Published in final edited form as:

Nat Immunol. ; 13(6): 587–595. doi:10.1038/ni.2286.

TGF- β and retinoic acid induce miR-10a, which targets Bcl-6 and constrains helper T cell plasticity

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Abstract

Distinct CD4⁺ T cell subsets are critical for host defense and immunoregulation. While these subsets can behave as terminally differentiated lineages, elements of plasticity are increasingly recognized. MicroRNAs are one factor that controls stability and plasticity. Herein, we report that miR-10a was highly expressed in naturally-occurring regulatory T (T_{reg}) cells and induced by retinoic acid and TGF- β in inducible T_{reg} cells. By simultaneously targeting Bcl-6 and a co-repressor, Ncor2, miR-10a attenuated phenotypic conversion of inducible T_{reg} cells to follicular helper T cells. miR-10a also limited T_H17 differentiation and therefore represents a new factor that can fine tune plasticity and fate decision of helper T cells.

INTRODUCTION

Appropriate differentiation of CD4⁺ T cell subsets is important for proper adaptive immune responses and is achieved by alteration of genetic programs controlled by transcription factors and/or repressors induced by extracellular cues¹. Cytokines in the milieu of differentiating T cells are the key exogenous factors in driving specification, such that multiple, distinct fates are possible for naïve T cells including T helper (T_H)1, T_H2 and T_H17 cells. Regulatory T (T_{reg}) cells are yet another subset of CD4⁺ T cells that serve to suppress activity of effector T cells, and thus maintain immune homeostasis. T_{reg} cells can be further divided into two populations, naturally-occurring T_{reg} (nT_{reg}) that arise in the thymus and inducible T_{reg} (iT_{reg}), which can arise in the periphery from naïve CD4⁺ precursors. Both of these subsets express Foxp3, a master transcription factor that defines this lineage and factors that promote expression of Foxp3 include: TGF- β , IL-2 and retinoic acid (RA)^{2–4}. T follicular helper (T_{FH}) cells are another functional subset, which is critical for providing help to B cells in germinal centers⁵. T_{FH} can arise from naïve CD4⁺ T cells; however, the relationship between T_{FH} cells and other subsets is the subject of considerable debate and ongoing investigation⁵.

There is evidence that these subsets behave as lineages with respect to expression of specific cytokines and lineage-defining master regulator transcription factors. However, there is also much emerging information on functional plasticity of helper T cells⁶. One example of plasticity is the generation of T_H1 cells from T_H17 cells⁷. Another particularly dramatic example is the conversion of polarized T_H2 cells to IFN- γ producers in the setting of viral infection⁸. Unlike nT_{reg} cells, iT_{reg} cells exhibit incomplete demethylation of the *Foxp3* locus⁹. While the latter may be intrinsically less stable, nT_{reg} cells are generally viewed as a more stable subset¹⁰. However, even for nT_{reg} cells, there is evidence that they can alter their phenotype. For instance, nT_{reg} cells can convert to T_{FH} cells in the environment in Peyer's patches¹¹ or become T_H17 cells upon stimulation with IL-6 *in vitro*¹². There are still other examples of conversion from other subsets such as T_H2 to T_{FH} cells¹³ and conversely conversion of T_{FH} to other effector subsets including T_H1, T_H2 and T_H17¹⁴. However, the molecular mechanisms underlying phenotype switch involving T_{reg} and other cells are incompletely understood.

MicroRNAs (miRNAs) are small non-coding RNAs, which regulate gene expression at post-transcriptional level by directly binding to mRNA of target genes. miRNAs are critically involved in a wide variety of biological processes of the cells from embryonic stem cell pluripotency to cancer tumorigenicity¹⁵. Evidence showing critical roles of miRNAs in immune cells continue to mount^{16–24}. Drosha and Dicer are two key components of the machinery responsible for miRNA generation and loss of these factors is associated with defects in lymphocyte differentiation and autoimmunity^{25–27}. With respect to the issue of CD4⁺ T cell plasticity, deficiency of Dicer resulted in helper T cell instability, unstable expression of *Foxp3* in T_{reg} cells and skewing to a T_H1 phenotype²⁸. Similarly, CD4⁺ T cells deficient in Drosha showed accelerated T_H25 differentiation and reduced iT_{reg} differentiation²⁵. Collectively, these data argue that miRNAs are important factors in preserving phenotypic stability of helper T cells. Furthermore, the data raise the question as to which specific miRNA(s) are responsible for maintaining lineage phenotype.

In this study, we investigated the function of miR-10a, which is highly expressed in nT_{reg} cells and induced by TGF- β and RA. We found that miR-10a controls the levels of expression of two key repressors, Bcl-6 and Ncor2, limits the conversion of iT_{reg} cells to T_{FH} cells. Under conditions in which naïve CD4⁺ T cells are exposed to RA, miR-10a inhibits T_H17 differentiation. This effect is dependent upon the induction of T-bet by RA. Our data demonstrate that miR-10a is one factor that preserves T_{reg} phenotype by targeting and constraining transcription factor pathways that promote alternate fates.

RESULTS

miR-10a is preferentially expressed in T_{reg} cells and induced by retinoic acid and TGF- β

To identify miRNAs with preferential expression in different T cell subsets, we obtained genome-wide miRNA expression profiles via massive parallel sequencing (miRNA-seq)²⁹. Among miRNAs that were differentially expressed and abundant in T cells, miR-10a-5p, referred to as miR-10a hereafter, was identified as the most selective marker of nT_{reg} cells versus other T cell subsets (Supplementary Fig. 1a). By contrast, another paralogous sequence, miR-10b-5p, was not detected in any of T_H subsets (data not shown). We next confirmed the selective expression of miR-10a by quantitative RT-PCR and found that T_H1, T_H2, T_H17, and naïve CD4⁺ T cells showed very low miR-10a expression, consistent with sequencing data (Fig. 1a and Supplementary Fig. 1b). By contrast, nT_{reg} cells expressed high levels of miR-10a. Because *in vitro* generated iT_{reg} cells share many of the functional properties of nT_{reg} cells, we next compared the expression of miR-10a in these two types of cells. Interestingly, we found that iT_{reg} cells expressed modest levels of miR-10a compared

to nT_{reg} cells; nonetheless, the levels were significantly higher in iT_{reg} cells compared to other T_H subsets.

Since the generation of iT_{reg} *in vitro* requires TGF- β , we next asked if this was a relevant factor for miR-10a induction. As shown in Fig. 1b and c, TGF- β induced miR-10a in a dose-dependent manner whereas IL-2 had no effect. Because the induction of miR-10a by TGF- β was modest, we considered other factors that might regulate its expression. Another means of inducing iT_{reg} cells is through exposure of T cells to RA^{3, 4}. This was notable as miR-10a resides within Hox gene cluster on murine chromosome 11, a genomic segment that is highly evolutionarily conserved among mammals. Hox genes are highly regulated by RA and in non-T cells miR-10a has been reported to be induced by RA³⁰. Consistent with these previous results, we noted that all-trans RA (ATRA) dramatically induced miR-10a expression in a dose-dependent manner. Importantly though, we also noted that the induction of miR-10a by ATRA was totally abolished by blocking TGF- β (Fig. 1d). This has not been appreciated previously and thus in contrast to previous findings, our data support the idea that RA and TGF- β are both required for induction of miR-10a in T cells. Along this line, we also found that the induction of miR-10a expression was blocked in the presence of the pan-RA receptor (RAR) inhibitor, LE540 (Fig. 1e).

