miR-126-3p inh.	-1.23
hsa-miR-425-5p inh.	-1.33	hsa-miR-31-5p inh.	-1.24	hsa-miR-142-3p inh.	-1.05
hsa-miR-21-5p inh.	-1.24	hsa-miR-155-5p inh.	-1.20	hsa-miR-361-3p inh.	-1.01
hsa-miR-342-5p inh.	-1.22	hsa-miR-126-3p inh.	-1.16	hsa-miR-223-3p inh.	-1.01
hsa-miR-590-3p inh.	-1.13	hsa-miR-28-5p inh.	-1.14	hsa-miR-769-5p inh.	-0.99
hsa-miR-361-3p inh.	-1.11	hsa-miR-223-3p inh.	-1.12	hsa-miR-28-5p inh.	-0.99
hsa-miR-1247-5p inh.	-1.02	hsa-miR-142-3p inh.	-1.10	hsa-miR-590-3p inh.	-0.97
hsa-miR-142-3p inh.	-0.94	hsa-miR-345-5p inh.	-1.04	hsa-miR-454-5p inh.	-0.93
hsa-miR-191-5p inh.	-0.92	hsa-miR-484 inh.	-1.02	hsa-miR-423-5p inh.	-0.92
hsa-miR-361-5p inh.	-0.90	hsa-miR-454-5p inh.	-0.97	hsa-miR-185-5p inh.	-0.88
hsa-miR-150-5p inh.	-0.87	hsa-miR-190 FI	-0.94	hsa-miR-340-5p inh.	-0.83
hsa-miR-155-5p inh.	-0.81	hsa-miR-361-5p inh.	-0.91	hsa-miR-345-5p inh.	-0.81
hsa-miR-126-3p inh.	-0.76	hsa-miR-494-3p inh.	-0.89	hsa-miR-191-5p inh.	-0.81
hsa-miR-128 inh.	-0.71	hsa-miR-425-5p inh.	-0.86	hsa-miR-425-5p inh.	-0.77
hsa-miR-484 inh.	-0.68	hsa-miR-21-5p inh.	-0.80	hsa-miR-132 FI	-0.66
hsa-miR-30 FI	-0.67	hsa-miR-185-5p inh.	-0.77	hsa-miR-155-5p inh.	-0.61
hsa-miR-223-3p inh.	-0.67	hsa-miR-128 inh.	-0.64	hsa-miR-652-3p inh.	-0.57
hsa-miR-132 FI	-0.60	hsa-miR-361-3p inh.	-0.63	hsa-miR-484 inh.	-0.57
hsa-miR-190 FI	-0.60	hsa-miR-192 FI	-0.60	hsa-miR-342-5p inh.	-0.53
hsa-miR-126-5p inh.	-0.60	hsa-miR-22-3p inh.	-0.56	hsa-miR-192 FI	-0.53
hsa-miR-421 inh.	-0.59	hsa-miR-17 FI	-0.46	hsa-miR-133 FI	-0.46
hsa-miR-339-5p inh.	-0.57	hsa-miR-191-5p inh.	-0.42	hsa-let-7 FI	-0.40
hsa-miR-362-5p inh.	-0.54	hsa-miR-140-5p inh.	-0.35	hsa-miR-21-5p inh.	-0.39
hsa-miR-652-3p inh.	-0.48	hsa-miR-133 FI	-0.35	hsa-miR-95-3p inh.	-0.39
hsa-miR-133 FI	-0.38	hsa-miR-150-5p inh.	-0.35	hsa-miR-17 FI	-0.28
hsa-miR-345-5p inh.	-0.38	hsa-miR-132 FI	-0.26	hsa-miR-1247-5p inh.	-0.27
hsa-miR-769-5p inh.	-0.36	hsa-miR-95-3p inh.	-0.26	hsa-miR-629-5p inh.	-0.25
hsa-miR-454-5p inh.	-0.36	hsa-miR-339-5p inh.	-0.23	hsa-miR-18 FI	-0.22
hsa-miR-342-3p inh.	-0.21	hsa-miR-18 FI	-0.21	hsa-miR-126-5p inh.	-0.19
hsa-miR-423-5p inh.	-0.16	hsa-miR-652-3p inh.	-0.17	hsa-miR-660-5p inh.	-0.07
hsa-miR-19 FI	-0.11	hsa-miR-199 FI	-0.15	hsa-miR-190 FI	-0.05
hsa-miR-99 FI	-0.06	hsa-miR-140-3p inh.	-0.12	hsa-miR-26 FI	-0.04

miRNA	Z score	miRNA	Z score	miRNA	Z score
hsa-miR-18 FI	-0.05	hsa-miR-1247-5p inh.	-0.11	hsa-miR-140-3p inh.	-0.03
hsa-let-7 FI	0.00	hsa-miR-194-5p inh.	0.00	hsa-miR-362-5p inh.	0.00
hsa-miR-194-5p inh.	0.03	hsa-miR-101 inh.	0.08	hsa-miR-140-5p inh.	0.01
hsa-miR-95-3p inh.	0.06	hsa-miR-126-5p inh.	0.11	hsa-miR-197-3p inh.	0.01
hsa-miR-197-3p inh.	0.14	hsa-miR-146 FI	0.13	hsa-miR-150-5p inh.	0.05
hsa-miR-140-5p inh.	0.19	hsa-miR-362-5p inh.	0.13	hsa-miR-103 FI	0.12
hsa-miR-22-3p inh.	0.19	hsa-miR-29 FI	0.16	hsa-miR-221/222-3p inh.	0.12
hsa-miR-29 FI	0.21	hsa-miR-99 FI	0.20	hsa-miR-128 inh.	0.18
hsa-miR-660-5p inh.	0.22	hsa-miR-421 inh.	0.20	hsa-miR-421 inh.	0.21
hsa-miR-140-3p inh.	0.25	hsa-let-7 FI	0.23	hsa-miR-22-3p inh.	0.21
hsa-miR-629-5p inh.	0.29	hsa-miR-30 FI	0.35	hsa-miR-27 FI	0.23
hsa-miR-185-5p inh.	0.32	hsa-miR-26 FI	0.39	hsa-miR-148 FI	0.25
hsa-miR-192 FI	0.38	hsa-miR-148 FI	0.39	hsa-miR-99 FI	0.28
hsa-miR-101 inh.	0.40	hsa-miR-660-5p inh.	0.40	hsa-miR-23 FI	0.31
hsa-miR-23 FI	0.48	hsa-miR-197-3p inh.	0.50	hsa-miR-101 inh.	0.35
hsa-miR-26 FI	0.51	hsa-miR-629-5p inh.	0.50	hsa-miR-378a-3p inh.	0.39
hsa-miR-186-5p inh.	0.51	hsa-miR-19 FI	0.51	hsa-miR-200 FI	0.41
hsa-miR-148 FI	0.54	hsa-miR-130 FI	0.55	hsa-miR-342-3p inh.	0.42
hsa-miR-200 FI	0.54	hsa-miR-342-5p inh.	0.55	hsa-miR-194-5p inh.	0.45
hsa-miR-27 FI	0.54	hsa-miR-221/222-3p inh.	0.59	hsa-miR-199 FI	0.56
hsa-miR-221/222-3p inh.	0.56	hsa-miR-200 FI	0.68	hsa-miR-125 FI	0.64
hsa-miR-103 FI	0.59	hsa-miR-23 FI	0.72	hsa-miR-130 FI	0.76
hsa-miR-17 FI	0.70	hsa-miR-342-3p inh.	0.73	hsa-miR-339-5p inh.	0.76
hsa-miR-199 FI	0.73	hsa-miR-186-5p inh.	0.77	hsa-miR-146 FI	0.79
hsa-miR-24-3p inh.	0.76	hsa-miR-378a-3p inh.	0.80	hsa-miR-10 FI	0.84
hsa-miR-142-5p inh.	0.81	hsa-miR-210-3p inh.	0.84	hsa-miR-30 FI	0.87
hsa-miR-532-5p inh.	0.83	hsa-miR-27 FI	0.92	hsa-miR-19 FI	1.29
hsa-miR-378a-3p inh.	0.83	hsa-miR-320 FI	0.93	hsa-miR-25+32 FI	1.29
hsa-miR-146 FI	0.84	hsa-miR-374 FI	0.93	hsa-miR-320 FI	1.29
hsa-miR-10 FI	0.89	hsa-miR-142-5p inh.	1.01	hsa-miR-142-5p inh.	1.35
hsa-miR-181 FI	0.89	hsa-miR-181 FI	1.05	hsa-miR-186-5p inh.	1.40
hsa-miR-125 FI	0.92	hsa-miR-24-3p inh.	1.05	hsa-miR-15 FI	1.54
hsa-miR-25+32 FI	0.94	hsa-miR-125 FI	1.11	hsa-miR-324-3p inh.	1.57
hsa-miR-130 FI	1.06	hsa-miR-103 FI	1.15	hsa-miR-574-5p inh.	1.77
hsa-miR-15 FI	1.09	hsa-miR-15 FI	1.57	hsa-miR-210-3p inh.	1.80
hsa-miR-374 FI	1.13	hsa-miR-25+32 FI	1.63	hsa-miR-532-5p inh.	1.85
hsa-miR-320 FI	1.25	hsa-miR-10 FI	2.08	hsa-miR-29 FI	2.14
hsa-miR-324-3p inh.	1.32	hsa-miR-324-3p inh.	2.21	hsa-miR-181 FI	2.19
hsa-miR-574-5p inh.	1.38	hsa-miR-532-5p inh.	2.37	hsa-miR-374 FI	2.25
hsa-miR-210-3p inh.	1.43	hsa-miR-574-5p inh.	2.77	hsa-miR-24-3p inh.	2.64

Chapter 4

Discussion, Conclusions, and Future Directions

Summary

Regulation of immune cell fate and function is crucial to the development of a properly functioning immune system. When lineage-fate decisions go awry, this can lead to improper immune responses that result in immunological disorders such as chronic inflammatory and allergic diseases, autoimmune diseases, and asthma. Asthma in particular results from a complex interaction between the environment and adaptive and innate immune systems. While much work has been done to study the Th2 responses in asthma, only recently has a role for Th17 cells been identified in this disease. It remains unclear whether and how these cells contribute to allergic asthma, and whether they may be responsible for the pathogenesis of a subset of treatment-resistant asthma that lacks clear evidence for Th2 cell involvement[127,128].

To better understand these types of diseases, it is important to characterize the molecular pathways regulating the effector functions of T helper cells, the main conductors of the adaptive immune response. MicroRNAs have recently emerged as critical mediators regulating the differentiation behavior and immune function of T helper cells. Their fine-tuning activity is particularly important in the regulation of T helper cells because the amount of competing regulatory factors present can dictate the lineage fate of activated T cells. In other words, small changes in gene expression can have large impacts on these decisions. Our work presented here aimed to provide a better understanding of the miRNA regulation of Th17 cell differentiation. From work like this we hope to gain a better appreciation of their dysregulation in diseases such as asthma.

We provide a comprehensive study in Chapter 2 dissecting the inhibitory role of one miRNA on Th17 cell differentiation *in vitro* and on Th17 responses *in vivo*. We analyzed the spectrum of effects of miRNAs within the miR-17~92 cluster on Th17 differentiation and through this work uncovered a distinct inhibitory function for miR-18a. Our detailed studies further characterized the role of miR-18a as a negative regulator of Th17 cell differentiation and immune function. Specifically, our *in vitro* studies showed an increase in $CCR6^{+}ROR\gamma t^{+}$ cells from $CD4^{+}$ T cells lacking miR-18a. Additionally, inhibiting the endogenous activity of miR-18a led to increased expression of CCR6 and RORγt in both human and mouse T cells. To uncover the functional significance of this finding, we performed experiments employing mouse models of airway inflammation and found increased Th17 cell frequencies in the lungs and lung-draining lymph nodes of miR-18 knockout mice. We also determined the network of target genes that mediate the miR-18 regulation of Th17 cell differentiation. miR-18a directly targets at least three transcription factors, *Smad4*, *Hif1a*, and *Rora*, all known to have a regulatory role in Th17 biology. These findings can be integrated into the already existing network of known regulatory factors modulating Th17 cells development and effector function[9,40,95,129].

In chapter 3, we used a technology that allowed for efficient and reliable screening of individual miRNAs in murine and human primary Th17 cells. Using this technique, we provide a broad systematic view of the miRNA regulation of Th17 cells. Our findings revealed novel miRNAs and families of miRNAs that can enhance and inhibit Th17 cells that were not previously studied in this context. The miRNA contribution to the

regulation of Th17 cell differentiation has been well defined by these studies and builds upon the existing literature. This work also creates opportunities for new studies to follow-up on interesting top candidate miRNAs to characterize them further to determine if there is a complementary phenotype predicted from these screening studies. Future experiments should also aim to identify the target genes of this miRNA-mediated regulation of Th17 differentiation. Together by analyzing the spectrum of effects on Th17 differentiation of all miRNAs expressed in T cells, and through the identification of target genes, we can gain a better understanding of gene expression networks that regulate Th17 cell differentiation. Through this, we may uncover novel avenues for therapy in Th17-driven inflammatory diseases like asthma.

Future Directions

One common miRNA cluster, different functional outcomes

The miR-17~92 cluster is important for the normal development of the immune system[130]. miRNAs from this cluster regulate the differentiation and function of several subsets of T helper cells, including Th17 cells. The miR-17~92 cluster is transcribed as a polycistronic transcript resulting in six individual mature miRNAs (miR-17, -18a, -19a, -19b, -20a, and -92a). There are also two miRNA cluster paralogs in mammals, the miR-106a~363 cluster and the miR-106b~25 cluster. miRNAs produced from all three of these paralogous clusters can be grouped together into four miRNA families based on similar seed sequences, the miR-17 family, miR-18 family, miR-19 family, and miR-25 family.

Studies using mice deficient in these miRNA clusters highlighted the potential functional redundancy of miRNAs within these homologous clusters when they showed the synergistic effects of deleting both the miR-17~92 and the miR-106b~25 clusters[131]. Additionally, deleting miR-106a~363 didn't have any further effect on the phenotype most likely due to its low abundance. Through our work with activity sensors, we know there is much residual activity of the miR-17 and miR-92 family miRNAs in miR-17~92 knockout mice presumably from miRNAs produced from these paralogous clusters. In contrast, most of the miR-19 family activity and basically all of the miR-18 family activity is accounted for by this miR-17~92 cluster. This is quite important, especially when studying phenotypic effects using miR-17~92 deficient cells. To truly isolate the limiting effects of individual miRNAs within these clusters, residual activity of similar functioning miRNA family members must be considered, since sequence homology suggests that miRNAs from the same family have similar target genes and thus potentially redundant functional effects. Additionally, miRNAs from different miRNA families produced from the same primary transcript can ultimately have antagonistic effects, such as in the case of the miR-17~92 cluster miRNAs. To help sort this out, more experiments should be done using the triple cluster knockout mice, which essentially are the only genetic knockout mice lacking all miR-17 and miR-92 miRNA family activity[72].

Even though miRNAs within the same family have been shown to provide distinct functions, it will still prove useful to study the effects of deleting each miRNA within the miR-17~92 cluster. Recently, novel transgenic mice lacking individual miRNAs within the miR-17~92 cluster were generated and can be used for this purpose[103]. These types

of studies are particularly important to help clarify the existing literature. Two recently published studies showed opposite functional effects for the miR-17 family miRNAs, miR-17 and miR-20b[74,75]. The Liu et al. group showed that miR-17 had a positive regulatory role in Th17 differentiation while the Zhu et al. group demonstrated an inhibitory role of miR-20b in Th17 cells. Future work should utilize mice deficient in all three miRNA clusters as well as novel mice with targeted deletions of each miRNA from the miR-17~92 cluster to help clarify the biological relevance of miR-17~92 miRNAs in various T helper cell subsets.

Intuitively, it might make sense for miRNAs transcribed from the same primary transcript to have similar functions, which is certainly evidenced by the role of miR-19 and miR-17 in promoting oncogenesis[68,69]. However, it is not unprecedented for miRNAs from this cluster to have opposing roles in the immune system. An example of these antagonistic effects was highlighted when overexpression of miR-92 in neonatal mice promoted erythroleukemia by targeting *Gata1* and *p53* but the phenotype was not recapitulated with retroviral overexpression of the entire miR-17~92 cluster[67]. In Th17 cells, miR-17~92 miRNAs have opposing roles. In addition to showing a positive regulatory role for miR-17, the Liu et al. group also showed that miR-19b targeted IKZF4 to promote Th17 cells[75]. Additionally, we show here that miR-18a has a distinct inhibitory role regulating Th17 cells. One mechanism for how miRNAs like miR-18 can exert unique functional effects different from that of miRNAs transcribed from the same cluster is through regulatory events that result in changes in miRNA expression.

Post-transcriptional regulation of miR-17~92 expression

Unlike most other miRNAs, the miRNAs of the miR-17~92 cluster are upregulated during T cell activation[109]. Our data in Chapter 2 suggests that there is differential upregulation of miRNAs from this cluster, resulting in large differences in expression levels during the activation process. This is particularly important because dynamic changes in miRNA expression can have critical effects on the outcome of the cell fate. It is certainly an interesting observation that needs to be parsed out further, and begs the obvious question of what accounts for the differential regulation of miRNAs within this cluster that allows them to exert unique or shared functional effects in different cellular contexts.

We know that miRNAs themselves can be regulated at multiple levels, including transcriptionally and post-transcriptionally through differential miRNA processing at several potential points in the miRNA biogenesis pathway. For miRNA clusters where multiple miRNAs are transcribed from the same primary transcript, the latter may be particularly important. Work done in ES cells has provided some insight into the regulation of the miR-17~92 cluster during biogenesis that is important to the expression of all mature miRNAs from the cluster except miR-92a[110]. Alternatively, miRNAs like miR-18a can be differentially regulated through the stabilization of pri-miR transcripts by RNA binding proteins, like hnRNPA1. Work by Guil and Cáceres showed that hnRNPA1 binds to pri-miR-18a and facilitates processing by Drosha[132]. It still remains to be determined what factors are important for the drastic upregulation of miR-18 in our study. The alluring possibility that hnRNPA1 or other RNA binding proteins may

contribute to the differential regulation of miRNAs within the miR-17~92 cluster by altering miRNA processing in T cells requires further consideration.

Complexity of miR-17~92 regulatory networks

The fact that miR-18 had antagonistic effects on Th17 cells compared to other members of the miR-17~92 cluster is particularly surprising when considering that miR-17 and miR-18a differ by only one nucleotide in their seed sequence, the key determinant in the recognition of target mRNAs. Partly because of this reason miR-18a hasn't been given considerable attention compared to other cluster miRNAs like miR-17. The dynamic signal-inducible expression of miR-18a together with a small shift in the repertoire of target genes is certainly one way miR-18 has evolved to make the miR-17~92 cluster a negative regulator of Th17 differentiation. Besides differential regulation of miRNAs in Th17 cells, the network of genes targeted by each individual miRNA and ultimately their availability in specific cellular contexts is another layer of regulation of miRNA-mediated effects on T helper biology. While miR-17 and miR-18a in particular share many common target genes, identifying unique target genes among miR-17 versus miR-18a is certainly an enticing future direction that could provide clarity in this context.

Another way these miRNAs may exert their antagonistic effects may have to do with the kinetics of their effects during Th17 differentiation (Fig. 1). When a naïve $CD4^+$ T cell develops into a Th17 cell, activation signals induce the Th17 cell to produce its effector cytokine, IL-17. This process can be blocked by factors like PTEN and miRNAs like miR-17 can promote IL-17 production through its inhibition of PTEN[75]. miR-18a,

however, may repress target genes that have effects at a different time point in the Th17 differentiation process, potentially further upstream to prevent differentiation in the first place. This possibility is in line with our work that showed miR-18a directly targeted *Smad4*, a component of TGFβ signaling pathway, *Hif1a* and *Rora*, key transcription factors that play important roles in the generation of Th17 cells[35,36,65,96]. Thus, miR-18a may be particularly important in regulating the initial induction of a naïve $CD4^+$ T cell to differentiate into a $CCR6^+RORγt^+$ cell that can produce IL-17 whereas the dominant effect of miR-17 may be to regulate signaling molecules that allow the $CCR6^+RORγt^+$ Th17 cell to deploy its effector function. Of course this is an over-simplification. We know there are many other identified miRNAs other than the miR-17~92 cluster that also mediate the regulation of Th17 cells that aren't accounted for with this type of explanation. This is why it is critical to not only identify the important mediators in this process but to start to build more global maps to better connect these networks.

miR-17 and miR-18a can also work together to regulate both shared and unique targets, and can have additive effects by targeting genes with overlapping functions. In a focused retesting of selected miR-17~92 predicted target genes, we included siRNAs against the mRNAs that encode RORγt, a known miR-17 target, and RORα, which we previously showed to be targeted by all 4 miRNA families in the miR-17~92 cluster, including miR-18a (Fig. 2). As expected, siRNAs against *Rorc* strongly inhibited Th17 cell differentiation, and siRNAs against *Rora* had a much more modest effect. However, combining siRNAs against both factors produced the greatest reduction in CCR6 and IL-17 expression, reflecting the redundancy of these factors[35], and illustrating one of the

ways that miR-17 and miR-18 may coordinately regulate the Th17 cell gene expression program. Taken together, our results and prior reports illuminate the complex regulation of Th17 differentiation and cytokine production, and indicate that the functional outcome of changes in miR-17~92 expression result from the integration of direct effects of each miRNA family on multiple transcriptional and signaling regulators.

The physiological role of miR-18a induction upon T cell stimulation

In T follicular helper (T_{FH}) cells, the miR-17~92 cluster restrains the expression of subset-inappropriate genes that are normally associated with Th17 cells, including *Ccr6*, *Il1r1*, *Il1r2*, the cytokine *Il22,* and the transcription factor *Rora*[34]. Although all 4 miRNA families represented in the cluster directly target conserved regions in the *Rora* 3'UTR, it still remains to be determined which individual miRNAs from the miR-17~92 cluster specifically repressed this exhibited increase in Th17-associated genes. One can imagine that at homeostasis, miR-18 functions to repress multiple genes involved in the regulatory network governing Th17 differentiation. As such, miR-18 may act as a buffer to prevent the expression of lineage-specific genes in inappropriate cellular contexts. In the environmental context of appropriate T_{FH}-inducing signals, miR-18 may aid in this process by inhibiting genes responsible for Th17 development allowing for robust T_{FH} cell differentiation. In situations that favor Th17 development, the Th17-inducing signals may be enough to overpower the miR-18 mediated inhibition of Th17 transcription factors allowing for fully differentiated Th17 cells. However, if miR-18 is not present, inappropriate gene signatures may be turned on in incorrect cell types leading to aberrant gene expression and cytokine production.

Alternatively, similar to miR-181 in human memory T cells acting through a self-regulatory mechanism[126], miR-18 could be induced in Th17 cells to dampen Th17 responses. This type of negative feedback can be crucial to prevent damage due to prolonged or excessive activation. Lastly, of course we cannot rule out the possibility that miR-18 is upregulated in activated T cells for some other unexplored reason that is independent from T helper cell differentiation. Sorting through the many unanswered questions posed here will help to clarify the major regulatory capacity provided by the individual miRNAs co-transcribed from the miR-17~92 cluster.

Beyond Th17 cells: miR-18 regulation of other (innate) immune cells?

Up until now, miR-18 has been studied in a wide range of biological processes including vascular biology[133], spermatogenesis[134], and various forms of cancer[135-141]. This is the first time however that miR-18 has been shown to be an important mediator of T cell biology. Several miRNAs, including miRNAs from the miR-17~92 cluster, can have effects on multiple T helper cell subsets. miR-19 has been shown to promote Th1, Th2, and Th17 differentiation[73,75,76]. miRNAs from the miR-17~92 cluster were also shown to promote robust T_{FH} cell differentiation[34]. Thus, it is not hard to imagine that miR-18 may also mediate the regulation of other effector T cell subsets.

Our finding that miR-18 deficiency increases the frequencies of tissue Th17 cells *in vivo* in airway inflammation models does not preclude this possibility. Studies using OVA-specific OTII transgenic miR-$18^{\Delta/\Delta}$ or control T cells that have been polarized under

Th17 conditions and transferred into wildtype mice can help to clarify this. These established cell transfer models of airway inflammation can test the role of Th17 cells while avoiding complicating factors from other T helper cell subtypes that may also require miR-18 for their optimal function (e.g. Th2 cells and regulatory T cells). Similarly, the effect of miR-18 on other immune cells should also be considered.

While we obtained similar results showing increased $CCR6^{+}ROR\gamma t^{+}$-expressing Th17 cells in the miR-$18^{\Delta/\Delta}$ mice *in vitro* and *in vivo,* the phenotypic effects we observe on IL-17A expression *in vivo* was not recapitulated *in vitro.* This leaves us to wonder what additional factors may contribute to the miR-18 regulation of Th17 effector cytokine production particularly *in vivo.* One can speculate that the regulation of miR-18 could be even further amplified in an *in vivo* setting. Alternatively, maybe other miR-17~92 miRNAs with opposing roles to miR-18a in Th17 cells, such as miR-17, do not contribute as much in certain biological contexts. One obvious possibility that must be considered is the role that miR-18a may play in other immune cells, particularly in the innate immune system, where there are several innate cellular sources of IL-17. Because the novel transgenic mice deficient in miR-18a used in these studies (miR-$18^{\Delta/\Delta}$) are full knockout mice[103] as opposed to having a conditional deletion in CD4-expressing T cells, the possibility that miR-18 regulates these other IL-17 producing immune cells should be examined further.

Innate immunity is critical for quick, immediate responses and important for the development of a lasting adaptive immune response. There is already work showing the

importance of the miR-17~92 cluster in invariant natural killer T (iNKT) cells which are immune cells similar to T lymphocytes but respond in an innate-like manner[142]. Using a reporter mouse, one study showed that iNKT cells are positioned inside the lung vasculature and can extravasate into the intraparenchymal lung tissue to promote inflammation upon antigen stimulation[143]. There are three types of iNKT cell subsets, including Th17-like iNKT cells[144]. Their master regulator RORγt is required for their development and they also express CCR6[145,146]. This subset produces IL-17A, IL-21, and IL-22 and is commonly identified as a $CD4^{-}NK1.1^{-}$ population[147148]. They have also been shown to contribute to neutrophilia in the lungs of mice exposed to allergen[149].

Other innate sources of IL-17 include a subset of γδ T cells and group 3 innate lymphoid cells. The IL-17-producing subset of γδ T cells express RORγt, CCR6, and the IL-23R[150]. They are activated by both IL-1β and IL-23, and can also produce the IL-21 and IL-22 cytokines[151]. They have also been shown to promote the $CD4^{+}$ Th17 production of IL-17A and IL-17F in an amplification feedback loop highlighting the influence of innate immunity on strengthening the adaptive immune response. There is also evidence that these IL-17-producing γδ T cells can reside in the lungs[152] and thus contribute to airway inflammation.

Innate lymphoid cells (ILCs) were originally identified at barrier sites and are another IL-17 source in the lungs. Similar to Th17 cells, the ILCs can be categorized into three major subgroups based on characteristic surface markers, transcription factors, and effector cytokines: Group 1 ILCs (ILC1), Group 2 ILCs (ILC2), and Group 3 ILCs

(ILC3)[153]. ILC3 cells also express RORγt and produce cytokines commonly associated with Th17 cells including IL-17A, IL-17F, IL-22, GM-CSF, and TNF[154]. They can be further separated into $CCR6^+$ and $CCR6^-$ subsets. They play roles in normal tissue homeostasis, combatting bacterial infections, and promoting inflammation[155]. Contrary to what might be expected based on other IL-17 studies in the lungs, one study showed that ILC3s actually reduced the airway hypersensitivity caused by type 2 inflammation induced by ILC2s[156]. One notable difference between ILC3s and Th17 cells is that unlike Th17 cells, ILC3s do not express RORα and in fact, it is the ILC2 subset that requires the RORα transcription factor for their development[157].

Ultimately, iNKT cells, ILC3s, and γδ T cells all produce the IL-17 cytokine and are expressed in the airways of mice. Because of this, it is quite possible that miR-18a is a limiting factor in one or more of these immune cell subsets and this should be explored further in the future.

Beyond studying the role of miR-18 in other immune cells, there are many avenues of investigation still left open to explore from our own work identifying miR-18 as a negative regulator of Th17 cells. In addition to regulating IL-17 *in vivo,* miR-18 may regulate other Th17-associated inflammatory cytokines, including IL-22, GM-CSF and TNF in certain biological contexts. The possibility that miR-18 regulates genes that promote IL-22 production is not hard to imagine since IL-22 was one of the genes upregulated in microarray studies in T_{FH} cells deficient in miR-17~92[34]. Additionally, *Hif1a*, one of the direct targets of miR-18a was found to regulate IL-22 production[158]. As

such, future experiments should focus on the physiological role of miR-18 in Th17-driven models where IL-17 and/or IL-22 are important mediators of disease pathogenesis.

In Conclusion

MicroRNAs are important small RNA mediators of gene regulation at the post-transcriptional level. miRNA-mediated regulation is critical to the proper development and function of the immune system. Much work has been done identifying a variety of functional roles for miRNAs in regulating biological processes in both innate and adaptive immune responses[48,159]. Over the last decade, there has been substantial progress specifically in the field of miRNA-mediated regulation of $CD4^+$ T helper cells[160].

$CD4^+$ T helper cells are categorized into distinct subsets (e.g. Th1, Th2, Th17, T_{FH}, TReg) that produce characteristic cytokines and express lineage-defining genes that can all be regulated by transcription factors and the surrounding cytokine milieu as well as through epigenetic alterations. miRNAs play such a crucial role as mediators in the differentiation process because they can modulate epigenetic factors and regulate the expression of these cytokines and transcription factors. Furthermore, miRNAs are well suited as post-transcriptional regulators of T helper cell differentiation and immune function because cell fate decisions can be greatly impacted by just minor changes in gene expression. In T cell biology, miRNAs not only regulate the differentiation and function of various $CD4^+$ T helper cell subsets but can also have an effect on processes like TCR signaling and T cell proliferation and survival[42].

$CD4^+$ T cells devoid of endogenous miRNAs exhibit decreased T cell proliferation and survival, and unexpectedly, these cells undergo unrestrained differentiation into effector T cell subsets and can produce aberrant cytokines[97,161-163]. This tells us that miRNAs play important roles in T cell survival and in maintaining the naïve state of $CD4^+$ T cells. Specifically, these miRNA-deficient $CD4^+$ T cells can differentiate into Th1 and Th2 cell subsets but cannot develop into T_{FH} cell subsets[108]. Work by others in Dicer knockout $CD4^+$ T cells[97] and our work shown here in Chapter 3 in DGCR8-deficient $CD4^+$ T cells show that while Th17 cells can be generated, their development is impaired compared to control (miRNA sufficient) $CD4^+$ T cells. This suggests that specific miRNAs are important to induce and/or sustain the Th17 cell subset.

To add to the complexity, while miRNAs mediate their regulatory effects through the repression of their target genes, miRNAs themselves can also be regulated[164]. The regulation of miRNAs can occur during several points in the miRNA biogenesis pathway, from signals that regulate their transcription to processes that stabilize or de-stabilize their processing[165]. Their functional effects can also be regulated after maturation by the availability of their target mRNA transcripts[166]. Upon T cell activation, most miRNAs are globally downregulated while a few specific miRNAs become quickly upregulated to exert their functions during the transition to the effector T helper state[109]. Understanding the events that regulate miRNAs and contribute to changes in miRNA profiles in specific cell lineages during T helper differentiation is important to deciphering the functional relevance of miRNA-mediated gene regulation.

Our work makes significant contributions to the study of miRNA-mediated regulation of Th17 cells. Specifically we show: (1) Th17 cells are differentially regulated by individual miRNAs produced from one common cluster, the miR-17~92 cluster; (2) An under-appreciated miRNA, miR-18a, within the cluster has a unique inhibitory function distinct from effects of all other miRNAs transcribed from the same polycistronic cluster; (3) miR-18a is dynamically upregulated during T cell activation, allowing it to implement its effects during a critical time in T cell fate decisions; (4) miR-18a mediates the regulation of Th17 cells by targeting multiple transcription factors involved in the development of the Th17 lineage, *Smad4*, *Hif1a*, and *Rora*; (5) miR-18a has functional consequences for Th17 responses in airway inflammation. Together, this work provides a comprehensive detailed analysis identifying a novel role for miR-18a as a negative regulator of Th17 differentiation. These results are important to our understanding of the molecular events governing Th17 cell fate and function and importantly, elucidate general principles of the evolution and regulation of miRNA clusters. Although the linear miRNA to target to function connection is much oversimplified, identifying specific target to function interactions by individual miRNAs is still crucial to advance the field and further develop our understanding of how miRNAs and their targets integrate into regulatory networks.

Our work also provides further support that miRNAs are important for the proper development and/or maintenance of the Th17 effector lineage, as exemplified by the impaired Th17 differentiation of miRNA-deficient $CD4^+$ T cells. We contribute to the

existing literature providing additional support of some previously identified miRNAs that regulate Th17 cells. Importantly, we also identify novel miRNAs that can regulate Th17 cells that were not previously studied in this cellular context. The multiple miRNAs that inhibited or enhanced Th17 cells help define the broad scope of miRNA-mediated regulation of Th17 cells. While more detailed characterization still needs to be performed, this type of work can be integrated into our existing frameworks for the known miRNA regulatory networks of murine and human Th17 cells.