To help explain the interaction of RA and TGF- β , we considered the possibility that these factors might be impinging upon one another's signaling pathways. Indeed, we found that TGF- β induced RAR α expression in a dose-dependent manner (Fig. 1f). Accordingly, in conjunction with TGF- β , we found that a RAR α agonist but not a RAR γ agonist induced miR-10a expression to levels equivalent to those induced by ATRA (Fig. 1g). Collectively these results demonstrated that RA-induced miR-10a expression is mediated through RAR α in a TGF- β -dependent manner, with the latter inducing expression of the former.

miR-10a directly targets Bcl-6 and Ncor2 and down-regulates protein expression

Since identification of target genes by miR-10a is indispensable to understanding how the miRNA functions in helper T cells, we next performed *in silico* analysis to predict target mRNAs based on nucleotide sequences using PicTar (<http://pictar.mdc-berlin.de/>). Among the predicted targets, mRNAs for Bcl-6 and Ncor2 were identified as carrying potential miR-10a target sequences in their 3' untranslated regions (3'UTR) (Supplementary Fig. 2a). The target genes were also predicted by another algorithm, TargetScan (<http://www.targetscan.org>), and the target sequences in 3'UTR of Bcl-6 and Ncor2 are highly conserved among various species (Supplementary Fig. 2b). We reasoned that if miR-10a were involved in fate decision of helper T cell phenotype, these targets would provide good potential mechanisms for transmitting its effects. That is, Bcl-6 is as a key transcriptional repressor thought to be a master regulator for T_{FH} cells and Ncor2 is a co-repressor that complexes with Bcl-6 and RAR to suppress transcription of genes regulated by these factors^{31–35}.

To investigate whether miR-10a regulates Bcl-6 and Ncor2 expression, we first generated luciferase reporter constructs carrying 3'UTR of Bcl-6 or Ncor2 that comprised potential miR-10a target sequences linked to a luciferase gene (Fig. 2a). We found that over-expression of miR-10a significantly reduced activity of constructs expressing the Bcl-6 and Ncor2 target sequences (Fig. 2a: middle bars). However, miR-10a had no effect on the expression of constructs lacking these target sequences (Fig. 2a: right bars). These results demonstrate that, as predicted, miR-10a can directly target sequences in the 3'UTR of Bcl-6 and Ncor2.

We next sought to evaluate whether miR-10a influences endogenous Bcl-6 and Ncor2 protein levels. To this end, we first used CH12, a murine B cell lymphoma cell line that

constitutively expresses Bcl-6 and found that miR-10a over-expression decreased Bcl-6 protein levels as measured by flow cytometry and immunoblotting (Fig. 2b and c). To assess whether miR-10a regulates Bcl-6 in primary helper T cells, we over-expressed miR-10a in helper T cells using retroviral transduction and measured Bcl-6 protein levels. We found that miR-10a over-expression significantly decreased Bcl-6 expression as determined by flow cytometry and immunoblotting (Fig. 2d and e). Reduction in Bcl-6 protein levels was associated with compensatory increases in Bcl-6 mRNA levels (Fig. 2f). Conversely, over-expression of Bcl-6 protein using retroviral transduction suppressed endogenous Bcl-6 mRNA levels (Supplementary Fig. 3), consistent with previous data³⁶.

To simplify the complex regulation of Bcl-6 expression, we expressed a tagged Bcl-6 construct under a heterologous promoter and manipulated miR-10a levels by over-expressing or by sequestering miR-10a with “sponge target sequence” (Supplementary Fig. 4)³⁷. As shown in Fig. 2g, over-expression of miR-10a down-regulated Bcl-6 expression, whereas expression of sponge target sequence restored expression (Fig. 2g, red and green column). However, deletion of miR-10a target sequence from 3'UTR eliminated this regulation (Fig. 2g, blue column). Over-expression of miR-10a in helper T cells also reduced Ncor2 protein levels, but had little effect on mRNA levels (Fig. 2h and i).

Finally, to verify the effect of endogenous miR-10a in regulating its target proteins, we expressed the miR-10a sponge sequence in iT_{reg} cells. We found that expression of the miR-10a sponge sequence significantly up-regulated expression of Bcl-6 and Ncor2 compared to expression of a control vector (Fig. 2j and k).

Considering that Ncor2 is a co-repressor of RAR α , it seemed plausible to expect that reduction in Ncor2 levels would augment the ability of RA to induce miR-10a expression by relieving the inhibitory effects of Ncor2. To examine this possibility, we utilized retroviral expression of shRNA to knockdown Ncor2 expression (Supplementary Figure 5a and b) and cultured the cells with RA. Consistent with our previous results, RA induced expression of miR-10a in a dose-dependent manner; however, inhibiting Ncor2 shifted the dose-response curve to the left, making the cells more sensitive to RA and more effectively inducing miR-10a (Supplementary Figure 5c). Manipulating the levels of another miR-10a target, either by knocking down and over-expressing Bcl-6 had no effect on RA-induced miR-10a expression (Supplementary Figure 5c and Supplementary Figure 6). These results suggest that miR-10a amplifies its own expression during ATRA treatment by forming a positive-feedback loop through targeting Ncor2. A recent report that showed increase in miR-10a level by inhibition of Ncor2 in neuroblastoma cell line is in line with our results³⁸.

miR-10a constrains conversion of iT_{reg} to T_{FH}

Since Bcl-6 is pivotal for T_{FH} differentiation^{31–33}, we hypothesized that miR-10a might function to limit T_{FH} generation by suppressing Bcl-6. Since T_{reg} cells can convert to T_{FH} cells in Peyer's patches (PP), this is a circumstance in which miR-10a might be functionally relevant¹¹. In order to evaluate a potential effect of miR-10a on T_{reg}-T_{FH} conversion, we adapted this previously reported model, using iT_{reg} cells rather than nT_{reg} cells, since the former are more suited for effective retroviral transduction. To this end, naïve CD4⁺ and *in vitro* differentiated iT_{reg} cells derived from Foxp3^{GFP} mice were transferred into TCR α ^{-/-} mice and the appearance of T_{FH} in PP was assessed 6 weeks after adoptive transfer (Supplementary Fig. 7a and b). Immunofluorescence staining showed that transferred naïve CD4⁺ T cells resided predominantly in interfollicular regions, although a proportion of the cells localized to germinal centers (GC) of PP suggesting differentiation of the naïve T cells to T_{FH} (Supplementary Fig. 7c left panel). To further confirm T_{FH} differentiation of the naïve T cells, we used flow cytometry to assess expression of the characteristic surface markers PD-1 and CXCR5 (Supplementary Fig. 7d left panel). Consistent with the

immunofluorescence staining, it was clear that a portion of the transferred naïve CD4⁺ T cells acquired characteristics of T_{FH} cells. Similarly, transferred *in vitro*-generated iT_{reg} cells localized to GC of PP (Supplementary Fig. 7c middle panel) and they also acquired expression of PD-1 and CXCR5; in fact, iT_{reg} cells were more efficient in their ability to convert to T_{FH} cells than were naïve CD4⁺ T cells (Supplementary Fig. 7d). As a control, we also assessed GFP⁺ (Foxp3⁺) expression in transferred naïve and iT_{reg} cells. GFP⁺ (Foxp3⁺) cells were barely detectable in PP and spleens when naïve CD4⁺ T cells were transferred (Supplementary Fig. 7e). On the other hand, in mice that received GFP⁺ iT_{reg} cells, cells more easily lost GFP expression in PP compared to spleen, suggesting the PP environment promoted T_{FH} conversion (Supplementary Fig. 7e).