While we and others have identified miRNAs that regulate Th17 responses, there is still much to learn about the regulatory networks controlling Th17 cell development and function and the resulting impact on diseases like asthma. Future work should continue to identify miRNA functions in T cells and the effects from changes in the expression of these miRNAs and their targets during the differentiation of T helper subsets. This is crucial to broaden our understanding of their normal function at homeostasis and the resulting functional consequences of miRNA dysregulation in disease. To expand upon these findings, work should be done to integrate current literature on single miRNA-target interactions so that we can start to build large regulatory maps to better appreciate the combinatorial actions of miRNAs in mediating gene expression. Understanding the balancing effects of these miRNAs in certain cellular contexts is particularly important since miRNAs can have redundant and/or opposing roles on biological processes. Furthermore, identification of their relevant target gene networks may lead us to uncover novel gene interactions and molecular pathways not previously known to regulate T cell differentiation and function. Ultimately, we hope that new

insights into the molecular events that govern Th17 cell fate decisions and immune function may reveal new therapeutic solutions to target the chronic inflammation seen in diseases like asthma.

Figure 1. The role of miR-17~92 cluster miRNAs in Th17 cell differentiation.

Schematic of the effect of miR-17~92 miRNAs during Th17 cell differentiation. Th17 cells are generated from naïve $CD4^+$ T cells in the presence of their polarizing cytokines TGFβ and IL-6. Th17 cells express the chemokine receptor, CCR6, have characteristic transcription factors, including RORγt and RORα, and are defined by the effector cytokine, IL-17. The miR-17~92 miRNAs regulate multiple genes involved in this process.

Fig 1.

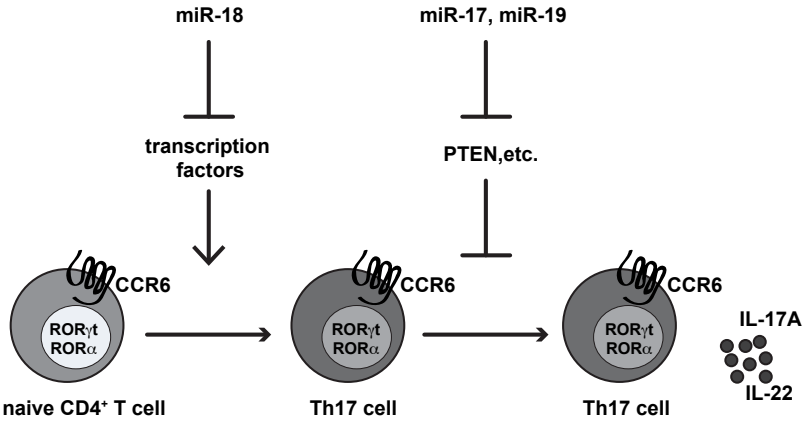
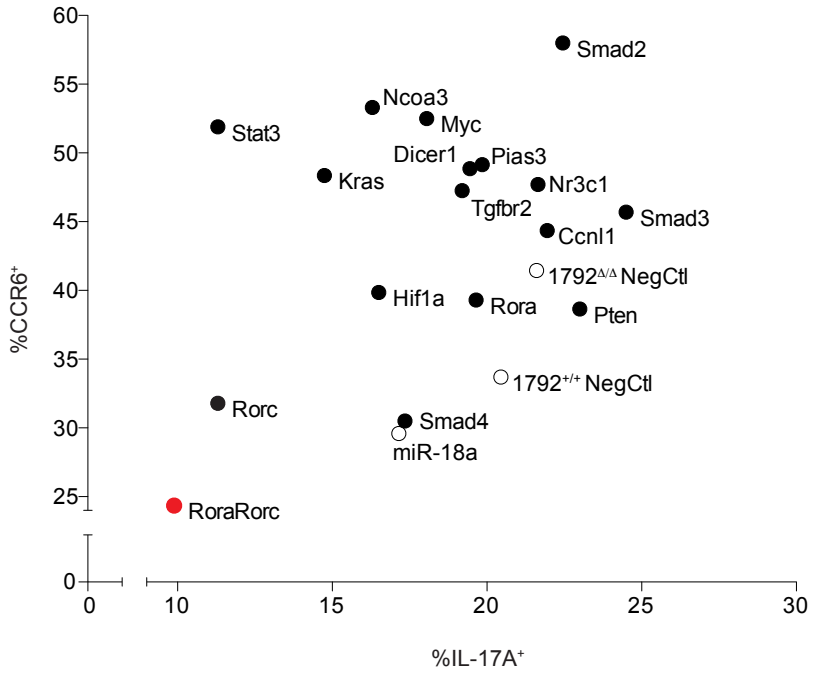


Figure 2. siRNAs against mRNAs that encode RORγt and RORα produced the greatest reduction in CCR6 and IL-17. 17~92$^{\Delta/\Delta}$ CD4^{+} T cells cultured under Th17-polarizing conditions and transfected with indicated miRNAs or siRNA pools against the indicated genes were analyzed by flow cytometry on day 3.5. Data presented as percentage of CCR6^{+} and IL-17A^{+} live singlet cells. Open circles indicate 17~92$^{+/+}$ and 17~92$^{\Delta/\Delta}$ CD4^{+} T cells transfected with siNegCtl or 17~92$^{\Delta/\Delta}$ CD4^{+} T cells transfected with miR-18a mimic as a positive control.

Fig 2.



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Appendix 1

Small RNA transfection in primary human Th17 cells by next generation electroporation

Small RNA Transfection in Primary Human Th17 Cells by Next Generation Electroporation

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Abstract

$CD4^+$ T cells can differentiate into several subsets of effector T helper cells depending on the surrounding cytokine milieu. Th17 cells can be generated from naïve $CD4^+$ T cells *in vitro* by activating them in the presence of the polarizing cytokines IL-1b, IL-6, IL-23, and TGFb. Th17 cells orchestrate immunity against extracellular bacteria and fungi, but their aberrant activity has also been associated with several autoimmune and inflammatory diseases. Th17 cells are identified by the chemokine receptor CCR6 and defined by their master transcription factor, RORgt, and characteristic effector cytokine, IL-17A. Optimized culture conditions for Th17 cell differentiation facilitate mechanistic studies of human T cell biology in a controlled environment. They also provide a setting for studying the importance of specific genes and gene expression programs through RNA interference or the introduction of microRNA (miRNA) mimics or inhibitors. This protocol provides an easy to use, reproducible, and highly efficient method for transient transfection of differentiating primary human Th17 cells with small RNAs using the Neon® next generation electroporation device.

Introduction

$CD4^+$ T cells are crucial orchestrators of the adaptive immune response. Naïve $CD4^+$ T cells are capable of developing into several different effector T cells (*e.g.* Th1, Th2, Th17, *etc.*), each with their own set of characteristic cytokines and transcription factors, depending upon the local microenvironment[1]. The lineage decisions that T cells make are critical for both protective immunity and tolerance to self. Th17 cells are one subset of T cells known to combat extracellular bacteria and fungi, but their improper responses are also implicated in the pathogenesis of multiple autoimmune and inflammatory diseases such as multiple sclerosis and psoriasis[2,3]. Human Th17 cells can be generated from naïve $CD4^+$ T cells *in vitro* by providing them with an appropriate polarizing environment[4]. Various combinations of the cytokines IL-1b, IL-23, TGFb, and IL-6 have been used for the development of human Th17 cells. Human Th17 cells express CCR6, a chemokine receptor that is commonly used to identify this cell population and are defined by the expression of their principal transcription factor, RORγt (encoded by *RORC*) [5,6]. Th17 cells have the ability to express multiple cytokines, but IL-17A is the lineage-defining effector cytokine produced by these cells. We examined the expression of all three Th17-associated markers (CCR6, RORγt, IL-17A) to assess the robustness of our human Th17 *in vitro* differentiation assay. Additionally, we cultured human $CD4^+$ T cells under non-polarizing conditions, where no cytokines or blocking antibodies were added to the culture media to use as a negative control since expression of these Th17 markers should be very low or absent.

One way to study normal human T cell development and biology is to manipulate gene

expression during their development. Short-interfering RNA (siRNA) are synthetic small RNA molecules that target protein-coding mRNAs and can be utilized to reduce specific gene expression. MicroRNAs (miRNAs) are endogenous non-coding small RNAs known to modulate gene expression post-transcriptionally. miRNAs have been shown to play an important role in both murine and human T cell biology, including in Th17 cells[7-9]. It is crucial to have reliable methods of manipulating small RNA activity in human T cells to study their effects on gene expression and ultimately on human T cell biology. Here, we describe an easy-to-use, consistent and reliable protocol that we developed for introducing small synthetic RNAs and locked nucleic acids (LNAs, chemically modified nucleic acids with increased stability) into immune cells, and specifically into human Th17 cells.

There are several alternative methods of introducing small RNAs into mammalian cells, which generally fall into chemical, biological, or physical categories[10]. Commonly used chemical methods, including lipid-based transfections and calcium-phosphate transfections, rely on creating chemical-DNA complexes that are more efficiently taken up by cells. In general, chemical methods are not as efficient for the transfection of primary T cells. The most common biological method is to use a viral vector (e.g. retrovirus or lentivirus), which directly inserts foreign RNA into a host as a part of its natural replication cycle. Viral transduction typically takes longer to complete, especially when one factors in time for molecular cloning of proviral plasmids. Additionally, viral transduction vectors can be potentially harmful to human researchers. Electroporation is a physical method of inducing membrane permeabilization by subjecting cells to high

voltage pulses, allowing nucleic acids to transiently enter into the cell where they can act on their target. Traditional electroporation instruments were not effective for transfecting primary lymphocytes. However, optimized 'next generation' electroporation has proven to be capable of transfecting T cells at very high efficiency, especially when the material to be transfected is small RNA. The term next generation is loosely used to differentiate the two newer platforms including the Thermo Fisher Scientific Neon® Transfection System, presented here in this protocol, and the Amaxa™ Nucleofector™ devices from traditional electroporation machines. Additionally, this method is easily scalable for moderate throughput screens with up to approximately 120 small RNAs in a single experiment, often using validated synthetic reagents. Importantly, successful transfections can be achieved in as little as 16 hours after T cell activation. The disadvantage of this method, however, is that it does not result in stable genomic incorporation, and is therefore transient. Therefore, it is worth the extra effort to create a stable expression construct that can be packaged intro a viral vector and successfully expressed in T cells in cases where long-term expression of a small RNA is required.

We have used the Neon® next generation transfection to deliver diverse synthetic single or double-stranded RNA or LNA oligonucleotide tools for different purposes[11-13]. Efficient RNA interference can be induced in primary mouse and human T cells using double-stranded short-interfering RNA (siRNA). This protocol describes optimized conditions for using this technique in human Th17 cells. In addition to siRNAs, commercially available synthetic miRNA mimics and inhibitors can be used to study miRNA gain and loss of function. miRNA mimics are double-stranded RNA molecules

very similar to siRNAs, but designed with the sequence of endogenous mature miRNAs. miRNA inhibitors are chemically-modified RNA and/or LNA based single stranded oligonucleotides that bind to native miRNAs and antagonize their function. We have found that all of these tools can be used effectively in cultured primary T lymphocytes, including but not limited to human Th17 cells.

Protocol

1. **Preparation of T cell culture, Isolation of $CD4^+$ T cells, and Th17 polarization**

1.1. On Day 0, coat 6-well tissue culture plates with 1.5 mL per well of anti-human CD3 (2 µg/mL) and anti-human CD28 (4 µg/mL) in PBS with calcium and magnesium for at least 2 h at 37 °C.

1.1.1. Alternatively, coat the plates overnight at 4 °C. Wrap plates in parafilm.

1.2. Prepare the cord blood mononuclear cells (CBMCs) by density gradient centrifugation[14] per manufacturer's instructions.

Caution: Work carefully and ensure proper personal protective equipment (PPE) is worn when handling human blood to avoid any risk of exposure to blood-borne pathogens.

1.3. Once the mononuclear cells have been isolated and washed, perform human $CD4^+$ T cell isolation by negative selection using the DynaBeads® Untouched™ Human $CD4^+$ T cell kit.

Note: If there is red blood cell contamination after mononuclear cell isolation, an optional red blood cell lysis may be performed prior to the $CD4^+$ T cell isolation steps. Resuspend the mononuclear cells in 1 ml of isolation buffer (2% FBS in PBS). Add 5 ml of 1X BD Pharm Lyse™ lysing solution. Incubate for 15 min at room temperature. Then add 5 ml of isolation buffer and centrifuge at 300 x g for 5 min at 4 °C to pellet the cells.

1.3.1 Resuspend the mononuclear cells at a density of $50x10^6$ per 500 µl in the isolation buffer in a new 5 ml tube.

1.3.2 Add 100 µl of FBS and 100 µl of the Antibody Mix per tube then incubate each tube at 4 °C for 20 min on an orbital shaker to mix well.

1.3.3 After the incubation, add 3-4 ml of isolation buffer to wash the cells. Centrifuge the cells at 300 x g for 8 min at 4 °C to pellet the cells. Carefully aspirate the supernatant.

Note: During this spin, pre-wash the Depletion MyOne™ Dynabeads®. Transfer the desired amount of Dynabeads® into a new tube (used at 1:1 with the cells) and add an equal volume of isolation buffer to wash the beads. Mix well using a 1 mL micropipette and then place the tube in the magnet for at least 1 min. Carefully aspirate the supernatant. Resuspend the Dynabeads® in the same volume initially transferred prior to the wash.

1.3.4 Resuspend the cells in each tube with 500 µl of the isolation buffer and add 500 µl of pre-washed Depletion MyOne™ Dynabeads®.

1.3.5 Incubate the cells with the Dynabeads® for 15 min on an orbital shaker at room temperature (18 °C to 25 °C) to mix well.

1.3.6 After the incubation, thoroughly pipet the cells using a 1 mL micropipette at least 10 times. Then add 3-4 ml of isolation buffer and place each tube in the magnet for 2 min.

1.3.7 Carefully transfer the negatively selected $CD4^+$ T cells that are in the supernatant to a new tube.

1.4. Once $CD4^+$ T cell isolation is completed, count the cells with a hemocytometer and keep the cells on ice.

1.5. Wash the antibody-coated plates two times with PBS. Then add 1.5 mL of 2X mix of Th17-polarizing media to each well: anti-human IFNg (20 µg/mL), anti-human IL-4 (20 µg/mL), human TGFβ (10 ng/mL), human IL-1β (40 ng/mL), human IL-23 (40 ng/mL), human IL-6 (50 ng/mL) all diluted in a serum-free base media (supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10mM HEPES, 1 mM sodium pyruvate and 100 µM 2-mercaptoethanol).

Note: Transfection and RNA interference does work in serum-containing media. The purpose of using serum-free media with this protocol is to achieve better IL-17A production.

1.6. Centrifuge the human $CD4^+$ T cells at 500 x g for 5 min at 4 °C to pellet cells. Carefully aspirate the supernatant. Then resuspend the cells in serum-free base media and plate the cells at a density of $3x10^6$ in 1.5 mL per well so that the final volume is 3 mL per well and polarizing cytokines are now at a 1X final concentration.

1.6.1. Place the plates in 5% CO_2, 37 °C incubator for two days.

2. **Electroporation of *in vitro* Polarized Human Th17 cells**

2.1. On Day 2, coat 48-well tissue culture plates with 250 µL per well of anti-human CD3 (2 µg/mL) and anti-human CD28 (4 µg/mL) in PBS with calcium and magnesium for at least 2 h at 37 °C.

2.1.1. Alternatively, coat the plates overnight at 4 °C. Wrap plates in parafilm.

2.2. Prepare small RNAs for transfection. For each transfection, aliquot 1 µL of a 5 µM stock solution of siRNA into a 1.5 mL microcentrifuge tube. Include appropriate chemistry-matched small RNA control. Keep all tubes on ice.

2.3. Wash the antibody-coated plates two times with PBS. Then add 500 µL of 1X Th17-polarizing media to each well: anti-human IFNγ (10 µg/mL), anti-human IL-4 (10 µg/mL), human TGFβ (5 ng/mL), human IL-1β (20 ng/mL), human IL-23 (20 ng/mL), human IL-6 (25 ng/mL) all diluted in a serum-free base media (supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, 10mM HEPES, 1 mM sodium pyruvate and 100 µM 2-mercaptoethanol).