We next evaluated the effect of inhibiting miR-10a function on the conversion of iT_{reg} to T_{FH} cells. To this end, we generated iT_{reg} cells (97.7 ± 0.4 % Foxp3⁺, data not shown), which expressed the miR-10a sponge sequence or a control sequence. We then adoptively transferred the transduced iT_{reg} cells into TCRα^{-/-} mice and assessed the acquisition of T_{FH} markers on this cell population. Compared to cells transduced with the control vector, cells transduced with the miR-10a sponge vector more readily acquired the T_{FH} markers PD-1 and CXCR5 in PP (Fig. 3a). This was associated with significantly more germinal center B cells in this tissue (Fig. 3b).

To verify this finding, we next determined the effect of over-expressing miR-10a in iT_{reg} cells, reasoning that enforced expression of miR-10a should limit T_{FH} conversion by attenuating levels of Bcl-6. As shown in Fig. 3c, we observed that over-expression of miR-10a significantly reduced the efficiency of transferred iT_{reg} cells to convert into T_{FH} cells in PP, underscoring the crucial roles of miR-10a in regulating iT_{reg} to T_{FH} conversion.

We next measured the levels of miR-10a and Bcl-6 in T_{FH} (CD4⁺Vβ⁺PD-1^{hi}CXCR5^{hi}) cells and non-T_{FH} (CD4⁺Vβ⁺PD-1⁻CXCR5⁻) cells from PP (Supplementary Fig. 8). As expected, Bcl-6 was preferentially expressed in T_{FH} cells compared to non-T_{FH} cells (Fig. 3d), whereas the opposite was the case for miR-10a (Fig. 3e). Recently a new population of helper T cells designated follicular regulatory T cells (T_{FR}) has been identified. These cells express both Foxp3 and Bcl-6, and exert a regulatory function in germinal center^{39–41}. To assess miR-10a expression levels in this interesting subset, we isolated three populations: T_{FR} (Foxp3⁺CD4⁺Vβ⁺PD-1^{hi}CXCR5^{hi}), T_{reg} (Foxp3⁺CD4⁺Vβ⁺PD-1⁻CXCR5⁻) and Foxp3⁻T_{FH} (Foxp3⁻CD4⁺Vβ⁺PD-1^{hi}CXCR5^{hi}) (Supplementary Fig. 9a). As shown in Supplementary Fig. 9b, T_{FR} cells expressed higher levels of miR-10a than T_{reg} cells. Taken together, these results argue that one function of miR-10a is to constrain iT_{reg} to T_{FH} conversion.

Biphasic effects of RA on T_H17 differentiation

Deficiency of Bcl-6 has also been reported to influence IL-17A production³². Consequently, miR-10a might also regulate IL-17A production; however, effects of miR-10a would only come in to play in the presence of RA and in previous work the effect of RA was not considered. Additionally though, alterations in Ncor2 levels could affect sensitivity to RA through effects on RAR³⁴. Thus, miR-10a might alter T_H17 differentiation in the context of exposure to RA, but it was not obvious as to what that effect might be. Moreover, whereas RA was originally reported to induce iT_{reg} and suppress T_H17 differentiation³, more recent work suggests that RA is necessary for normal immune responses and can enhance inflammation^{42, 43}.

These findings led us to re-examine the effect of RA on T_H17 differentiation to elucidate potential effects of miR-10a. Consistent with previous reports, we found that 10 nM ATRA inhibited IL-17A production and enhanced Foxp3 expression (Fig. 4a and b). However,

physiologic levels of RA (1 nM)⁴⁴ enhanced the proportion of IL-17A-producing T cells (Fig. 4b; blue triangles). Previous work has demonstrated that IL-2 inhibits T_H17 differentiation⁴⁵ and the positive effect of RA on IL-17A production was even more evident in situations in which IL-2 was neutralized (Fig. 4b; green circles). As a control, cells cultured under iT_{reg} condition without IL-6 did not produce IL-17A even with low dose of RA (Fig. 4b; black diamonds). Interestingly, the magnitude of IL-17A production, assessed by MFI, increased at all doses of ATRA (Supplementary Fig. 10), despite the reduction in the proportions of IL-17A-producing cells. The discrepancy between MFI and percentage of IL-17A⁺ cells on ATRA response underscores the complex biological effects of ATRA on T_H17. Under these culture conditions, ATRA significantly up-regulated miR-10a expression in a dose-dependent manner (Supplementary Fig. 11).

TGF- β is another factor that regulates IL-17A production, with low doses of TGF- β promoting IL-17A production and high doses inhibiting⁴⁶. However, as indicated above, TGF- β also enhances T cell responsiveness to ATRA by up-regulating RAR α expression (Fig. 1f). We therefore assessed the combined effects of RA and TGF- β on *in vitro* T_H17 differentiation (Fig. 4c and d). Consistent with previous reports, T_H17 differentiation was more efficient at lower concentrations of TGF- β and inhibited by higher concentrations. The ability of RA to enhance IL-17A production was more dramatic at high concentrations of TGF- β , but regardless of the concentration of TGF- β , the biphasic effects of RA were evident (Fig. 4c and d). These results indicate that T_H17 differentiation can be fine-tuned by ATRA and TGF- β ; inefficient induction of T_H17 response that might occur with higher levels of TGF- β can be compensated by the presence of RA.

miR-10a can constrain T_H17 differentiation

Given that the ability of RA to influence T_H17 differentiation and induce miR-10a, we next sought to analyze the impact of miR-10a expression on T_H17 differentiation *in vivo* using a disease model dependent upon these cells. To this end, we transduced naïve CD4⁺ T cells from 2D2 transgenic mice and assessed whether miR-10 over-expression influenced the severity of experimental autoimmune encephalomyelitis. Following immunization with MOG peptide, mice that received 2D2 CD4⁺ T cells that over-expressed miR-10a had significantly delayed onset of neurological disease (Fig. 5a). Consistent with this finding IL-17A production in LN, spleen and CNS was significantly reduced in miR-10a-expressing 2D2 cells compared to cells transduced with the control vector, whereas there was no significant effect of miR-10a on IFN- γ production (Fig. 5b). These results argue that miR-10a can function as a factor that limits T_H17 responses *in vivo*. It is notable however, that although the onset of disease was delayed, disease was not abrogated. On the contrary, disease eventually occurred at the same severity as controls, suggesting that miR-10a fine tunes IL-17.

Having found that miR-10a over-expression can limit T_H17 differentiation *in vivo*, we set out to better understand the circumstances in which miR-10a can influence T_H17 differentiation. As shown in Figure 6a, when RA was used to generate T_H17 cells that expressed miR-10a, (data not shown), sequestering miR-10a with the sponge vector significantly increased the proportion of IL-17A producing cells compared to a control vector. Conversely, when miR-10a was over-expressed under the same conditions, T_H17 differentiation was inhibited (Fig. 6b). The alterations in IL-17A production were not due to changes in the levels of Foxp3 (Supplementary Figure 12). Interestingly though, when miR-10a was over-expressed in T_H17 cells cultured without RA, there was no significant effect (Fig. 6c). Thus, miR-10a appears to be a regulator of T_H17 differentiation, but only in circumstances in which RA is present.

T-bet-dependent regulation of IL-17A expression by Bcl-6 and Ncor2

The finding that miR-10a inhibited T_H17 differentiation was surprising to us. We had anticipated that since miR-10a inhibits Bcl-6 expression and Bcl-6 has been reported to inhibit IL-17A production³², miR-10a expression would promote T_H17 differentiation. We therefore next sought to clarify potential mechanisms by mimicking its effects and directly down-regulating expression of Bcl-6 and Ncor2. In contrast to previous reports, knocking down Bcl-6 resulted in reduced T_H17 differentiation (Fig. 7a). The same result was observed with knocking down Ncor2 levels (Fig. 7a). Thus, inhibiting Bcl-6 and Ncor2 expression recapitulated the effect of over-expressing miR-10a. While this result may seem counter-intuitive, it needs to be borne in mind that T_H17 differentiation occurred in the presence of RA in these experiments.

While modulating sensitivity of cells to RA by changing Ncor2 levels can alter T_H17 differentiation, it was less obvious why down-regulation of Bcl-6 might inhibit T_H17 differentiation. In this regard, a relevant Bcl-6 target is T-bet, an important negative regulator of T_H17 differentiation^{32, 33, 47}. Therefore we speculated that deletion of T-bet might reveal the positive action of Bcl-6 with regard to IL-17A production. In other words, if Bcl-6 antagonizes the action of T-bet, reducing the expression of the former would be expected to enhance the latter's function and thereby suppress T_H17 differentiation. To test the possible involvement of T-bet in Bcl-6-dependent regulation of T_H17, we evaluated the effect of Bcl6-KD in T-bet^{-/-} CD4⁺ T cells on T_H17 differentiation and found that decreasing Bcl-6 levels had no effect on T_H17 differentiation in the absence of T-bet (Fig. 7b). Thus, the reduction in IL-17A production associated with reduced expression of Bcl-6 is entirely regulated upon T-bet.

In addition to forming a complex with RAR α , Ncor2 forms a repressor complex with Bcl-6³⁵. We therefore wondered whether the effects of inhibiting Ncor2 might also be dependent upon T-bet. Of note, we found that the effect of knocking down Ncor2 on IL-17A production was also T-bet dependent (Fig. 7a and b; middle panels).

The unexpected involvement of T-bet in mediating regulation of T_H17 differentiation by miR-10a targets, Bcl-6 and Ncor2, prompted us next to evaluate the effect of RA on T-bet expression. As shown in Fig. 7c and d, RA was a very effective inducer of T-bet under T_H17 conditions. This result then helps explain the negative effect of miR-10a on T_H17 differentiation in the presence of RA and the lack of effect in the absence of RA (Fig. 6c).

DISCUSSION

In the present study, we investigated role of a microRNA that was found to be preferentially expressed in T_{reg} cells. We found that miR-10a was induced by RA and TGF- β and targets Bcl-6 and Ncor2. Functionally, we found that miR-10a limited the ability of iT_{reg} cells to convert to T_{FH} cells in PP, which was confirmed by both loss-of-function and gain-of-function experiments. We also found that miR-10a limited T_H17 differentiation *in vitro* and *in vivo* and that the effect of miR-10a could be phenocopied by reducing expression of its targets Bcl-6 and Ncor2. Strikingly though, the negative effects of Bcl-6 and Ncor2 knock-down on T_H17 differentiation were dependent upon T-bet, a transcription factor that we also found to be induced by RA. Thus, miR-10a appears to be a factor that limits conversion to T_{FH} and T_H17 cells in the setting of RA and TGF- β by regulating levels of Bcl-6 and Ncor2.

The importance of miRNA in T cell biology is rapidly becoming apparent. It is clear that mouse and human T_H subsets express distinct miRNAs^{16, 29}. Although there is clear evidence that miRNAs in general are critically involved in T cell stability^{25–27}, the precise roles of individual miRNAs have been less clear. However, this gap in knowledge is rapidly

being filled and several studies reported the roles of individual miRNAs in helper T cells. Such reports include miR-125b in maintenance of the naïve helper T cells, miR-182 in clonal expansion^{16, 19}, miR-326 in T_H17 differentiation, miR-29 in T_H differentiation^{17, 18, 21}, miR-146a in T_{reg}²⁰, miR-155 in T_H2 differentiation and T_{reg} development^{22–24}. It appears that miR-10a can be added to this list as a factor that influences T_{FH}/T_{reg} plasticity.

miR-10a is highly expressed in nT_{reg} but does not seem to directly regulate Foxp3 or other factors involved in T_{reg} homeostasis. Rather, it appears that by targeting Bcl-6, a relevant action of miR-10a with respect to T cell biology is to limit the ability of T_{reg} cells to acquire features of T_{FH} cells. That is, its function is to reduce the plasticity of T_{reg} cells rather than directly preserving Foxp3 expression or promoting suppressive activity of this subset. Of note in this regard is that iT_{reg} cells generated with RA were more likely to retain Foxp3 expression and were more resistant to conversion to other lineages compared to iT_{reg} cells generated by other methods⁴. The differential expression of miR-10a among nT_{reg} and RA-treated iT_{reg} cells helps explain the stability of these subsets and is consistent with the functional data presented in the present study.

Very recently, it has been shown that a subset of GC T cells express Foxp3 and Bcl-6^{39–41}. Functionally, it appears that these cells exhibit suppressive function. Our data show that miR-10a is highly expressed in T_{FR} cells compared to T_{reg} cells (Supplementary Figure 9b). Consistent with our view that miR-10a can limit T_{reg}/T_{FH} conversion, it is possible that miR-10a might have particularly important functions in this subset.

In our hands, miR-10a did not reduce Bcl-6 mRNA levels. While a previous report demonstrated that many miRNAs function by destabilizing their target mRNAs⁴⁸, it is also apparent that it is not always the case. However, the regulation of Bcl-6 expression is quite complex and subject to autoregulation³⁶. Given this complexity, it is difficult to say with certainty that miR-10a does or does not affect Bcl-6 mRNA. Nevertheless, the preponderance of data clearly indicate that miR-10a can influence Bcl-6 protein levels. There is less information on Ncor2 mRNA regulation, but in any case it does appear that miR-10a has immunologically relevant functions that can be linked to altering the levels of Ncor2 and Bcl-6.

An unexpected finding in the present study was that miR-10a also controls IL-17A production and that this may also be related to its ability to modulate expression of Bcl-6. We did not anticipate this result as Bcl-6 has been previously reported to itself negatively regulate IL-17A³². What was not taken into account in the previous *in vitro* experiments were the complex actions of RA as it pertains to T_H17 differentiation.

RA is a ubiquitous metabolite of vitamin A with broad actions on cellular differentiation. Vitamin A and RA are abundantly present in the liver and the gut and it is very clear that RA plays major roles in local homeostasis of mucosal immunity. It is important for trafficking and gut tropism of conventional T cells and T_{reg} cells^{4, 49}. RA can induce expression of Foxp3 and inhibit IL-17A production³. Herein, we confirmed that high doses of RA inhibit IL-17A production and found accordingly, that over-expression of miR-10a, which is induced by RA, likewise inhibits IL-17A. It is of interest in this regard to note that RA appears to directly regulate miR-10a. Recent work shows that RA receptor directly binds the miR-10a locus in ES cells⁵⁰.

Recent work, however, has demonstrated that even though RA can promote Foxp3 expression, it is also necessary for normal gut immune responses. That is, vitamin A-deficient mice exhibited poor T_H17 responses during *T. gondii* infection⁴². Our results showing the biphasic effects of RA on T_H17 differentiation are consistent with these

findings. At present, we do not have a precise explanation for how RA enhances IL-17 production. The induction of miR-10a also does not provide a ready explanation for the positive effects of RA, as over-expressing it inhibits IL-17A production. As Ncor2 is a regulator of RA signaling though, it is conceivable that fine-tuning the levels of Ncor2 could have divergent effects on IL-17A production since RA's effects are clearly not simple.