2.4. After the culture plates and transfection reagents are prepared, resuspend the cells with a 1 mL micropipette, pipetting gently but ensuring that all the cells are detached from the bottom of the wells. Pool the cells into a conical tube and centrifuge at 500 x g for 5 min at 4 °C.

Note: For culture periods longer than the four-day protocol presented herein, the cells should be transfected approximately every 3 days using this same protocol. Step 2.4 should be modified however since the cells treated with different small RNAs should not be pooled. All of the conditions being tested must be collected, counted, and transfected separately.

2.5. Carefully aspirate the supernatant, and then resuspend the cells in at least 1 mL of PBS to wash. Count the live Th17 cells (*e.g.* with a hemocytometer using Trypan Blue exclusion to assess viability) then transfer the cells to a microcentrifuge tube.

2.6. Centrifuge at 500 x g for 5 min at room temperature to pellet the cells.

2.7. Carefully aspirate the supernatant, and then resuspend the cells using the provided Resuspension buffer T from the Neon® Transfection System 10 µL kit at a density of 2.5-4 × 10^7 cells per mL. Keep cells at room temperature.

2.7.1 Alternatively, the Neon® Transfection System 100 µL kit may also be used for larger scale transfections.

Note: As a guideline, try to prepare only as many cells as can be transfected within 30 min. Multiple batches can be compared.

2.8. Add 9 µL of cells to 1 µL of small RNA in each microcentrifuge tube. Pipet once to mix the cells and small RNAs for transfection then load into the provided Neon® pipette electrode tip.

Note: We often add 9.5 µL of cell suspension to ensure there is enough of the mixture to prevent creating bubbles in the pipet electrode tip prior to transfection.

2.9. Fill the provided cuvette with 3 ml of room temperature Electrolytic Buffer E. Place the cuvette inside the Neon pipette station then place the pipette into position inside the cuvette.

2.10. Immediately electroporate each 10 µL mix of cells and small RNA using the

following parameters: pulse voltage 1500-1550 V, pulse width 10 ms, and 3 pulses total on the Thermo Fisher Scientific Neon® Transfection device.

Note: Everything necessary for transfection is provided in the Thermo Fisher Scientific Neon® Transfection kits. Here is a provided link to their website: https://www.thermofisher.com/us/en/home/life-science/cell-culture/transfection/transfection---selection-misc/neon-transfection-system.html

2.11. After the electroporation is complete, directly add the cell mixture to 500 µL of 1X Th17-polarizing media in prepared wells of a culture plate. Place plates in a 5% CO_2, 37 °C incubator for two more days.

Note: Wash the Neon® pipette electrode tip by pipetting up and down in PBS in between each transfection.

3. **Harvesting Human Th17 Cells**

3.1. Resuspend the cells in culture media with a micropipet, pipetting gently but ensuring that all the cells are detached from the bottom of the wells. Prepare the cells for functional and/or gene expression assays.

Note: Routinely, enough cells are yielded from a single well of transfected Th17 cells to be used for flow cytometric analysis of surface marker and intracellular cytokine and transcription factor staining or for preparation of RNA for gene expression analysis.

Representative Results

The first step to developing a reliable system of successfully electroporating human Th17 cells was to generate robust *in vitro* differentiated human Th17 cell cultures. T cells cultured under Th17-polarizing conditions expressed the chemokine receptor CCR6 and the transcription factor RORγt (**Figure 1A, left**). These markers were not expressed when T cells were cultured under non-polarizing (ThN) conditions (**Figure 1A, right**). T cells cultured under Th17-polarizing conditions also produced IL-17A upon restimulation (**Figure 1B, left**) but not under ThN conditions (**Figure 1B, right**). Th17 differentiation still occurs after transfection with small RNAs but there is an observed reduction in the frequency of IL-17A production (**Figure 1C**). The effect of small RNA transfection on cytokine production demonstrates the importance of having a chemistry-matched small RNA control for comparison within each experiment. Importantly, viability is maintained after transfection with small RNAs and is typically greater than 70% (**Figure 1D**).

To determine the efficiency of this protocol, we used RNA interference. The CD45 antigen is encoded by the *PTPRC* (protein tyrosine phosphatase, receptor type C) gene. CD45 is expressed on all hematopoietic cells and therefore human Th17 cells should robustly express this surface marker. Transfection of human Th17 cells on day 2 with an siRNA pool targeting *PTPRC* strongly reduced the expression of CD45 compared to a chemistry matched control siRNA (**Figure 2A**). This unimodal population shift in CD45 expression indicates near 100% transfection efficiency, typical of this protocol for small RNAs. Thus, this is an important tool that can be used to assess

transfection efficiency during protocol optimizations. *RORC* encodes RORγt, the predominant transcription factor of human Th17 cells, which directly regulates IL-17A production. Transfecting developing human Th17 cells with an siRNA pool against *RORC* strongly reduced RORγt expression as expected (**Figure 2B**). Reducing RORγt expression by RNAi had a functional effect, as these cells exhibited reduced IL-17A production (**Figure 2C**) compared to cells transfected with a control siRNA. Non-polarized $CD4^+$ T cells should not express any RORγt or IL-17A and are shown as a negative control in this figure.

Acknowledgements

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Discussion

This protocol provides an improved method for the delivery of small RNAs into human Th17 cells. Although human Th17 cells were used here, this method of electroporation with small RNAs can be used with other primary human T helper subsets, such as Th1, Th2, and Tregs. It has not worked well for naïve $CD4^+$ T cells so the cells must be activated in culture prior to transfection. For this protocol, we first optimized the *in vitro* culture system for better IL-17A production. The biggest factor was base media. We found that serum-free media was superior to traditional serum-containing media for better induction of IL-17A producing human Th17 cells. Since Th17 cells can be generated with subsets of the cytokines TGFb, IL-1b, IL-23, and IL-6, we tested different cytokine mixes. In this study, we achieved more robust IL-17A production when all four polarizing cytokines were included. Additionally, we optimized shortening the length of the culture to ensure only one transfection was necessary during the culture period. While this four-day protocol allows results to be generated faster and necessitates only one transfection, culture length could be lengthened to further increase IL-17A production. A longer culture period may even be necessary to achieve optimal differentiation of other primary T cell subsets. Since these transfections are transient, multiple transfections may be required over time for lengthy culture periods. We suggest replenishing small RNA reagents after approximately 3 days by re-electroporating the cells with the reagent to prevent dilution over multiple cell divisions. We have routinely done this for mouse $CD4^+$ T cells. Although it is more technically challenging since the

cells in each condition must be collected and counted separately, the transfection is successful and there are minimal effects on cell viability.

In these experiments, human Th17 cells were generated through *in vitro* culture of human umbilical cord blood. Importantly, an alternative option for this differentiation assay would be to use human peripheral blood mononuclear cells (PBMCs). For both CBMCs and PBMCs, it is necessary to isolate $CD4^+$ T cells prior to the start of the culture. We present here our method for purifying $CD4^+$ T cells by negative selection, which yielded robust transfection efficiency, but we expect other methods of preparing primary human $CD4^+$ T cells would work equally well. We have tried several kits that use either positive or negative selection for the isolation of mouse $CD4^+$ T cells and both methods have resulted in similar transfection efficiency. An additional consideration is the importance of using naïve T cells in order to optimally differentiate into Th17 cells. Using human cord blood is advantageous for this reason because it already has a higher proportion of naïve T cells present and therefore it is not necessary to sort or pre-enrich for naïve T cells prior to the start of culture. However, a clear disadvantage of this approach is the limited availability of umbilical cord blood. Alternatively, sorting of naïve T cells from PBMCs[15] can be performed prior to culture since this resource is more readily available, although large quantities would have to be obtained.

While there are multiple methods for transfecting mammalian cells, primary T cells tend to be a more difficult cell type to transfect. Next generation electroporation is a successful physical method of transiently transfecting T cells that is technically easy to

perform and is reproducible. Once optimized, it has a very high efficiency of transfection for small RNAs that approaches 100%. This can be seen in Figure 2 where the entire unimodal population of $CD45^+$ cells (Figure 2A) and the population of all the cells expressing RORγt (Figure 2B) shift to a lower expression level. Importantly, optimized transfection with small RNAs does not compromise cell viability. We routinely observe greater than 70% viability post-transfection with small RNAs (Figure 1D). Although viability is not an issue with this protocol, the biology of other T cell characteristics, such as cytokine production, may be affected after transfection with small RNAs. For this reason, it is critically important to use a chemistry-matched small RNA control in every experiment.

We optimized the two electroporation parameters, pulse and voltage, for minimizing cell death and maximizing transfection efficiency. Higher voltages increase cell death during transfection. Some other technical considerations to prevent cell death and ensure successful transfection include minimizing the amount of synthetic reagents used and limiting the time between cell preparation and electroporation. Minimal concentrations necessary for synthetic transfection reagents should always be used to prevent potential T cell toxicity and off-target effects. Reducing cell preparation time prior to transfection to minimize the time of cells in transfection buffer at room temperature is crucial and can be achieved by preparing cells in batches, if necessary.

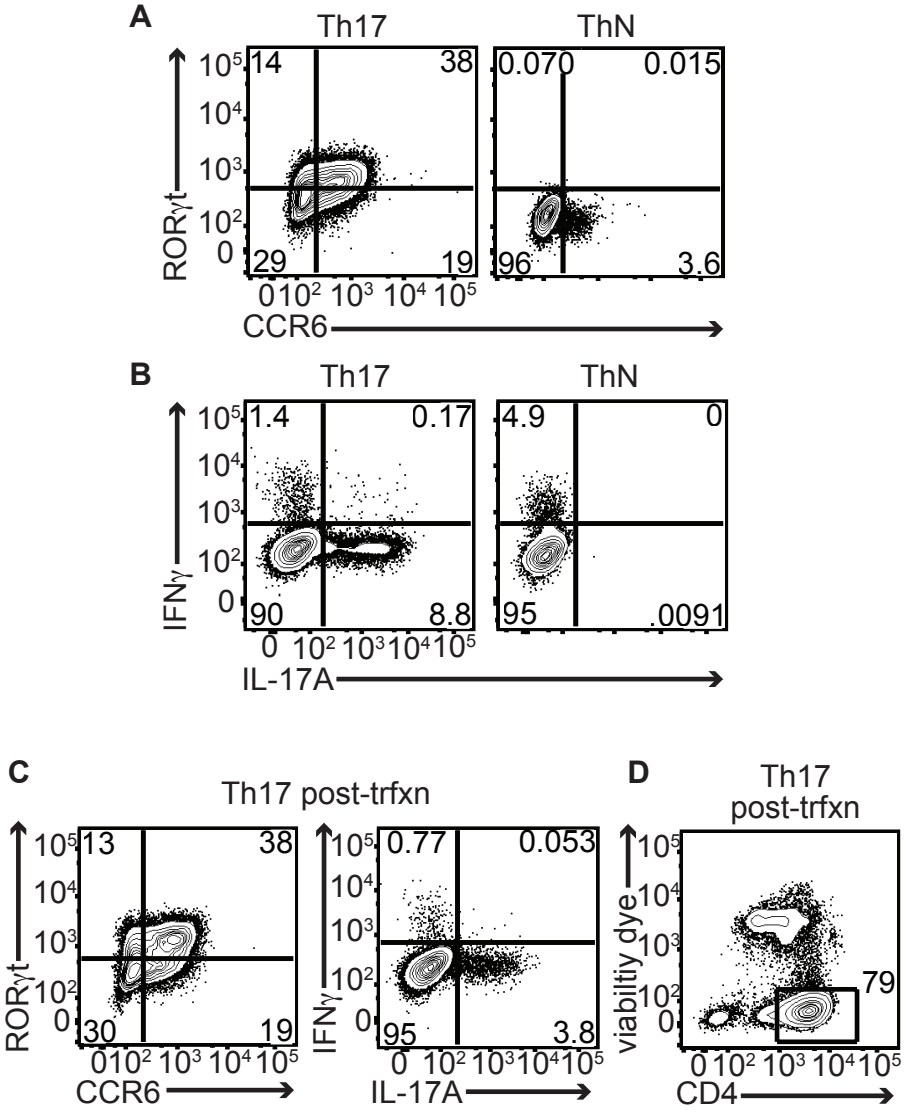
Currently, there are two commercially available next generation electroporation instruments. Both of these systems can efficiently transfect primary T cells. This

protocol was optimized using the Thermo Fisher Scientific Neon® Transfection system. While this transfection system is more cost-effective, the Amaxa™ Nucleofector™ system uniquely offers a 96-well adaptor for electroporation for higher throughput applications. We hope that additional commercial advancements will continue to increase the throughput and efficiency of these transfection systems, while keeping cost at a minimum

Next generation electroporation of human T cells can efficiently introduce small RNAs including siRNAs against target genes of interest as exemplified in this protocol as well as miRNA mimics or inhibitors. The high efficiency makes it unnecessary to use markers of transfection since nearly every cell is transfected with small RNAs. This is in contrast to the transfection of primary T cells with DNA plasmids where typical plasmid transfections result in only 20-30% expression of the marker gene with greater than 50% toxicity that increases with increasing amounts of plasmid. Introducing synthetic mimics using this method has allowed for dozens of genes to be screened for function in T cell biology in parallel without any need for molecular cloning[12,13]. It has also enabled testing for the function of all miRNAs expressed by T cells in parallel[11,13]. With this protocol, these types of moderate throughput screens are applicable to human T cells. Notably, this technique was recently used to introduce small RNA (gRNA)-Cas9 ribonucleoprotein complexes into primary human T cells for genome editing[16]. These diverse applications highlight the utility, flexibility and power of the protocol presented here as an important tool for immunologists seeking to study T cell biology.

Figure 1. *In-vitro* Differentiated Human Th17 Cells Express CCR6, RORγt, and IL-17A. Human $CD4^+$ T cells isolated from umbilical cord blood were cultured under Th17 conditions (IL-1b, IL-23, IL-6, and TGFb) or in non-polarizing (ThN) conditions (media only, no polarizing cytokines or blocking antibodies added) *in vitro* and analyzed on day 4 for Th17 marker expression by flow cytometry: A) Representative contour plots display surface CCR6 and intracellular RORγt co-staining of $CD4^+$ T cells. Numbers in quadrants indicate percent CCR6 and/or RORγt-positive live singlet $CD4^+$ cells. B) IL-17A and IFNγ production after restimulation with PMA/ionomycin. Numbers in quadrants indicate percent IL-17A and/or IFNγ-positive live singlet $CD4^+$ cells. C) Representative contour plot displays surface CCR6 and intracellular RORγt co-staining of $CD4^+$ T cells post-transfection with small RNA control. Numbers in quadrants indicate percent CCR6 and/or RORγt-positive live singlet $CD4^+$ cells (left). Representative contour plot displays IL-17A and IFNγ production after restimulation with PMA/ionomycin post-transfection with small RNA control. Numbers in quadrants indicate percent IL-17A and/or IFNγ-positive live singlet $CD4^+$ cells (right). D) Representative contour plot displays viability of $CD4^+$ cells post-transfection with small RNA control. Number indicates percent $CD4^+$ live singlet cells.

Fig. 1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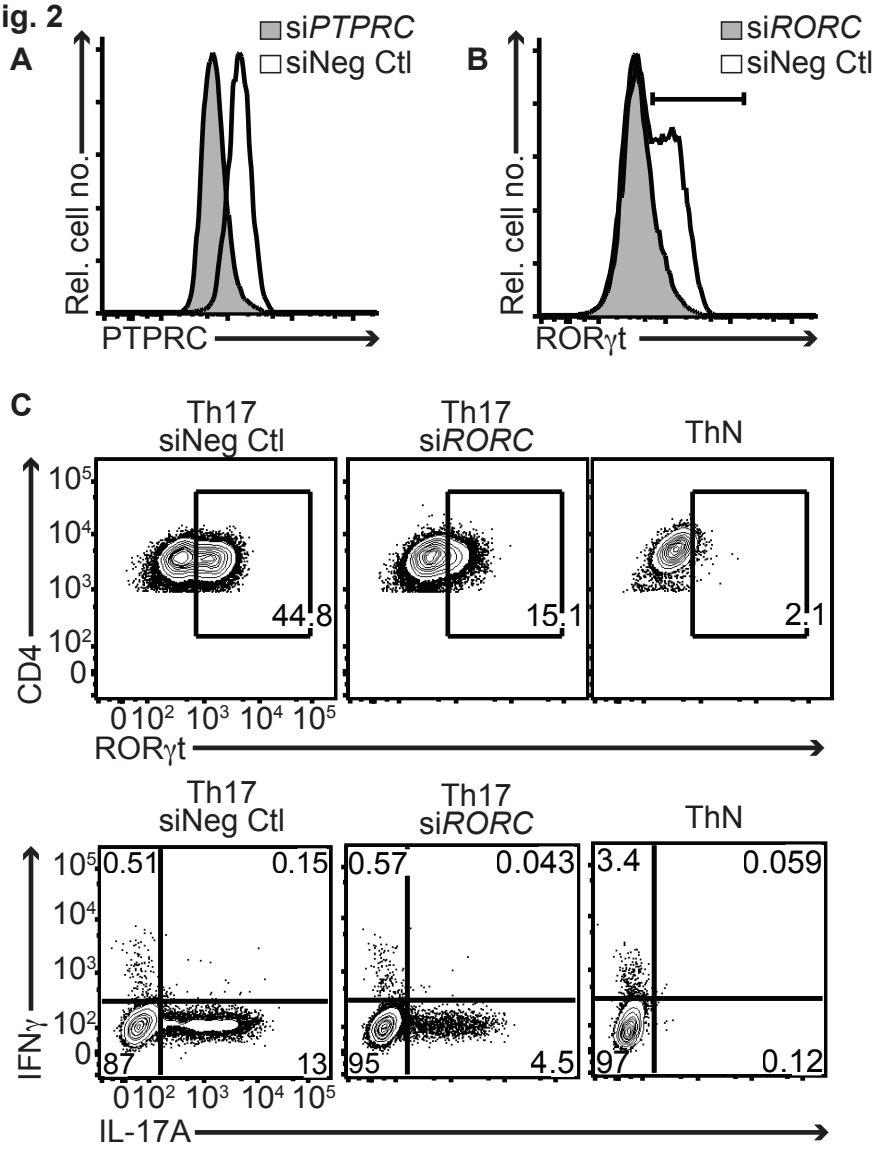
post-trfxn
79
viability dye
CD4

Figure 2. RNA Interference in Primary Human Th17 Cells. Human $CD4^+$ T cells from umbilical cord blood cultured under Th17 conditions were transfected on day 2 with an siRNA nontargeting control (siNeg Ctl) or targeting pool against *PTPRC* (si*PTPRC*) or *RORC* (si*RORC*) comprising 4 individual siRNAs. Cells were then analyzed by flow cytometry on day 4: A) Surface CD45 expression shown in a representative histogram after transfection with si*PTPRC* (grey tint) or siNeg Ctl (black line). B) Intracellular RORγt expression is shown in a representative histogram after transfection with si*RORC* (grey tint) or siNeg Ctl (black line). C) Representative contour plots display surface CD4 and intracellular RORγt co-staining of $CD4^+$ T cells (top panel) or IL-17A and IFNγ production after restimulation with PMA/ionomycin (bottom panel). Numbers in quadrants indicate percent $CD4^+ROR\gamma t^+$ or IL-17A and/or IFNγ-positive live singlet cells under ThN or Th17 conditions transfected with siNeg Ctl or si*RORC*.

Fig. 2


A
siPTPRC
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Rel. cell no.
PTPRC
B
siRORC
siNeg Ctl
Rel. cell no.
RORγt
C
Th17
siNeg Ctl
Th17
siRORC
ThN
CD4
RORγt
44.8
15.1
2.1
IFNγ
IL-17A
0.51
0.15
87
13
0.57
0.043
95
4.5
3.4
0.059
97
0.12

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Appendix 2

A miRNA upregulated in asthma airway T cells promotes Th2 cytokine production

Simpson LJ, Patel S, Bhakta NR, Choy DF, Brightbill HD, Ren X, Wang Y, Pua HH, Baumjohann D, Montoya MM, Panduro M, Remedios KA, Huang X, Fahy JV, Arron JR, Woodruff PG, and Ansel KM. A miRNA upregulated in asthma airway T cells promotes Th2 cytokine production. *Nature Immunology*. 2014;15(12):1162–1170.

nature immunology

A microRNA upregulated in asthma airway T cells promotes T_H2 cytokine production

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MicroRNAs (miRNAs) exert powerful effects on immunological function by tuning networks of target genes that orchestrate cell activity. We sought to identify miRNAs and miRNA-regulated pathways that control the type 2 helper T cell (T_H2 cell) responses that drive pathogenic inflammation in asthma. Profiling miRNA expression in human airway-infiltrating T cells revealed elevated expression of the miRNA miR-19a in asthma. Modulating miR-19 activity altered T_H2 cytokine production in both human and mouse T cells, and T_H2 cell responses were markedly impaired in cells lacking the entire miR-17~92 cluster. miR-19 promoted T_H2 cytokine production and amplified inflammatory signaling by direct targeting of the inositol phosphatase PTEN, the signaling inhibitor SOCS1 and the deubiquitinase A20. Thus, upregulation of miR-19a in asthma may be an indicator and a cause of increased T_H2 cytokine production in the airways.

Asthma is a respiratory disorder characterized by reversible airflow limitation, bronchial hyper-responsiveness and airway inflammation[1,2]. Although it is clear that asthma is a heterogeneous syndrome, a prominent subset of asthma is characterized by type 2 inflammation with infiltration of type 2 helper T cells (T_H2 cells) into the airways and lung parenchyma, as well as by a molecular signature of airway epithelial cell exposure to T_H2 cytokines, especially interleukin 13 (IL-13)[3,4]. IL-13 coordinates allergic lung inflammation through receptors on both structural cells and inflammatory cells. It induces epithelial cell hyperplasia and mucus production, airway smooth muscle cell hyper-responsiveness, and the recruitment of eosinophils[5]. IL-13 is a key driver of airway inflammation in mouse models of asthma[6], and studies of the biomarkers of type 2 inflammation predict enhanced clinical benefit from treatment with antibodies that block IL-13 signaling, such as lebrikizumab[7] and dupilumab[8].

The external signals and transcription factors that regulate T_H2 cell differentiation are well understood. The cytokine IL-4 is both the canonical product of T_H2 cells and a powerful driver of T_H2 cell differentiation. Naive $CD4^+$ T cells require concurrent signaling via the T cell antigen receptor (TCR) and cytokines to induce T_H2 differentiation. TCR ligation activates T cells through a broad signaling cascade that includes the kinase PI(3)K and transcription factor NF-κB pathways. Signaling via the receptor for IL-4 activates the transcription factor STAT6, which upregulates expression of the transcription factor GATA-3 in activated T cells. Together these two key transcription factors promote T_H2 differentiation and cytokine production[9]. Because T_H2 differentiation is governed by a positive feedback loop of cytokines and transcription factors, it is very sensitive to minor changes in cytokine production, the strength of TCR stimulation, and other intrinsic and environmental factors. Extensive knowledge of the signals that control T cell differentiation and of methods to reproducibly manipulate this process *in vitro* make it a useful system for the study of basic principles that govern gene-expression networks and cell identity.

miRNAs regulate gene-expression programs by reducing the translation and stability of target mRNAs[10]. miRNAs are grouped into families that each share a network of predicted mRNA targets. Although the quantitative effect produced by each miRNA-target interaction is small, the combined effect of the network of miRNA-target interactions produces substantial changes in cell activity. Several studies have attempted to elucidate miRNA functions in asthma by analyzing miRNA expression in whole lung, airway epithelial cells or mixed peripheral blood lymphocytes from humans with asthma or mouse models of allergic airway inflammation[11–14]. Such studies have provided insight into the effect of airway inflammation on miRNA expression patterns, but they have not defined cell-intrinsic effects of miRNA regulation on disease pathogenesis.

In T cells, miRNAs regulate proliferation, survival, activation, differentiation and cytokine production[15]. The miR-17~92 cluster has emerged as a particularly potent and pleiotropic regulator of T cell

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responses. This cluster is transcribed as a single primary miRNA (pri-miRNA) transcript that is processed to produce six mature miRNAs belonging to four miRNA families: the miR-17, miR-18, miR-19 and miR-92 families[16]. Primary miR-17~92 and the corresponding mature miRNAs are upregulated in activated CD4$^+$ T cells and can promote the proliferation and survival of T cells[17–20].

Although they are expressed without apparent cell-type specificity, miRNAs in the miR-17~92 cluster regulate the differentiation and function of several distinct T cell subsets. Both miR-17 and miR-19b promote differentiation into the T_H1 and T_H17 subsets of helper T cells[18,21]. These two miRNAs also inhibit inducible differentiation of regulatory T cells (T_{reg} cells) *in vitro*[18], but the cluster as a whole is required for normal T_{reg} cell function *in vivo*[22]. Responses of follicular helper T cells (T_{FH} cells) are reduced and dysregulated in the absence of miR-17~92 (refs. 23,24). However, the regulation of T_H2 cell differentiation and cytokine production by miRNA remains poorly understood.

We hypothesized that miRNAs that are expressed differently in airway-infiltrating T cells in asthma regulate T_H2 cell function and promote lung inflammation. We used a highly sensitive nanoscale microfluidic quantitative PCR (qPCR) approach to profile miRNA expression in CD4$^+$ T cells isolated from human asthmatic airways[25,26]. miR-19a, a member of the miR-17~92 cluster, was substantially upregulated in asthma. Through the use of genetically engineered mice, we found that miR-17~92 promoted T_H2 cytokine production *in vitro* and type 2 inflammation *in vivo*. We mapped the regulation of T_H2 cytokine production by miR-17~92 to the miR-19 family through the use of transfectable miRNA mimics and inhibitors in primary human and mouse T cells. A functional screen of the network of miR-19 targets in T_H2 cells revealed several signaling inhibitors that restrained IL-13 and IL-4 production. Together our data indicate that the observed increase in T cell miR-19a expression in humans with asthma may augment T_H2 responses in their airways.

RESULTS

miRNA expression in airway CD4 T cells

To investigate miRNA expression in airway-infiltrating T cells, we profiled the expression of a panel of 190 miRNAs in CD4$^+$ T cells sorted from bronchoalveolar lavage (BAL) fluid from 8 healthy, 13 steroid-naive asthmatic and 21 steroid-using asthmatic human subjects[12] (**Table 1**). We extracted RNA from sorted CD3$^+$CD4$^+$ T cells, and assessed miRNA expression by nanoscale microfluidic qPCR on 100 pg RNA from each subject (**Supplementary Fig. 1a** and **Supplementary Table 1**). Our analysis revealed few differences between asthmatic and healthy CD4$^+$ T cells from BAL fluid in their miRNA expression, with one notable exception (**Supplementary Fig. 1b** and **Supplementary Table 2**). Of the 89 miRNAs that we detected in at least 60% of the subjects, miRNA-19a had the most significantly elevated expression in asthma (**Fig. 1a** and **Supplementary Table 2**; $P = 0.0199$). miR-19a expression was consistently elevated in all of the steroid-naive asthmatic subjects, with very little variability, and was similarly elevated in the steroid-using asthmatic subjects, who were treated with the inhaled corticosteroid (ICS) budesonide (**Fig. 1b**). This miRNA remained elevated in CD4$^+$ T cells from steroid-naive asthmatics after 6 weeks of ICS treatment (**Fig. 1c**), which indicated that it was resistant to gene-expression changes induced by steroid treatment. Because miR-19a is a member of the miR-17~92 cluster, a highly conserved cluster of six miRNAs transcribed in one polycistronic pri-miRNA, we investigated the expression of other members of the cluster. Only miR-19a, and not miR-19b, miR-17, miR-18a or miR-20a, was expressed differently in CD4$^+$ T cells from asthmatic subjects than in CD4$^+$ T cells from healthy subjects (**Fig. 1d**). These data demonstrated that expression of miR-19a was specifically elevated in airway T cells in asthma and indicated that individual members of the miR-17~92 cluster were regulated differently in this setting.

miR-17~92 promotes T_H2 cytokine production

The miR-17~92 cluster regulates, with varying degrees of potency, the differentiation and effector functions of several helper T cell subsets, including T_H1 cells, T_H17 cells, T_{FH} cells and T_{reg} cells[19–24]. However, the cluster's role in T_H2 cell differentiation and cytokine production, important features of the asthmatic immune response, has not been explored. To investigate this, we cultured miR-17~92–deficient CD4$^+$ T cells (called '17~92$^{\Delta}$' cells here), miR-17~92–sufficient control CD4$^+$ T cells (called '17~92^{+}' cells here) and transgenic CD4$^+$ T cells overexpressing miR-17~92 (called '17~92^{tg}' cells here) in T_H2-polarizing conditions. Intracellular cytokine staining revealed that 17~92^{tg} cells produced more of the type 2 cytokine IL-13 than did their 17~92$^+$ counterparts (**Fig. 2a**). Conversely, fewer 17~92$^{\Delta}$ cells than 17~92$^+$ control cells produced the type 2 cytokines IL-13, IL-5 and IL-4 (**Fig. 2a**,**b**). This defect in T_H2 differentiation and cytokine production did not result in increased production of T_H1 cytokines, as measured by production of interferon-γ (IFN-γ) (**Fig. 2c**). 17~92$^{\Delta}$ cells produced substantially more tumor-necrosis factor, a known direct target of miR-19 (**Fig. 2c**), which suggested that these cells were capable of efficient cytokine production in T_H2-polarizing conditions and that the defect was limited to type 2 cytokines. 17~92$^{\Delta}$ cells produced fewer T_H2 cytokines, even among established GATA-3^{hi} T_H2 cells (**Fig. 2d**). Thus, the miR-17~92 cluster positively regulated T_H2 cytokine production.

To determine whether the T_H2 cytokine defect was cell intrinsic, we cultured congenically marked 17~92$^{\Delta}$ CD4$^+$ T cells together with 17~92$^+$ CD4$^+$ T cells in T_H2 conditions. After 5 d in culture, the CD45.1$^+$17~92$^{\Delta}$ T cells produced less IL-13 and IL-4 than did CD45.2$^+$17~92$^+$ cells (**Fig. 3a**), which demonstrated that the cytokine defect was cell intrinsic and did not entirely depend on feedback from IL-4 or other products produced by the mutant T cells in culture.

In T_H2 cell cultures, we observed a slight proliferation defect in 17~92$^{\Delta}$ and slightly greater proliferation of 17~92^{tg} cells, compared with the proliferation of 17~92$^+$ cells (**Fig. 3b**). To determine whether the frequency of T_H2 cytokine–producing cells among 17~92$^{\Delta}$, 17~92$^+$ or 17~92^{tg} CD4$^+$ T cells was an indirect result of their rate of proliferation, we labeled the cells with the division-tracking dye CellTrace Violet and analyzed cytokine production at each division after 5 d of culture in T_H2-polarizing conditions. The production of IL-13 and

Table 1 Subject characteristics

	Healthy	Steroid-naive asthma	Steroid-using asthma
Sample size	8	13	21
Sex	4 male, 4 female	5 male, 8 female	9 male, 12 female
Age (years)	40.3 (25–56)	30.1 (20–56)	37.3 (18–55)
FEV1% predicted	100 (88–128)	90 (61–103)	80 (59–110)
Methacholine PC_{20} (mg/ml)	>10	0.8 (0.08–4.3)	2.1 (0.14–8.1)
10^3 sorted CD4$^+$ T cells	215 (24–440)	243 (29–1,250)	277 (45–2,200)

Characteristics of healthy and asthmatic human subjects providing BAL fluid for CD4$^+$ T cells for miRNA expression analysis. Averages are followed by ranges in parentheses. 'FEV1% predicted' is a measure of respiratory capacity; PC20 is the concentration that elicits a 20% drop in FEV1.

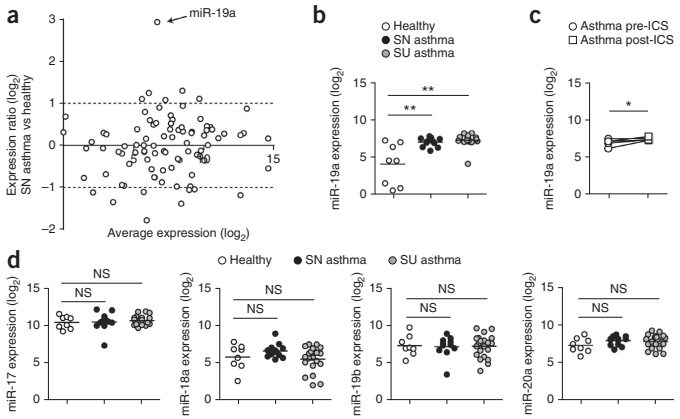


Figure 1 miR-19a expression is elevated in CD4+ T cells from asthmatic lungs. (**a**) qPCR analysis of miRNA expression in sorted CD4+ T cells: circles represent global-mean-normalized average miRNA expression in steroid-naive (SN) asthmatic subjects compared with that of healthy subjects; dashed lines indicate twofold higher or lower expression. (**b**) qPCR analysis of miR-19a expression in healthy subjects or steroid-naive or steroid-using (SU) asthmatic subjects, normalized to the global mean. (**c**) qPCR analysis of miR-19a expression in CD4+ T cells from five individual asthmatic subjects before (pre-ICS) and after (post-ICS) 6 weeks of ICS treatment. (**d**) qPCR analysis of the expression of miR-17, miR-18a, miR-19b and miR-20a (members of the miR-17~92 cluster; miR-92a was not detected in this experiment), normalized to the global mean: each symbol represents an individual subject; horizontal lines indicate the mean. NS, not significant; $*P < 0.05$, $**P < 0.0001$ (one-way analysis of variance (ANOVA) with Dunnett's post-test (compared with healthy controls; **b**,**d**) or paired two-tailed *t*-test (**c**)). Data were pooled from two experiments with 8 healthy subjects, 13 steroid-naive asthmatic subjects and 21 steroid-using asthmatic subjects (**c** includes subset of the steroid-naive asthmatic subjects in **a**,**b**,**d** for which data before and after ICS were available).