RA has also been found to enhance T_H1 responses^{42, 43}. This was attributed to enhanced IL-12 production from dendritic cells; however, our results indicate that under certain circumstances RA can also induce expression of T-bet in helper T cells. This is also a mechanism by which RA can inhibit IL-17A production, as T-bet inhibits ROR γ t function⁴⁷.

What was initially very perplexing to us was how miR-10a's ability to down-regulate Bcl-6 would result in decreased IL-17 production. This seemed at odds with the reported ability of Bcl-6 to inhibit IL-17A³² but in the context of RA's ability to induce T-bet, a potent inhibitor of IL-17A, the result now makes more sense. While in some circumstances reducing the level of Bcl-6 might amplify IL-17A production, in circumstances in which T-bet is present, reduction in Bcl-6 levels has the opposite effect. That is, inhibition of Bcl-6 expression results in an opposed action of T-bet to more efficiently inhibit IL-17A production. By modulating Bcl-6 levels, under the conditions employed in the present study, this is how we believe miR-10a inhibits IL-17A production. Although the effect of miR-10a on *in vitro* T_H17 differentiation was only evident in the presence of RA, we were able to discern an effect *in vivo* when miR-10a was over-expressed in adoptively transferred 2D2 T cells. However, our data also show that miR-10a modulated but did not abrogate disease. Thus, like other miRs, miR-10a is best thought of as “tuning” responses. The data also make clear that the effects of RA on T_H17 differentiation are complex and its effects are influenced by another ubiquitous regulator of T cell differentiation, namely TGF- β . Thus, the action of RA is by no means simple, as it can affect the levels of T-bet, Foxp3 and Bcl-6, depending upon the context.

In this study, we sought to dissect the function of miR-10a, a miRNA that is highly expressed in nT_{reg} cells and inducible by RA and TGF- β . By targeting Bcl-6, the miRNA appears to have functionally attenuated T_{FH} and T_H17 cell differentiation. As such, it is one factor that appears to contribute to maintenance of cell specific phenotype in T_{reg} cells by targeting factors that could lead to conversion to other fates (Fig. 8). It is quite conceivable that the regulatory role of miR-10a would be most effective under the circumstances where cells are poised for phenotypic transition, the gut being one place where this occurs. Thus, regulation of miR-10a levels would appear to be a reasonable mechanism for fine-tuning factors that influence fate decision versus plasticity in T_H subsets.

METHODS

Mice

Foxp3-IRES-GFP knock-in mice on C57BL/6 background, referred to as Foxp3^{GFP} mice, were described previously¹. B6.129S2-*Tcr α ^{tm1Mom}*/J mice (TCR α ^{-/-} mouse), B6.129S6-*Tbx21^{tm1Glm}*/J mice (*Tbx21*^{-/-} mice) and C57BL/6-Tg (Tcr α 2D2, Tcr β 2D2)1Kuch/J mice (2D2 mice)² were purchased from Jackson Laboratory. C57BL/6J mice, B6.CD45.1 mice and B6.129S6-*Rag2^{tm1Fwa}* N12 mice (*Rag2*^{-/-} mice) were purchased from Taconic. All animals were handled and housed in facilities in accordance with National Institutes of Health Animal Care and Use Committee guidelines.

Antibodies

CD44-FITC (IM7), B220-PE (RA3-6B2), CD25-PE (PC61), CD45.1-PE (A20), IL-17A-PE (TC11-18H10), CD62L-APC (MEL-14), TCR β -APC (H57-597), B220-Alexa488, GL-7-Alexa647, hNGFR-biotin (C40-1457), and streptavidin-PE/Cy7 were purchased from BD Bioscience. hNGFR-FITC (ME20.4), hNGFR-PE, hNGFR-APC, CD4-APC/Cy7 (RM4-5), TCR β -Pacific blue (H57-597) were purchased from Biolegend. Foxp3-FITC or APC (FJK-16s), AID (mAID-2), T-bet-eFluor660 (4B10) and Bcl6-PE (GI191E) were purchased from eBioscience. Rabbit anti-Bcl-6 antibody (#4242) and anti-Flag-Alexa647 antibody (#3916S) were purchased from Cell Signaling Technology. Rabbit anti-Ncor2 antibody (06-891) and mouse anti-actin antibody (C4) were purchased from Millipore. Anti-rat IgG-Alexa647, anti-rabbit IgG-Alexa488 and streptavidin-Alexa594 were purchased from Invitrogen. Anti-rabbit IgG-IRDye800 and anti-mouse IgG-IRDye700 were purchased from Rockland.

Cell isolation and sorting

Peripheral T cells were obtained from spleen and LNs of 8- to 12-week-old mice. Naïve CD4⁺ T cells (CD4⁺CD25⁻CD62L^{hi}CD44^{lo}) were isolated by FACS Aria II (BD) or MoFlo (Beckman Coulter) after enrichment of CD4⁺ T cells by using AutoMACS with mouse CD4⁺ T Cell Isolation Kit (Miltenyi Biotec). For some experiments, CD4⁺CD25⁻CD11b⁻CD11c⁻CD62L^{hi}CD44^{lo} population was isolated as naïve CD4⁺ T cells as mentioned. To isolate naïve CD4⁺ T cells and nTreg cells from Foxp3^{EGFP} mouse, CD4⁺GFP⁻CD62L^{hi}CD44^{lo} cells and CD4⁺GFP⁺ cells were isolated, respectively. To isolate iTreg cells, GFP⁺ cells were differentiated from naïve T cells of Foxp3^{EGFP} mouse and isolated. To analyze lymphocytes from Peyer's patches (PP), PP were enucleated from the small bowels and subjected to 15-minute incubation at 37 °C in HBSS containing 10% FBS, 5mM EDTA, 15mM HEPES, and 1mM DTT. Supernatant containing cell debris was removed at every vigorous vortex and wash with HBSS containing 5% FBS, 25mM HEPES until supernatant becomes clear. Then Peyer's patch was mechanically smashed to make single cell suspension. For some experiments, T_{FH} cells (CD4⁺V β ⁺PD-1^{hi}CXCR5^{hi}) and non-T_{FH} cells (CD4⁺V β ⁺PD-1⁻CXCR5⁻) were isolated from PP by FACS. For some experiments, T_{FR} cells (CD4⁺V β ⁺Foxp3⁺PD-1^{hi}CXCR5^{hi}), T_{reg} cells (CD4⁺V β ⁺Foxp3⁺PD-1⁻CXCR5⁻), T_{FH} cells (CD4⁺V β ⁺Foxp3⁻PD-1^{hi}CXCR5^{hi}) were isolated by FACS from spleen of Foxp3^{GFP} mice which were immunized with SRBC as previously described³.

Cell culture

All culture for T cells were performed in RPMI 1640 (Invitrogen) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 10 mM HEPES, 1 mM sodium pyruvate and non-essential amino acid, and 2 β M β -mercaptoethanol. Cells were activated with plate-bound anti-CD3 (10 μ g/ml; 145-2C11) and CD28 (10 μ g/ml; 37.51, both from eBioscience) Ab. The following culture conditions were used unless mentioned elsewhere. T_H17 condition contained 2 ng/ml TGF- β (R&D systems), 10 ng/ml IL-6 (R&D), 10 μ g/ml anti-IFN- γ (BD) and 10 μ g/ml anti-IL-2 (BD). For Figure 7 a and b, anti-IL-4 antibody was additionally used. iT_{reg} condition contained 5 ng/ml TGF- β , 50 U/ml hIL-2, and 10 μ g/ml anti-IFN- γ . ATRA, A7980, LE540 and AM580 were used as described previously⁴. NIH3T3 cells were maintained in DMEM (Invitrogen) supplemented with 10% calf serum, 2 mM glutamine, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 1 mM sodium pyruvate and non-essential amino acid. For HEK293T cells, DMEM supplemented with 10% FBS, 2 mM glutamine, 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 1 mM sodium pyruvate and non-essential amino acid was used.