IL-5 in 17~92+ cells did increase with each division (**Fig. 3c**,**d**). However, 17~92Δ cells produced significantly less IL-13, IL-5 and IL-4 at each cell division than did 17~92+ cells (**Fig. 3c**,**d**). In contrast, 17~92tg cells produced more IL-13 and IL-5 at each division, but produced an amount of IL-4 equal to that produced by 17~92+ cells (**Fig. 3c**,**d**). Together these data indicated that the miR-17~92 cluster promoted type 2 cytokine production in a cell-intrinsic and proliferation-independent manner.

miR-19 augments T_H2 differentiation

To understand the mechanism by which miR-17~92 controls cytokine production in T_H2 cells, we needed to examine the functions of individual miRNA members of the cluster. Therefore, we transfected mature miRNA mimics corresponding to each of the six miRNAs in the miR-17~92 cluster into 17~92Δ CD4+ T cells on days 1 and 4 of T_H2-polarizing cultures. Transfection of either miR-19a or miR-19b was sufficient to completely restore T_H2 cytokine production (**Fig. 4a–c**). Other miRNAs within the cluster conferred only partial restoration of T_H2 cytokine production compared with the restoration achieved by miR-19a or miR-19b. These data indicated that miR-19 was the primary component of the miR-17~92 cluster that augmented T_H2 differentiation.

All of the miR-19a and a large majority of the miR-19b expressed in T cells derived from the miR-17~92 cluster (**Supplementary Fig. 2**). Consistent with that finding, specific retroviral sensors of the activity of miR-19a and miR-19b were strongly repressed in 17~92+ cells but not in 17~92Δ cells (**Supplementary Fig. 3**). T cell activation induces increased transcription of the miR-17~92 cluster, and all of

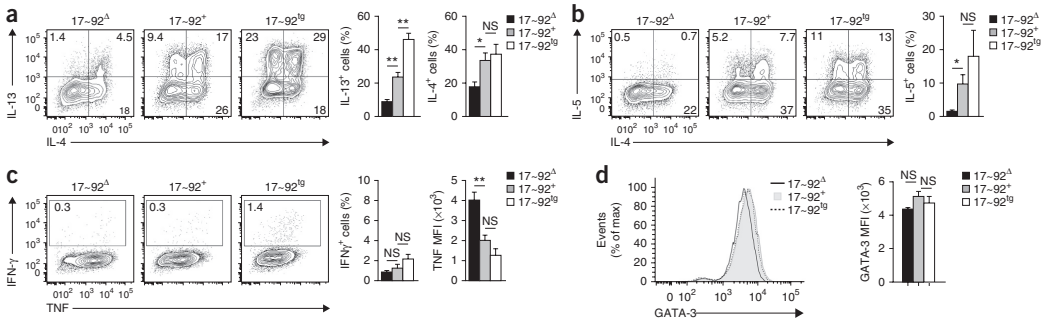


Figure 2 The miR-17~92 cluster promotes T_H2 cytokine production. (**a**–**c**) Intracellular staining of cytokines (along axes) and pooled analysis of day-5 T_H2-polarized CD4+ T cells from 17~92Δ mice (left), 17~92+ mice (middle) and 17~92tg mice (right). Numbers in quadrants indicate percent cytokine-producing live singlet cells. Far right, total cytokine-positive cells as a percentage of live singlets (mean ± s.e.m.). TNF, tumor-necrosis factor. (**d**) Flow cytometry analysis of GATA-3 expression in day-5 T_H2-polarized CD4+ T cells from 17~92Δ , 17~92+ and 17~92tg mice, presented as mean fluorescence intensity (MFI) of GATA-3 expression in live singlets (mean ± s.e.m.). $*P < 0.05$, $**P < 0.001$ (one-way ANOVA with Dunnett's post test (**a**,**c**,**d**) or one-way ANOVA Kruskal-Wallis test (for groups with unequal variance) and Dunn's post-test (**b**)). Data were pooled from 11 independent experiments with 12 17~92Δ mice, 17 17~92+ mice and 6 17~92tg mice (**a**), 7 independent experiments with 8 17~92Δ mice, 10 17~92+ mice and 4 17~92tg mice (**b**) or 11 independent experiments with 12 17~92Δ mice, 17 17~92+ mice and 7 17~92tg mice (**c**) or are from two independent experiments with four mice per genotype (two per group with duplicate cultures; **d**).

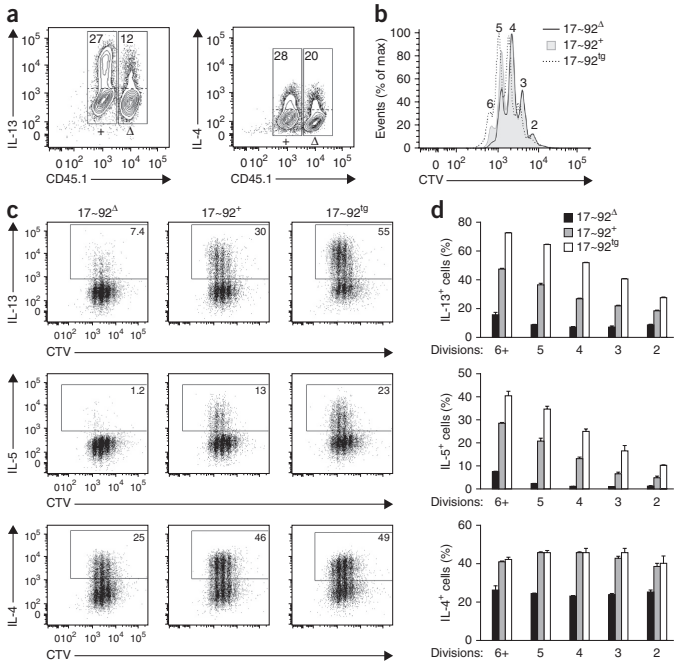


Figure 3 The miR-17~92 cluster promotes T_H2 cytokine production in a cell-intrinsic and proliferation-independent manner. (**a**) Intracellular cytokine staining of 17~92$^{\Delta}$ (CD45.1$^+$) and 17~92$^+$ (CD45.2$^+$) CD4$^+$ T cells cultured together for 5 d in T_H2-polarizing conditions. Numbers in outlined areas indicate percent cytokine-producing cells (above the dashed line) in either the CD45.1$^+$ gate or the CD45.1$^-$ gate (solid outlines). (**b**) CellTrace Violet (CTV) stain showing proliferation of day-5 T_H2-polarized 17~92$^{\Delta}$, 17~92$^+$ and 17~92^{tg} CD4$^+$ T cells. Numbers above peaks indicate division number relative to that of an undivided control. (**c**) Intracellular cytokine staining of CTV-labeled 17~92$^{\Delta}$ (left), 17~92$^+$ (middle) and 17~92^{tg} (right) day-5 T_H2-polarized CD4$^+$ T cells. Outlined area indicates cytokine-positive gate used for quantification in **d**; numbers within indicate total percent cytokine-producing live singlet cells. (**d**) Quantification of data in **c** showing cytokine-positive cells (as a percentage of live singlets) at each division. $P < 0.0001$ (two-way ANOVA comparing IL-13, IL-5 and IL-4 in 17~92$^{\Delta}$ and 17~92$^+$ mice). $P < 0.0001$ (two-way ANOVA comparing IL-13 and IL-5 in 17~92$^+$ and 17~92^{tg} mice). $P = 0.3153$ (two-way ANOVA comparing IL-4 in 17~92$^+$ and 17~92^{tg} mice). Data are from one experiment representative of two independent experiments with two mice each and duplicate cultures (**a**) or are from one experiment representative of six independent experiments with 17~92$^{\Delta}$ and 17~92$^+$ mice ($n = 6$ each) or two experiments with 17~92$^+$ and 17~92^{tg} mice ($n = 2$ each) (**b**–**d**), with $n = 2$ replicate cultures from one mouse of each genotype (mean and range).

the miRNAs in the cluster continued to have high expression during T_H2 cell differentiation[27] (data not shown). A single transfection either early (day 1) or late (day 4) in the culture did not restore T_H2 cytokine production (**Fig. 4d**,**e**). We concluded that miR-19 was required throughout T_H2 polarization to support robust T_H2 differentiation and cytokine production. miR-19 mimics also modestly increased proliferation of 17~92$^{\Delta}$ cells in T_H2 conditions (**Supplementary Fig. 4**).

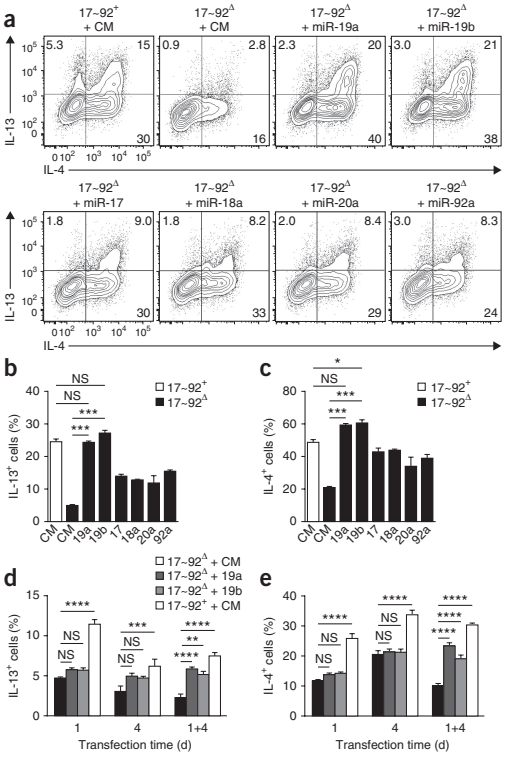
We next investigated whether modulating the activity of miR-19 alone was sufficient to alter type 2 cytokine production in miR-17~92–sufficient T cells with normal endogenous expression of the other miRNAs in the cluster. Transfection of inhibitors of miR-19a and miR-19b into 17~92$^+$ T cells specifically increased the expression of corresponding sensors[20] (Online Methods) of the activity of miR-19a and miR-19b and decreased expression of IL-13 (**Supplementary Fig. 3**). These inhibitors also significantly reduced production of IL-13, but not IL-4, when transfected into CD4$^+$ T cells obtained from human cord blood and cultured in T_H2-polarizing cultures (**Fig. 5a**,**b**). Conversely, transfection with the miR-19a mimic was sufficient to increase the expression of IL-13, but not that of IL-4, in CD4$^+$ T cells from human cord blood (**Fig. 5c**,**d**). Similarly, 17~92$^+$ mouse CD4$^+$ T cells had increased production of IL-13, but not of IL-4, when transfected with mimics of miR-19a or miR-19b in nonpolarizing conditions (**Supplementary Fig. 3**). We concluded that changes in miR-19 expression, such as those seen in human asthmatic airway T cells, were sufficient to modulate the abundance of IL-13 produced in primary human and mouse CD4$^+$ T cells.

miR-19 targets *Pten*, *Socs1* and *Tnfaip3*

Because a large number of direct miR-19 targets have been validated in B cell lymphomas and other cell types, we took a candidate approach to investigate how miR-19 augments T_H2 differentiation. We individually inhibited the 38 previously confirmed miR-19 targets[28–30] that are expressed in T cells[31] through the use of small interfering RNA (siRNA) in 17~92$^{\Delta}$ CD4$^+$ T cells during T_H2 polarization (**Supplementary Table 3**). We calculated *z*-scores for IL-13 and IL-4 for each of the transfections (**Fig. 6a**,**b**). We further tested the top eight candidate genes whose inhibition increased IL-13 and IL-4 by inhibition with three individual siRNAs per gene in 17~92$^{\Delta}$ CD4$^+$ T cells during T_H2 polarization to confirm the 'rescue' of IL-13 and IL-4 (**Fig. 6c**,**d**). The type 2 cytokine defect of 17~92$^{\Delta}$ cells was 'rescued' by inhibition of *Pten*, *Socs1* and *Tnfaip3* (which encodes A20) with at least two of three individual siRNAs (**Fig. 6e**). To confirm that miR-19 inhibited each of the top eight candidate genes, we analyzed gene expression by qPCR in 17~92$^{\Delta}$ cells transfected with miR-19a, miR-19b or control mimics, compared with that in 17~92$^+$ cells (**Fig. 6f**). Furthermore, to determine whether genetic depletion of PTEN expression in 17~92$^{\Delta}$ cells was able to restore T_H2 cytokine production, we deleted one allele of *Pten* in 17~92$^{\Delta}$ cells (17~92$^{\Delta}$*Pten*$^{+/-}$). Genetic depletion of PTEN moderately 'rescued' the cytokine defect of 17~92$^{\Delta}$ T cells (**Fig. 6g**).

Pten, Socs1 and *Tnfaip3* encode negative regulators of T cell signaling pathways that are important for all helper T cell subsets. To determine whether each of these targets regulated other helper T cell cytokines, such as IFN-γ, IL-17A and IL-17F, we transfected 17~92$^{\Delta}$ cells with individual siRNAs targeting *Pten, Socs1* and *Tnfaip3*

Figure 4 miR-19a and miR-19b 'rescue' the T_H2 cytokine defect in 17~92$^{\Delta}$ cells. (**a**) Intracellular staining of cytokines (along axes) in 17~92^{+}CD4^{+} or 17~92$^{\Delta}$CD4^{+} T cells transfected with control mimic (CM) or mimics of miR-19a, miR-19b, miR-17, miR-18a, miR-20a or miR-92a on days 1 and 4 of T_H2 polarization and analyzed on day 5. Numbers in quadrants indicate percent cytokine-producing cells. (**b**,**c**) Quantification of the production of IL-13 (**b**) or IL-4 (**c**) at day 5 after transfection with miRNA mimics. (**d**,**e**) Quantification of the production of IL-13 (**d**) or IL-4 (**e**) at day 5 of T_H2 polarization in 17~92^{+} CD4^{+} or 17~92$^{\Delta}$ CD4^{+} T cells transfected with CM, miR-19a or miR-19b on day 1 only, day 4 only or both days 1 and 4. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ and $^{****}P < 0.0001$ (one-way ANOVA with Dunnett's post-test (comparing each column to 17~92$^{\Delta}$ + CM; **b**,**c**) or two-way ANOVA with Bonferroni post-test (comparing each column to 17~92$^{\Delta}$ + CM at each time point; **d**,**e**)). Data are representative of three (**a**–**c**) or two (**d**,**e**) independent experiments, each with one mouse per genotype (mean and s.e.m. of three individual transfections per condition in **b**–**d**).



under nonpolarizing (T_HN), T_H17-polarizing and T_H2-polarizing conditions (**Fig. 6h**–**j**). Consistent with published reports[18,21], inhibition of *Pten* increased the production of all T helper cytokines tested, whereas reducing the expression of *Socs1* or *Tnfaip3* enhanced the production of T_H2 cytokines but not that of IFN-γ or IL-17. Thus, miR-19 specifically regulated T_H2 responses through distinct limiting mRNA targets.

miR-17~92 augments T_H2 cell function *in vivo*

We next investigated whether the diminished production of T_H2 cytokines by 17~92$^{\Delta}$ T cells would result in altered type 2 inflammation in airways *in vivo*. We transferred 17~92$^{\Delta}$ or 17~92^{+} ovalbumin (OVA)-specific OT-II T_H2 cells (cells with transgenic expression of a major histocompatibility complex class II–restricted TCR) into *Cd28*$^{-/-}$ mice and challenged the recipient mice every 24 h oropharyngeally with OVA for 3 d (**Supplementary Fig. 5a**). At 18 h after the final challenge, we analyzed pulmonary resistance in response to increasing doses of acetylcholine. Mice that received either no OT-II cells or 17~92$^{\Delta}$ OT-II T_H2 cells had significantly lower pulmonary resistance than that of mice that received 17~92^{+} OT-II T_H2 cells (**Fig. 7a**), which indicated that T_H2 cells that lacked the miR-17~92 cluster were less capable of inducing allergic airway hyper-responsiveness. Histological analysis of lung sections from these mice revealed a greater abundance of mucus-secreting goblet cells and more severe inflammation in mice that received 17~92^{+} OT-II T_H2 cells than in those that received 17~92$^{\Delta}$ OT-II T_H2 cells (**Fig. 7b**–**d**). Mice that received 17~92$^{\Delta}$ OT-II T_H2 cells transfected with the miR-19a mimic showed a trend toward increased inflammation and mucus secretion (**Fig. 7b**–**d**). To better characterize and quantify airway inflammation, we analyzed BAL fluid from the recipient mice by flow cytometry (**Supplementary Fig. 5b**). Mice that received 17~92$^{\Delta}$ OT-II T_H2 cells had significantly less eosinophilia in the airways than did mice that received 17~92^{+} OT-II T_H2 cells (**Fig. 7e**). However, transfection of 17~92$^{\Delta}$ OT-II T_H2 cells with miR-19a induced airway eosinophil infiltration similar to that seen in recipients of 17~92^{+} OT-II T_H2 cells (**Fig. 7e**). Macrophage and neutrophil numbers were relatively similar in all three groups of recipients (**Fig. 7e**), which suggested that the differences in airway inflammation were restricted to effects induced by type 2 cytokines. We concluded that the miR-17~92 cluster, and miR-19a specifically, had a role in the *in vivo* function of T_H2 cells as inducers of the allergic inflammatory phenotype associated with asthma.

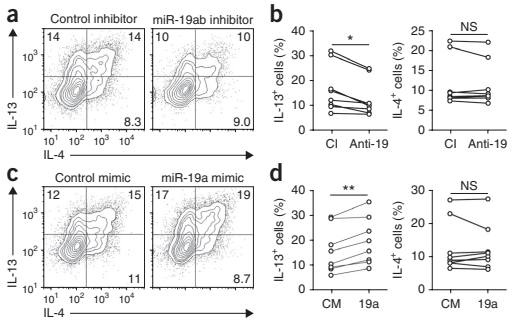


Figure 5 miR-19a promotes IL-13 production in human CD4^{+} T cells. (**a**) Intracellular cytokine staining of CD4^{+} T cells isolated from human cord blood and transfected with control inhibitor (CI) or miR-19ab inhibitor on days 1 and 4 in T_H2-polarizing conditions, analyzed on day 5. (**b**) Quantification of IL-13- and IL-4-producing cells in **a**. (**c**) Intracellular cytokine staining of CD4^{+} T cells isolated from human cord blood and transfected with control mimic (CM) or miR-19a mimic (19a) on days 1 and 4 in T_H2-polarizing conditions, analyzed on day 5. (**d**) Quantification of IL-13- and IL-4-producing cells in **c**. Numbers in quadrants (**a**,**c**) indicate percent cytokine-positive cells. Circles (**b**,**d**) represent the mean of two individual transfections of each inhibitor or mimic; lines connect individual cord blood donors receiving either inhibitors or mimics. $^{*}P < 0.01$ and $^{**}P < 0.001$ (two-tailed paired *t*-test). Data are representative of three experiments with eight samples ($n = 8$).

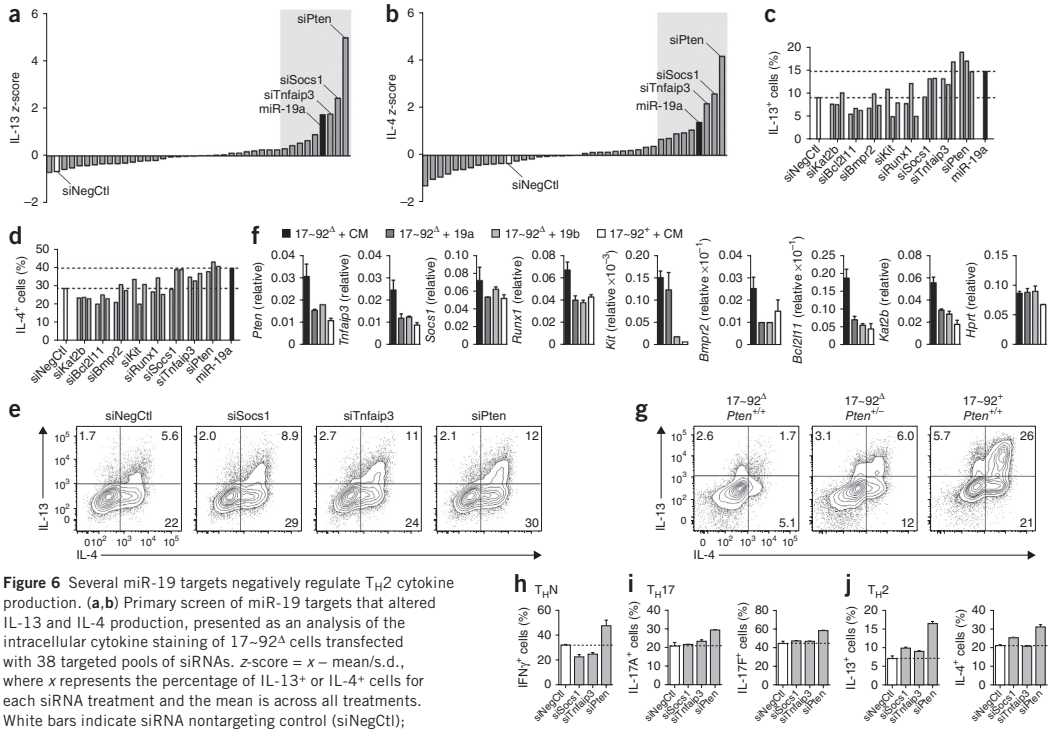


Figure 6 Several miR-19 targets negatively regulate T_H2 cytokine production. (**a**,**b**) Primary screen of miR-19 targets that altered IL-13 and IL-4 production, presented as an analysis of the intracellular cytokine staining of 17~92$^{\Delta}$ cells transfected with 38 targeted pools of siRNAs. *z*-score = *x* – mean/s.d., where *x* represents the percentage of IL-13$^+$ or IL-4$^+$ cells for each siRNA treatment and the mean is across all treatments. White bars indicate siRNA nontargeting control (siNegCtl); black bars indicate miR-19a mimic, a positive control; shaded gray boxes highlight the eight genes analyzed further in **c**,**d**. (**c**,**d**) Analysis of intracellular cytokine staining in 17~92$^{\Delta}$ cells transfected with three individual siRNAs against each of the eight candidate genes identified in **a**,**b**. Bars quantify IL-13$^+$ (**c**) and IL-4$^+$ (**d**) cells as a percentage of live singlets. Dashed lines indicate the range between the negative and positive controls. (**e**) Intracellular cytokine staining in 17~92$^{\Delta}$ cells transfected with individual siRNA targeting *Socs1*, *Tnfaip3* or *Pten*, compared with that in 17~92$^{\Delta}$ cells transfected with siNegCtl. Numbers in each quadrant indicate percent cytokine-positive cells. (**f**) qPCR analysis of expression of the eight candidate miR-19a targets in 17~92$^{\Delta}$ cells transfected with control (CM), miR-19a or miR-19b mimics, and in 17~92$^+$ cells transfected with CM; results are normalized to those of the control gene *Gapdh*. (**g**) Intracellular cytokine staining in day-5 'low T_H2'-polarized cells (Online Methods) from 17~92$^{\Delta}$ *Pten*$^{+/+}$, 17~92$^{\Delta}$ *Pten*$^{+/-}$ and 17~92$^+$*Pten*$^{+/+}$ mice. Numbers in quadrants indicate percent cytokine-positive cells. (**h**–**j**) Analysis of intracellular cytokine staining of 17~92$^{\Delta}$ cells in nonpolarizing conditions (**h**), T_H17-polarizing conditions (**i**) or T_H2-polarizing conditions (**j**) transfected with individual siRNA against *Socs1*, *Tnfaip3* or *Pten*, compared with that of cells transfected with siNegCtl. Data are representative of two (**a**–**d**,**f**–**j**) or four (**e**) independent experiments (error bars represent mean with range of $n = 2$ technical replicates (**f**) or of $n = 2$ transfections for each condition (**h**–**j**)).

DISCUSSION

Guided by miRNA expression in T cells present in the airways in human asthma, we identified a miRNA that augmented T_H2 cytokine production and allergic inflammation via coordinated regulation of cytokine and antigen receptor signaling pathways. Our data demonstrated that the miR-17~92 cluster, and specifically miR-19a, promoted T_H2 cytokine production by simultaneously targeting inhibitors of the NF-κB, STAT and PI(3)K pathways. In the context of published studies of the miR-17~92 cluster, our findings have illustrated basic principles of miRNA regulation, including the network 'logic' of miRNA function and the way that complex biological processes such as effective T cell–mediated immune responses emerge from coordinated miRNA regulation of diverse aspects of cell activity.

The miR-17~92 cluster has many established functions in lymphocytes[32]. The cluster as a whole promotes the proliferation and survival of T cells as well as the differentiation and function of several committed effector T cell subsets[18,19,21–24], which makes it an important coordinator of T cell responses. T_{FH} cell responses involve the concerted action of all four miRNA families in the miR-17~92 cluster[23,24]. miRNAs of the miR-17 and miR-92 families support T cell proliferation in the absence of other miRNAs[20]. In our experiments, miRNAs of the miR-19 family, as well as those of the miR-17 family and miR-92 family, partially restored the proliferation of 17~92$^{\Delta}$ T cells. Both miR-17 and miR-19b promote survival of T cells and differentiation of T_H1 and T_H17 cells while limiting differentiation of induced T_{reg} cells[18,21]. However, no specific functions have previously been attributed to miR-19a in helper T cells. We mapped the T_H2-cytokine-promoting activity of the miR-17~92 cluster to miR-19, as both miR-19a and miR-19b restored the production of IL-13 and IL-4 in 17~92$^{\Delta}$ T cells, but other miRNAs in the cluster did not.

Our study has emphasized an important concept about the mechanism by which miRNAs regulate cell activity: a single miRNA can be expressed in and regulate many different cell types through distinct but overlapping networks of targets. Each target may have a limiting

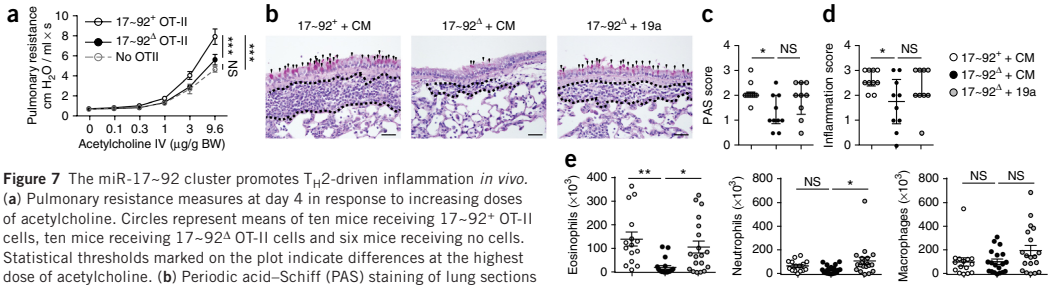


Figure 7 The miR-17~92 cluster promotes T_H2-driven inflammation *in vivo*. (**a**) Pulmonary resistance measures at day 4 in response to increasing doses of acetylcholine. Circles represent means of ten mice receiving 17~92$^+$ OT-II cells, ten mice receiving 17~92$^\Delta$ OT-II cells and six mice receiving no cells. Statistical thresholds marked on the plot indicate differences at the highest dose of acetylcholine. (**b**) Periodic acid–Schiff (PAS) staining of lung sections from recipient mice with 17~92$^+$ OT-II cells transfected with control mimic (CM) (left; $n = 10$), 17~92$^\Delta$ OT-II cells transfected with CM (middle; $n = 10$), or 17~92$^\Delta$ OT-II cells transfected with miR-19a mimic (right; $n = 9$). Black arrowheads mark PAS$^+$ cells. The area between the dotted lines indicates infiltration with cells of the immune system. Scale bars, 20 μm. (**c**,**d**) Histological scores of PAS staining in **b** to quantify mucus-secreting cells (**c**), and hematoxylin and eosin staining (**d**) to quantify airway inflammation. Scores use a scale of 0–4 and were assessed by an observer blinded to sample identity. (**e**) Flow cytometry of inflammatory cells in BAL fluid 4 d after cell transfer, quantifying eosinophils (CD11b$^+$Siglec F$^+$, left), neutrophils (CD11b$^+$Ly6G$^+$, middle) and alveolar macrophages (CD11c$^+$Siglec F$^+$, right) as the product of their frequency among live cells, and the total cell count in BAL fluid. $n = 15$, 19 and 19 mice, respectively. $^*P < 0.05$, $^{**}P < 0.001$ and $^{***}P < 0.0001$ (two-way ANOVA with Bonferroni post-tests (**a**) or one-way ANOVA with Dunnett's post-test (**c**–**e**)). Data were pooled from two (**a**,**c**,**d**) or four experiments (**e**; mean ± s.e.m. (**a**,**e**) or median with interquartile range (**c**,**d**) or are representative of two experiments (**b**).

role in distinct differentiation environments or may have a similar role in a variety of contexts. For example, miR-19 regulated the production of T_H2 cytokines in part through PTEN, a target that has widespread effects on effector programs of helper T cells[18,19,21,23]. In contrast, SOCS1 and A20 were limiting factors for the production of T_H2 cytokines but not for that of T_H1 or T_H17 cytokines. The same concept applies to transcription factors and their target genes. For example, c-Maf regulates production of both T_H2 cytokines and T_H17 cytokines through distinct molecular pathways. Similarly, GATA-3 is the principal determinant of T_H2 cell differentiation and a direct regulator of *Il13* and *Il5*, but it also regulates T cell development in the thymus and survival in the periphery. Our findings have demonstrated that careful delineation of target networks might reveal not only the mechanisms through which transcription factors and miRNAs mediate their functions but also novel or unexpected limiting requirements for downstream genes and pathways that coordinate T cell–mediated immunity.

We identified at least three important pieces of the miR-19 target network in T_H2 cells: *Pten*, *Socs1* and *Tnfaip3*. Each of these target mRNAs encodes an inhibitor of a distinct signaling pathway, which indicated that miR-19 augmented the production of T_H2 cytokines by simultaneously amplifying signaling via PI(3)K, Jak-STAT and NF-κB. However, our analysis revealed a limiting independent role for each target, as depleting each individually was sufficient to markedly increase T_H2 cytokine production. These pathways are all essential components of the signaling via receptors for antigens and cytokines that induces the differentiation and effector function of T cells.

PTEN inhibits the PI(3)K pathway, which promotes T cell proliferation and cell survival[33], and deletion of one allele of *Pten* results in autoimmunity and lymphoproliferative disease in mice[34]. PTEN is an important target of miR-17~92 in the differentiation of T_H1 cells, T_H17 cells and T_{FH} cells[18,21,23,24], but its effects on cytokine production by T_H2 cells have remained unknown. Genetic 'rescue' of PTEN overexpression in 17~92$^\Delta$ cells partially restored the production of T_H2 cytokines, which indicated that both PTEN and other targets contributed substantially to the T_H2 phenotype.

SOCS1 inhibits the Jak-STAT pathway downstream of cytokine receptors[35] and favors T_H17 differentiation over T_H1 differentiation by repressing signaling downstream of IL-12 and IFN-γ[36,37]. Production of both IFN-γ and IL-4 is greater in CD4$^+$ T cells in SOCS1-deficient mice than in wild-type mice[38], and SOCS1 inhibits IL-4 signaling in macrophages[39]. Such findings have led to speculation that SOCS1 may inhibit T_H2 responses[40], but this possibility had remained untested before our experiments, to our knowledge. Furthermore, SOCS1 has not been reported to be an important target of miR-17~92 in regulating the differentiation and effector functions of any helper T cell subset.

Tnfaip3 encodes A20, a constitutively expressed negative regulator of the NF-κB pathway in T cells. A20 has been identified as an important target of miR-19 in macrophages[30]. Inhibition of A20 increases IL-2 production in Jurkat cells[41], but its effect on T_H2 cytokine production had not yet been described, to our knowledge. Identification of these three targets will improve understanding of the intracellular components that regulate T_H2 cytokine production. Genome-wide approaches to determine the full miR-19 target network in T_H2 cells would probably reveal additional targets involved in helper T cell biology.