Experimental autoimmune encephalomyelitis (EAE)

Naïve CD4⁺Vβ11⁺CD25⁻CD62L^{hi} CD44^{lo} cells isolated from 2D2 mice were stimulated with plate-coated anti-CD3 and CD28 antibodies for 16 hours and retrovirally transduced with miR-10a over-expression vector or control vector and then stimulated under the neutral condition (anti-IFN-γ and anti-IL-4 antibodies; 10 μg/ml for each) for further 2 days. After that, cells were maintained in the RPMI medium supplemented with 50 U/ml hIL-2 for 2 days. Transduced hNGFR⁺ cells which hardly expressed IFN-γ (< 0.5 %) and IL-17A (undetectable; data not shown), were isolated by magnetic beads and 1.0 × 10⁶ cells were adoptively transferred to Rag2^{-/-} mice followed by subcutaneous immunization with 100 μg MOG35-55 peptide (Peptides international) in CFA on day 0. These mice were scored daily according to the criteria as previously mentioned⁵. For analysis of cytokine production in transferred cells, mononuclear cells were isolated from the spinal cord, lymph nodes and spleen and analyzed by intracellular staining with flow cytometry.

Retroviral vector

To express miR-10a-5p, genomic DNA of mmu-mir-10a were cloned by using primers, 5'-ACCCACAGTGACTTTTCTGCTCC-3' and 5'-GGACACCTCAGGTAGATGAGATTT-3' and inserted between CD19 exon 10 and 11 sequences following Orange2 sequence on pLenti vector (Invitrogen), referred to as pLenti-Orange2-miR-10a, so that miR-10a locus will be transcribed and spliced out as a mirtron (Supplemental Figure 4a). Then miR-10a-5p is expected to be produced from mirtron through endogenous processing mechanism of mature miRNA. pLenti-Orange2-Control that transcribes only a part of CD19 sequence was generated as a control. Orange2 was replaced by truncated hNGFR and whole essential component of interest was transferred into pMYs-puro retroviral vector (Cell Biolabs) generating pMY-hNGFR-miR-10a and pMY-hNGFR-miR-NC (Supplemental Figure 4d). Target sequence for miR-10a-5p, 5'-cacaattcggttaaacagggtta-3', sequence was repeated 8 times with linker sequence between them following Δ4GFP sequence on LV-SFFV vector⁶, referred to as LV-SFFV-Δ4GFP-miR-10a-5pT (Supplemental Figure 4a). LV-SFFV-Δ4GFP-scT was generated by using 8-time repeated scrambled target sequence as a control. Δ4GFP was replaced by truncated hNGFR and whole essential component of interest was transferred into pMY-puro vector generating pMY-hNGFR-miR10a-5pT and pMY-hNGFR-scT from corresponding lentiviral vectors (Supplemental Figure 4d). To express shRNA specific for Bcl-6 and Ncor2, BLOCK-iT Lentiviral Pol II miR RNAi Expression System (Invitrogen) was utilized. shRNA sequences for Bcl-6 (Mmi505152) and Ncor2 (Mmi520051) were purchased from Invitrogen. pcDNA6.2-GW/EmGFP-miR-neg Control was used to make negative control vectors. Gene segments including EmGFP and shRNA were transferred into pMYs-puro vector generating pMY-EmGFP-iBcl6, pMY-EmGFP-iNcor2 and pMY-EmGFP-Control (negative control) from corresponding original vectors. EmGFP was replaced by truncated hNGFR to generate pMY-hNGFR-iBcl6, pMY-hNGFR-iNcor2 and pMY-hNGFR-Control. To over-express Bcl-6, we first made pMY-IRES-hNGFR vector as a control vector by replacing EGFP of pMYs-IRES-GFP vector (Cell Biolabs) with hNGFR. Then Bcl-6 cDNA was appropriately subcloned into pMY-IRES-hNGFR vector to generate pMY-Bcl-6-IRES-hNGFR vector (RV-Bcl-6) for Bcl-6 over-expression. To express Flag-tagged Bcl-6 protein from mRNA including 3'UTR of Bcl-6 or that lacking miR-10a-seed sequence, we generated pMYs-based expression vectors, Bcl-6 and Bcl-6-del described in Figure 2g, which also express hNGFR, using the 3'UTR sequences derived from luciferase reporter construct.

Retroviral transduction

Retroviral vector was transfected into PlatE cells to generate recombinant retrovirus. To perform retroviral transduction of CD4⁺ T cells, naïve CD4⁺ T cells were stimulated with plate-bound anti-CD3 and CD28 Ab + soluble anti-IFN-γ Ab for 16 hours. Culture medium

was replaced with retroviral soup and 4 μ g/ml polybrene, followed by centrifugation at 2500 rpm for 2 hours. After 4-hour incubation at 37 °C, viral supernatant was replaced with T cell culture medium containing cytokines and antibodies of interest. 3 days later, T cell differentiation was evaluated by flow cytometry. For retroviral transduction to HEK293T cells and CH12, we spinoculated cells with retroviral soup at 1800 rpm or 2500 rpm, respectively for 90 min and incubate them at 37 °C for 4 hours.

Adoptive transfer for analysis of T_{FH} in PP

Naïve CD4⁺GFP⁻CD62L^{hi} CD44^{lo} cells were isolated from Foxp3^{EGFP} mice and transferred to TCR α ^{-/-} mice. Some naïve T cells were stimulated with 10 ng/ml TGF- β and 50 U/ml rhIL-2 for 6 days and then Foxp3⁺ (GFP⁺) cells were isolated by FACS and transferred to TCR α ^{-/-} mice. 6 – 7 days after retroviral transduction of naïve CD4⁺ T cells, hNGFR⁺ cells were isolated by FACS sorting or magnetic beads and transferred into TCR α ^{-/-} mice. 5 or 6 weeks later, spleen and Peyer's patches were collected and analyzed by flow cytometry or immunohistochemistry.

Flow cytometry

Cytokines, transcriptional factors, and surface markers were evaluated by FACS Canto, Calibur or Verse (BD). In brief, for cytokine detection, cells were stimulated for 2 h with PMA and ionomycin with the addition of GolgiPlug (BD). To exclude dead cells, cells were stained 7-AAD (BD) and washed with PBS twice before fixation. Then cells were fixed and permeabilized with Foxp3 Staining Buffer Set (eBioscience) or BD Cytofix/Cytoperm (BD) and stained with fluorescent antibodies. Events were collected and analyzed with FlowJo software (Tree Star).

RT-PCR

Total RNA was isolated by mirVana miRNA Isolation Kit (Applied Biosystems). cDNA synthesis was performed with TaqMan Reverse Transcription Reagents (Applied Biosystems). Quantitative PCR was performed with ABI 7500 Fast Real-Time PCR System using Taqman site-specific primers and probes (Applied Biosystems). For reverse transcription and quantification of miRNA, TaqMan Reverse Transcription Kit was used in combination with TaqMan miRNA assays for snoRNA202 and hsa-mir-10a. Results were properly normalized to β -actin or snoRNA202 levels.

Western blotting

Cell lysate was fractionated on 4–12 % Bis-Tris gel (Invitrogen) SDS-polyacrylamide gel electrophoresis, followed by transfer to a nitrocellulose membrane. The membrane was blocked in blocking buffer (Odyssey) for 30 min and subjected to immunoblots with appropriate antibodies. Bands were visualized using Odyssey infrared imaging system (LI-COR Biosciences). Band intensities were analyzed by ImageJ (available at <http://rsbweb.nih.gov/ij/>) and normalized by the action intensity and shown as in ratio.

Luciferase assay

3'UTR of Bcl-6 and Ncor2 were cloned into pmirGLO vector (Promega) from genomic DNA by using the following primers, referred to as Luc-Bcl6 and Luc-Ncor2, respectively: forward; 5'-atgaagcatggagtgctcctcgccctt-3' and reverse; 5'-atctgcaggcagacacggatctgaga-3' for Bcl-6, forward; 5'-tgagacactctcgacagcgagtga-3' and reverse; 5'-aaatgacagaatgccgcgtgcaca-3' for Ncor2. Predicted target sequences of both 3'UTR were removed from each vector by using QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies) and referred to as Luc-Bcl6-del and Luc-Ncor2-del. These constructs were transfected in NIH3T3 cells with expression vector for miR-10a or a control vector. Then

firefly luciferase and Renilla luciferase activity were measured with Dual-Glo Luciferase Assay System (Promega).

Immunohistochemical analysis

Peyer's patches were embedded in OCT compound (Sakura Finetechnical). Frozen sections were made by cryostat at 20 μ m and stored at -80°C until use. After fixation with 4 % PFA or acetone, sections were stained with antibodies and analyzed by Leica SP5 NLO Confocal Microscope.

Transfection to NIH3T3 cells

Three vectors to express miR-10a, miR-10a sponge sequence, and Flag-tagged Bcl-6 were transfected to NIH3T3 cells by Lipofectamine 2000 (Invitrogen) as described in the manufacturer's instruction. Two days later, the cells were collected with PBS + 2 mM EDTA and analyzed by flow cytometry.

Statistical analysis

Appropriate analyses were used and stated in each figure legend. P-value less than 0.05 was considered as significant difference. All error bars show SEM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Y. Belkaid, W. Chen and A. Villarino for their careful reading of this manuscript; K. Moro (Keio University, Tokyo, Japan) and T. Tamachi (NIH) for their excellent advice on manipulating PP; L. Naldini (San Raffaele Institute) for the LV-SFFV lentiviral vector; and J. Simone (NIH) and J. Lay (NIH) for cell sorting. This study was supported by JSPS Research Fellowship for Japanese Biomedical and Behavioral Researchers at NIH (H.T.) and the NIAMS Intramural Program.

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Figure 1.

miR-10a is highly expressed in nT_{reg} cells and is induced by RA and TGF- β . **(a)** miR-10a expression was evaluated by isolating total RNA from different T cell subsets and assessing expression by quantitative RT-PCR. **(b–g)** Naïve CD4⁺ T cells were stimulated with anti-CD3 and anti-CD28 antibodies and miR-10a expression was determined in cells cultured in **(b)** medium alone, IL-2 (50 U/ml) or TGF- β (20 ng/ml), **(c)** with varying concentrations of TGF- β , **(d)** varying concentrations of ATRA with TGF- β (20 ng/ml, solid circles) or anti-TGF- β antibody (solid squares), or **(e)** anti-TGF- β antibody, TGF- β (20 ng/ml) or LE540 (pan-RAR antagonist, 1 μ M). **(f)** Expression of RAR α was assessed by quantitative RT-PCR after stimulating cells with anti-CD3 and anti-CD28 antibodies and varying concentrations of TGF- β . **(g)** miR-10a levels were determined in isolated naïve CD4⁺ T cells that were stimulated with anti-CD3 and anti-CD28 antibodies in the presence of TGF- β alone or with ATRA (1 μ M), AM580 (RAR α agonist, 1 μ M) or A7980 (RAR γ agonist, 1 μ M) as indicated. All experiments were performed in triplicate. Statistical significance was determined by t-test. * indicates $p < 0.05$, ** indicates $p < 0.01$. Similar results were obtained in two (a, c, e, f, g) or three (b, d) independent experiments.

Figure 2. miR-10a directly targets Bcl-6 and Ncor2 and down-regulates protein expression. (a) miR-10a target sequences (in red) of the 3'UTR of the Bcl6 (upper) and Ncor2 (lower) mRNAs, which were included in luciferase reporter vectors (left panels). Also shown are the mutant vectors, in which the miR-10a seed complementary sequences were deleted as indicated by asterisks (Luc-Bcl6-del and Luc-Ncor2-del). These luciferase reporter constructs were transfected with miR-10a or a control construct in NIH3T3 cells and luciferase expression was determined and normalized by Renilla luciferase activity. (b, c) CH12, a B cell lymphoma cell line that expresses Bcl-6, was transduced with retroviral vectors encoding hNGFR and miR-10a (blue) or a control vector (red). Bcl-6 expression was measured by flow cytometry after gating hNGFR⁺ population (b) or by immunoblotting after transduced (hNGFR⁺) cells were isolated by magnetic beads (c). Blotting of lysates with anti-actin antibody is shown below with quantification of Bcl-6/actin ratios determined by densitometry. (d – h) Naïve CD4⁺ T cells were transduced with control vectors (red) or vectors expressing miR-10a (blue) and stimulated with anti-CD3 and CD28 antibodies with TGF- β (5 ng/ml) and IL-2 (50 U/ml) and anti-IFN- γ antibody (iT_{reg} condition). Bcl-6 levels were assessed by flow cytometry after gating on transduced cells (d). A representative result is shown on the left panel and levels of Bcl-6 expression expressed as the average mean fluorescence intensity are shown on the right. (e, h) The effect miR-10a on levels of Bcl-6 and Ncor2 in activated CD4⁺ T cells transduced with miR-10a and control vector was confirmed by immunoblotting and quantified as in (c). (f, i) Bcl-6 and Ncor2 mRNA expression was measured by quantitative RT-PCR and normalized to β -actin mRNA level. (g) NIH3T3 cells were transduced with different combinations of three vectors: a Flag-tagged Bcl-6 expression vector that includes the Bcl-6 3'UTR and hNGFR; a vector encoding miR-10a and Orange2; and a vector encoding the miR-10a sponge target sequence and Δ 4GFP. Cells were also transduced with corresponding control vectors as delineated in the figure. Changes in Bcl-6 levels were measured by flow cytometry using anti-Flag

antibody after gating on GFP⁺Orange2⁺hNGFR⁺ cells and are expressed as MFI. (**j**, **k**) Naïve CD4⁺ T cells were transduced with a control vector or a vector expressing a miR-10a sponge target sequence (miR-10a-5pT) and stimulated under iT_{reg}-inducing conditions. Bcl-6 expression in CD4⁺hNGFR⁺ population was evaluated by flow cytometry and the MFI is shown in the right panel (**j**). Transduced cells were isolated and Ncor2 expression was assessed by immunoblotting. Pooled data from three independent experiments were shown in the right panel (**k**). Similar results were obtained from three (a, e – k) or two (b, c, d) individual experiments.