We hypothesized that miRNA-expression profiling in airway-infiltrating T cells might reveal functionally relevant miRNAs and pathways that are directly involved in asthma pathogenesis. Indeed, we identified miR-19a as a candidate regulator of T_H2 responses through miRNA profiling[25,26] in the small number of CD4$^+$ T cells that could be recovered from BAL fluid. Published studies have identified miRNAs of interest by profiling expression in complex cell mixtures, such as whole lungs in animal models[14], epithelial cell brushings[12] or mixed lymphocytes from peripheral blood of asthmatic subjects[11,42]. Interpretation of data from unseparated tissues can be ambiguous. Differences in miRNA expression might reflect changes in the cellular composition of samples from asthmatic subjects, and the formation of mechanistic hypotheses about disease pathogenesis will require further work to identify which cell type(s) exhibit differences in the expression of any miRNA of interest. Additional studies are needed to confirm the observed increase in miR-19a in airway CD4$^+$ T cells in asthma and to determine whether this change is limited to T_H2 cells in this context. Larger studies may also identify correlations between miR-19a expression and asthma severity, lung function, response to corticosteroid treatment, or biomarkers of T_H2 inflammation that stratify asthma phenotypes[1,43].

Although clusters of miRNAs are transcribed as a single polycistronic pri-miRNA transcript, each mature miRNA in the cluster is not necessarily expressed at the same abundance in a given cell type and condition. Nevertheless, we were surprised to find an increase specifically in miR-19a, not other members of the miR-17~92 cluster, in airway-infiltrating T cells in asthma. miR-19a and miR-19b are also 'preferentially' increased in premalignant cells in a mouse model of B cell lymphoma and in human Burkitt's lymphoma cell lines[16]. A specific increase in miR-19a might be mediated by 'preferential' processing from the polycistronic miR-17~92 pri-miRNA transcript or by a sequence-specific increase in the efficiency of some other step in miR-19a biogenesis. Alternatively, mature miR-19a might be specifically stabilized in T cells in inflamed lungs. Regardless of the mechanism, the observed increase in miR-19a abundance should have a substantial effect on the secretion of IL-13, as altering miR-19a activity by overexpression or depletion in both human and mouse primary T cells altered IL-13 production *in vitro*.

The identity and function of miRNAs that regulate type 2 inflammation in a T cell–intrinsic manner have remained uncertain. miR-155 has been a major focus because miR-155–deficient T cells have a modest bias toward T_H2 differentiation *in vitro*[44,45] and miR-155–deficient mice develop partially penetrant spontaneous airway remodeling with some of the characteristics of asthma[44]. However, these mice are resistant to experimentally induced airway inflammation[46], which suggests that miR-155 might have a role in cell types other than T_H2 cells in this model. Our data suggested that upregulation of miR-19a in T cells in asthmatic airways might be an indicator and a cause of increased IL-13 production and probably contributed to type 2 inflammation in asthma. Indeed, 17~92$^{\Delta}$ T_H2 cells induced far less airway eosinophilia than did 17~92^{+} T_H2 cells in an allergic airway inflammation model, and restoration of miR-19a was sufficient to 'rescue' this defect.

Our study has linked miR-19a activity with human asthma and has identified mechanisms of miR-19a function in T cells, which suggests that miR-19 might be a useful drug target. Published animal model studies have confirmed the concept of miRNA-based therapy through the use of intranasal administration of sequence-specific miRNA inhibitors to ameliorate allergic airway inflammation[13,47–49]. In addition, identifying the target networks through which miRNAs act may be an effective path to the development of novel therapies. For example, our findings lend weight to the argument that NF-κB inhibitors might be effective in asthma and other allergic diseases[50]. Through the coordinated repression of several mRNA targets, miR-19 amplified signals that augmented production of T_H2 cytokines, the principle drivers of asthma pathogenesis.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

L.J.S. designed, performed and analyzed most experiments. S.P., D.F.C., H.D.B., X.R., Y.W., H.H.P., D.B., M.M.M., M.P. and K.A.R. helped design and perform some experiments. X.H. helped design animal airway allergy experiments. N.R.B. analyzed human miRNA expression data. P.G.W., J.V.F. and J.R.A. designed and helped perform the clinical study for human miRNA expression analysis. K.M.A. helped design, analyze and interpret all experiments. L.J.S. and K.M.A. wrote the manuscript. All authors reviewed and approved the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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ONLINE METHODS

Human subjects. Bronchoalveolar lavage (BAL) fluid was obtained from three groups of subjects: asthmatic subjects that had not been treated with inhaled corticosteroids (ICS) for 6 weeks before the study start date, called 'steroid-naive' here; asthmatic subjects that were treated continuously with ICS, called 'steroid-using' here; and healthy control subjects. BAL was obtained from steroid-naive subjects at baseline and again after 8 weeks of ICS treatment with 200 µg budesonide twice a day. BAL was obtained from steroid-using subjects after their ICS treatment was standardized to a regimen of 200 µg budesonide twice a day for 8 weeks.

This study, called the Study of the Mechanisms of Asthma (MAST), was registered on clinicaltrials.gov: NCT00595153. A power calculation was used to determine the number of subjects needed to measure differences in expression of 3 biomarker genes of IL-13 exposure in the airway. We used all sorted T cell samples available from this completed study. Inclusion and exclusion criteria for human subjects are provided online (clinicaltrials.gov). Written informed consent was obtained from all subjects, and all studies were performed with approval from the UCSF Committee on Human Research.

Sorting of clinical BAL samples. Investigators were blinded to the group allocation of subjects during cell sorting and sample processing. Live $CD3^+CD4^+$ T cells were sorted with a FACSAria flow cytometer (BD Biosciences) from BAL fluid cells transported in ice-cold PBS with 5% FCS within approximately 4 h of collection. Anti-CD3–PE (MHCD03044) was from Invitrogen. Anti-CD4–Cy7 (557852), anti-CD8–APC-Cy7 (557834) and PI (51-66211E) were from BD Biosciences. The average purity of the sorted cells, confirmed by flow cytometric analysis, exceeded 95%. Sorted cells were washed in ice-cold PBS with 2% FCS and 2 mM EDTA and then lysed in RLT buffer (Qiagen) before being 'snap-frozen' and stored at −80 °C.

High-throughput multiplex qPCR. High-throughput multiplex qPCR was performed according to a published protocol[26]. Briefly, $CD4^+$ T cells sorted by flow cytometry from clinical samples were lysed and stored in RLT buffer (Qiagen) at −80 °C until all samples were collected. Frozen lysates were thawed and loaded onto QIAshredder homogenizer columns (Qiagen). Using a low concentration of ethanol (35% v/v), the large fraction of RNA (>150 nt) was precipitated and captured on collection columns (RNeasy; Qiagen). The small fraction of RNA (<150 nt) does not bind in a low concentration of ethanol, and so the flow-through from the first column was brought up to a concentration of 75% v/v ethanol and RNA was precipitated and collected on a fresh column (MinElute; Qiagen). Both RNA fractions were treated with DNase per manufacturer's recommendations (Qiagen). 2 µl of RNA from each large fraction, as well as a standard curve of RNA from *in vitro*-derived T_H2 cells, were reverse transcribed using the SuperScript III kit (Invitrogen). RNA was then quantified by qPCR with primers for the gene encoding β_2-microglobulin and comparison to to the standard curve of RNA. We calculated 100 pg RNA from the small fraction for each sample for multiplex reverse transcription using 2 separate mixes of 96 miRNA stem-loop reverse primers at a final concentration of 1nM (**Supplementary Table 1**). Reverse transcription and 20 cycles of preamplification were performed according to published protocols[25,26]. Excess primers were removed from samples using 9 µl ExoSAP-IT (USB) per sample, and then 15 µl of each sample was purified on Illustra AutoScreen-96 Well Plates (GE Healthcare). MiRNA qPCR data was collected on the BioMark system (Fluidigm) using a 96.96 Dynamic Array Integrated Fluidic Circuit (IFC).

Fluidigm Biomark qPCR data analysis. Data was first analyzed on Real Time PCR Analysis software (Fluidigm) with the quality threshold set at 0.5, baseline correction set to linear (derivative), and Ct threshold method set to auto (detectors). The data was further analyzed in R (version 2.14.1) through the following pipeline: Ct values <5 or >28 were removed per Fluidigm recommendations; miRNAs that were detected in less than 60% of patient samples were removed from analysis; missing Ct values for each miRNA were replaced with the limit of detection (highest Ct value within the standard curve) plus 0.1; and the data were global mean-normalized per plate to account for inter-plate differences.

Mice. Mice with *lox*P-flanked alleles encoding miR-17~92 (*Mirc1*$^{fl/fl}$, 008458, The Jackson Laboratory), and *Rosa26*–miR-17~92–transgenic mice (Gt(ROSA)26Sor$^{tm3(CAG–MIR17–92,–EGFP)Rsky}$, 008517, The Jackson Laboratory) were crossed to CD4-Cre mice (4196, Taconic) to generate CD4-Cre;*Mirc1*$^{fl/fl}$ (called '17~92$^{\Delta}$' here), CD4-Cre;Gt(ROSA)26Sor$^{tm3(CAG–MIR17–92,–EGFP)Rsky}$ (called '17~92^{tg}' here) and CD4-Cre; *Mirc1*$^{+/+}$ or *Mirc1*$^{fl/fl}$ (collectively called '17~92^{+}' here). OT-II mice (004194) were from The Jackson Laboratory. Mice with *lox*P-flanked *Pten* alleles have been described[51]. *Cd28*$^{-/-}$ mice (002666) were from The Jackson Laboratory. All mice were housed and bred in the specific pathogen-free barrier facility at the University of California, San Francisco. The Institutional Animal Care and Use Committee at the University of California, San Francisco, approved all animal experiments.

***In vitro* human cord blood T cell polarization.** Peripheral blood mononuclear cells (PBMCs) from anonymous human cord blood donors were isolated by Lymphoprep gradient (1114545; Accurate Chemical & Scientific). $CD4^+$ T cells were isolated from PBMCs using the Dynabeads Untouched Human $CD4^+$ T Cell Isolation Kit (Invitrogen). Cells were stimulated for ~65 h on plates coated with 1 µg/ml anti-CD3 (UCSF Monoclonal Antibody Core; clone OKT-3) and 2 µg/ml anti-CD28 (UCSF Monoclonal Antibody Core; clone 9.3) at an initial density of 0.7×10^6 cells/ml. After stimulation, the cells were rested for 2 d in media containing 20 units/ml recombinant human IL-2 (NCI). For T_H2-polarizing conditions, 12.5 ng/ml recombinant human IL-4 (200-04; PeproTech) was added throughout the 5 d. T cell culture was in RPMI-1640 media with 10% FCS, pyruvate, nonessential amino acids, L-arginine, L-asparagine, L-glutamine, folic acid, beta mercaptoethanol, penicillin and streptomycin.

***In vitro* mouse primary T cell polarization.** $CD4^+$ T cells were isolated from spleen and lymph nodes of young male and female mice (4–8 weeks old) with a Mouse CD4 Dynabeads Isolation kit (L3T4; Invitrogen). Mice of different genotypes were matched by age and sex in experiments. For experiments including CellTrace Violet (Invitrogen), cells were stained at 5µM in PBS for 20 min at 37 °C, quenched with 5 volumes of media with 10% FCS for 5 min, and washed twice in media before culture. For all conditions, cells were activated with hamster antibody to mouse CD3 (0.25 µg/ml; 2C11; UCSF Monoclonal Antibody Core) and mouse CD28 (1 µg/ml; 37.51; UCSF Monoclonal Antibody Core) on a plate coated with goat antibody to hamster IgG (0.3 mg/ml in PBS; MP Biomedicals) for ~65 h at an initial density of 0.7×10^6 cells/ml, and then were allowed to 'rest' for 48-72 h in medium with 20 units/ml recombinant IL-2 (National Cancer Institute). For T_H2-polarizing conditions, 500 units/ml IL-4 supernatant and 5 µg/ml anti-IFN-γ (XMG1.2; BioXCell) were added to the culture throughout the 5–6 d. 'Low T_H2' conditions included only 5 units/ml IL-4, and 'T_HN' conditions received no polarizing cytokines or antibodies. Cells subjected to T_H17-polarizing conditions received 5 ng/ml recombinant human TGF-β (PeproTech), 25ng/ml recombinant murine IL-6 (PeproTech), 10 µg/ml anti-IL-4 (11B11; BioXCell) and 10 µg/ml anti-IFN-γ (XMG1.2; BioXCell) throughout the 5 d of culture, as well as 20 ng/ml recombinant mouse IL-23 (R&D Systems) on days 3–5. All cultures used DMEM high glucose media supplemented with 10% FCS, pyruvate, nonessential amino acids, MEM vitamins, L-arginine, L-asparagine, L-glutamine, folic acid, β-mercaptoethanol, penicillin and streptomycin.

miRNA mimics, miRNA inhibitors, siRNA, and miRNA sensors. During *in vitro* polarization, human or mouse primary $CD4^+$ T cells in culture were transfected with miRNA mimics, inhibitors, or siRNAs (Dharmacon) at 24 and 96 h of culture. miRIDIAN miRNA mimics were used at 500 nM per transfection. miRIDIAN miRNA Hairpin Inhibitors were also used at 500 nM. siGENOME SmartPools and ON-TARGETplus Individual siRNA were used at 500 nM. Cells were transfected using the Neon Transfection system by Invitrogen as described[20]. Retroviral miRNA sensors containing 4 perfectly complementary binding sites for miR-1, miR-19a, and miR-19b were constructed and transduced at 48 h as described[20].

Intracellular cytokine and transcription factors stains. For cytokine staining, cells were restimulated with 20 nM PMA and 1 µM ionomycin for

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4 h, and 5 μg/ml brefeldin A was added during the last 2 h of restimulation. Cells were stained with the viability dye eFluor780 (65-0865-14; eBiosciences), and then fixed in 4% paraformaldehyde for 8 min at room temperature. Cytokine staining was done in permeabilization buffer containing 0.5% saponin (Invitrogen). For transcription factor staining, unstimulated cells were fixed, permeabilized, and stained using the FoxP3 Staining Kit (00-5523-00; eBiosciences). Samples were analyzed with a flow cytometer (BD LSR II). Mouse antibodies were as follows: anti-IL-13–PE (eBio13A; eBiosciences), anti-IL-4–APC (11B11; eBiosciences), anti-IL-5–PE (TRFK5; Biolegend), anti-IFN-γ–FITC (XMG1.2; eBiosciences), anti-TNF–Alexa Fluor 700 (MP6-XT22; BD Biosciences), anti-IL-17A–eFluor 450 (eBio17B7; eBiosciences), anti-IL-17F–PE (079-289; BD Pharmingen) and anti-GATA-3–PE (eBiosciences; E50-2440). Human antibodies were as follows: anti-IL-13–FITC (eBiosciences; PVM13-1) and anti-IL-4–APC (BD Biosciences; 8D4-8).

***In vivo* allergic airway inflammation model.** For each *in vivo* experiment, we used five mice per group to gain power for statistical analysis, and repeated the model to pool data and determine reproducibility. Mice received 3 different cell types in rotation such that each cage had one to two recipients for each cell type. Mice were challenged in random order and were tested for pulmonary resistance in rotating order through each group. One mouse was removed from analysis owing to low body weight and lack of movement; histological analysis confirmed signs of emphysema. Six- to eight-week old sex-matched littermate *Cd28*$^{-/-}$ mice were injected retro-orbitally with 2×10^5 *in vitro* T_H2-polarized 17~92$^+$ or 17~92$^\Delta$ OT-II cells. The mice were challenged oropharyngeally with 50 μg ovalbumin (OVA) in PBS at 24, 48, and 72 h post transfer to induce allergic airway inflammation. 18 h after the final challenge, mice were anesthetized with ketamine and xyalazine, and pulmonary resistance was measured by trachea cannulation in response to intravenous acetylcholine, as described[52]. BAL was collected by 5 lavages of 0.8 ml of PBS for flow cytometry, and lungs were filled with 10% buffered formalin and fixed for histology. BAL cells were washed, counted, and surface stained for analysis by flow cytometry. Antibodies used for quantification of myeloid cells were as follows: anti-CD11b–Alexa Fluor 488 (M1/70; eBiosciences), anti-CD11c-PE-Cy7 (N418; Biolegend), anti-Ly6G-V450 (1A8; BD Biosciences) and anti-Siglec F-PE (E50-2440; BD Biosciences). Two paraffin-embedded 5-μm sections of the whole lung from each mouse were stained with H&E and PAS. To quantify inflammation, H&E-stained lung sections were de-identified for blinding and scored for peribronchial and perivascular inflammatory cell infiltration: grade 0, no infiltration; grade 1, <25% of examined area; grade 2, 25–50%; grade 3, 51–75%; and grade 4, >75%. To quantify goblet cell hyperplasia, PAS stained lung sections were de-identified for blinding and scored for the percentage of PAS positive cells among airway epithelial cells: grade 0, none; grade 1, <25% of airway epithelial cells; grade 2, 25–50%; grade 3, 51–75%; and grade 4, >75%.

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