Figure 3.

miR-10a constrains conversion of iT_{reg} to T_{FH} cells. **(a, b)** Naïve CD4⁺ T cells were transduced with a control vector or a vector encoding the miR-10a sponge target sequence (miR-10a-5pT) and hNGFR, and cultured with 10 ng/ml TGF- β , 50 U/ml IL-2, and 1 nM ATRA for 5 days. This generated a population of cells that were 97.7 $\pm$ 0.4 % Foxp3⁺. Transduced hNGFR⁺ cells were isolated and transferred into TCR α ^{-/-} mice. Five weeks later, the acquisition of surface markers indicative of T_{FH} conversion (CD4⁺V β ⁺hNGFR⁺CD44^{hi}PD-1^{hi}CXCR5^{hi}) was measured by flow cytometry **(a)**. Concomitantly, the appearance of B220⁺IgD^{lo}Fas⁺GL-7⁺ germinal center B cells was also determined **(b)**. Pooled data from a representative experiment are shown in the right panel ($n = 5$ mice). Similar results were obtained from three **(a)** and two **(b)** independent experiments using 3 to 5 mice in each experiment. **(c)** Naïve CD45.1⁺CD4⁺ T cells were transduced with a vector encoding miR-10a and hNGFR, and differentiated into iT_{reg} cells. A vector expressing hNGFR but not miR-10a was used as a control. Transduced cells were purified by FACS cell sorting and transferred into TCR α ^{-/-} mice. Mice were sacrificed and the presence of T_{FH} in Peyer's patches (PP) was assessed by flow cytometry 6 weeks after transfer. Cells isolated from PP were gated on the CD4⁺CD45.1⁺hNGFR⁺CD44^{hi}7-AAD⁻ population and T_{FH} were enumerated by expression of PD-1 and CXCR5. Pooled data from an experiment were shown in the right panel. Similar results were obtained from three independent experiments. **(d, e)** T_{FH}(CD4⁺V β ⁺PD-1^{hi}CXCR5^{hi}) and non-T_{FH} (CD4⁺V β ⁺PD-1⁻CXCR5⁻) cells were isolated from PP by cell sorting and Bcl-6 expression was assessed by flow cytometry **(d)**. miR-10a expression was measured by quantitative RT-PCR **(e)**.

Figure 4.

Retinoic acid has biphasic effects on T_H17 differentiation. **(a – d)** Naïve CD4⁺ T cells cultured under conditions to generate T_H17 cells (IL-6, TGF-β and anti-IFN-γ antibody) or (IL-6, TGF-β, anti-IFN-γ antibody and anti-IL-2 antibody) and iT_{reg} cells (IL-2, TGF-β and anti-IFN-γ antibody) along with varying concentrations of ATRA and analyzed for expression of IL-17A and Foxp3 by flow cytometry. The effect of ATRA on the proportion of Foxp3 expressing- **(a)** and IL-17A producing- **(b)** cells over a wide range of doses. Representative results from three independent experiments are shown in the left panel **(a and b)**. Each data point represents the mean ± SEM (n ≥ 3). A representative experiment is shown in the middle panels. Pooled data from five (iT_{reg} and T_H17 (anti-IL-2) conditions) or seven (T_H17 condition) independent experiments are shown in the right panels. P values were determined by paired sample t-test. **(c, d)** The effects of ATRA and varying concentrations of TGF-β on IL-17A production. **(c)** Isolated naïve CD4⁺ T cells cultured under conditions (IL-6, anti-IFN-γ antibody and anti-IL-2 antibody) to generate T_H17 cells with varying concentrations of ATRA and TGF-β. * indicates P < 0.05 for all conditions. **(d)** The increase over basal expression represents a ratio determined by the difference in the percentage of IL-17A producing cells in the presence and absence of ATRA (10 nM) for each concentration of TGF-β divided by the percentage of IL-17A-producing cells generated without ATRA. P values were determined by t-test (c, d). Each data point represents the mean ± SEM (n ≥ 3) and similar results were obtained in two independent experiments.

Figure 5.

miR-10a delays the onset, but does not abrogate experimental autoimmune encephalomyelitis. Naïve CD4⁺ were isolated from 2D2 TCR transgenic mice and transduced with a vector expressing miR-10a and hNGFR or a control vector expressing only hNGFR. Cells were then stimulated with anti-CD3 and anti-CD28 antibodies, anti-IFN-γ and anti-IL-4 antibody and no exogenous cytokines for 3 days and expanded with IL-2 (50 U/ml) for additional 2 days. Transduced cells were isolated and transferred into Rag2^{-/-} mice that were then immunized with MOG peptide (100 μg). **(a)** The appearance of neurological disease was assessed daily and shown in the left panel. The data represent pooled data from 5 independent experiments (n = 29 mice for control, 24 mice for miR-10a group). The difference in the two groups was analyzed by two-way ANOVA. Area under the curve (AUC) from each experiments were calculated and shown in the right panel. P value was determined by t-test. **(b)** IL-17A and IFN-γ expression in 2D2 T cells in CNS, LN, and spleen at day 11 after immunization was analyzed by flow cytometry, gating on CD4⁺Vβ⁺hNGFR⁺7-AAD⁻ cells. Representative expression is shown on the left and pooled data are depicted on the right (n=5). Similar results were obtained from two independent experiments.

Figure 6.

miR-10a constrains TH17 differentiation in the presence of RA. **(a)** Naïve CD4⁺ T cells were transduced with a retroviral construct expressing a miR-10a-5p “sponge” target sequence (miR10a-5pT) or a scrambled sequence (control) and cultured under TH17 conditions with anti-IL-2 antibody and ATRA (1.5 nM). **(b, c)** Naïve CD4⁺ T cells were transduced with a retroviral construct expressing miR-10a or control vector under TH17 conditions and anti-IL-2 with **(b)** or without ATRA (1.5 nM) **(c)**. Cells were analyzed for Foxp3 and IL-17A expressions by flow cytometry. A representative experiment is depicted in the left panel and pooled data from multiple independent experiments are shown in the right panels. Statistical significance was determined by paired sample t-test.

Figure 7.

Ncor2 and Bcl-6 regulate IL-17A in a T-bet-dependent manner. **(a, b)** Naïve CD4⁺ T cells from wild-type mice **(a)** or *Tbx21*^{-/-} mice **(b)** were transduced with retroviral vectors encoding shRNAs for Ncor2 (iNcor2), Bcl-6 (iBcl6) or a control vector and cultured under T_H17 conditions with anti-IL-2 antibody + ATRA (1.5 nM). IL-17A and Foxp3 expression was assessed by flow cytometry. Representative contour plots are shown on the left and pooled data (n = 4) from this experiment are shown on the right. **(c, d)** Naïve CD4⁺ T cells were cultured under T_H17 conditions and anti-IL-2 antibody with or without ATRA at the indicated concentrations. Cells were analyzed for T-bet expression by immunoblotting **(c)** and flow cytometry **(d)**. Experiments were repeated twice (a, b) or three (c, d) times with similar results.

Figure 8.

Model depicting actions of miR-10a in differentiating T cells. **(a)** Retinoic acids induce expression of miR-10a, which suppresses the Bcl-6 and Ncor2 complex. **(b)** Environments such as Peyer's patch promote the instability of T_{reg} to become T_{FH}. However, the presence of miR-10a inhibits expression of Bcl-6 and serves to limit T_{FH} conversion. **(c)** Retinoic acid also induces expression of T-bet and by limiting Bcl-6 expression, miR-10a relieves the antagonistic effect of the latter on the former. The net effect is T-bet more efficiently interferes with IL-17A production. In this manner, miR-10a inhibits both T_{FH} and T_H17 differentiation.