Accumulation of IL-17–producing Th17 cells is associated with the development of multiple autoimmune diseases; however, the contribution of microRNA (miRNA) pathways to the intrinsic control of Th17 development remains unclear. Here, we demonstrated that miR-21 expression is elevated in Th17 cells and that mice lacking miR-21 have a defect in Th17 differentiation and are resistant to experimental autoimmune encephalomyelitis (EAE). Furthermore, we determined that miR-21 promotes Th17 differentiation by targeting and depleting SMAD-7, a negative regulator of TGF-β signaling. Moreover, the decreases in Th17 differentiation in miR-21–deficient T cells were associated with defects in SMAD-2/3 activation and IL-2 suppression. Finally, we found that treatment of WT mice with an anti–miR-21 oligonucleotide reduced the clinical severity of EAE, which was associated with a decrease in Th17 cells. Thus, we have characterized a T cell–intrinsic miRNA pathway that enhances TGF-β signaling, limits the autocrine inhibitory effects of IL-2, and thereby promotes Th17 differentiation and autoimmunity.
MicroRNA-21 promotes Th17 differentiation and mediates experimental autoimmune encephalomyelitis

Gopal Murugaiyan,1,2 Andre Pires da Cunha,1,2 Amrendra K. Ajay,3 Nicole Joller,1,2,4 Lucien P. Garo,1,2 Sowmya Kumaradevan,1,2 Nir Yosef,5 Vishal S. Vaidya,5 and Howard L. Weiner1,2

1Ann Romney Center for Neurologic Diseases, 2Evergrande Center for Immunologic Diseases, and 3Renal Division, Department of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, Massachusetts, USA. 4Institute of Experimental Immunology, University of Zurich, Zurich, Switzerland. 5Broad Institute of MIT and Harvard, Cambridge, Massachusetts, USA.

Accumulation of IL-17–producing Th17 cells is associated with the development of multiple autoimmune diseases; however, the contribution of microRNA (miRNA) pathways to the intrinsic control of Th17 development remains unclear. Here, we demonstrated that miR-21 expression is elevated in Th17 cells and that mice lacking miR-21 have a defect in Th17 differentiation and are resistant to experimental autoimmune encephalomyelitis (EAE). Furthermore, we determined that miR-21 promotes Th17 differentiation by targeting and depleting SMAD-7, a negative regulator of TGF-β signaling. Moreover, the decreases in Th17 differentiation in miR-21–deficient T cells were associated with defects in SMAD-2/3 activation and IL-2 suppression. Finally, we found that treatment of WT mice with an anti–miR-21 oligonucleotide reduced the clinical severity of EAE, which was associated with a decrease in Th17 cells. Thus, we have characterized a T cell–intrinsic miRNA pathway that enhances TGF-β signaling, limits the autocrine inhibitory effects of IL-2, and thereby promotes Th17 differentiation and autoimmunity.

Introduction

IL-17–producing Th17 cells contribute to protection against microbial pathogens but also play a critical role in the development of autoimmunity (1), including multiple sclerosis (MS) and its animal model experimental autoimmune encephalomyelitis (EAE) (2–5). Multiple cytokines including TGF-β, IL-6, IL-1β, and IL-21 have been shown to induce the differentiation of naïve T cells toward the Th17 phenotype (6–11). The differentiation of Th17 cells requires expression of the transcription factor ROR-γt (12). The induction of ROR-γt is dependent on STAT-3, which is preferentially activated by IL-6. In addition, other transcription factors including ROR-α, IRF-4, BATF, and HIF-1α are also involved in the control of Th17 lineage commitment (13–16).

Among the cytokines, TGF-β critically promotes Th17-mediated immune responses. Altered TGF-β and TGF-β receptor (TGF-βR) signaling have been implicated in Th17-mediated autoimmune pathogenesis (6, 7). For example, mice expressing dominant-negative TGF-βR1I confer resistance to EAE through a reduction in Th17 cells (17). TGF-β signals are transduced through activation of the SMAD proteins SMAD-2 and SMAD-3 (18), and enhanced generation of naive Th17 cells is associated with increased TGF-β–induced SMAD-2/3 activation (19–21). In addition, TGF-β signaling has been shown to downregulate IL-2 expression and abrogate IL-2–mediated suppression of Th17 differentiation (20–23). However, the role that microRNAs (miRNAs) play in the activation of TGF-βR signaling in driving Th17 cell development and, consequently, Th17-mediated autoimmunity remains unknown.

miRNAs are a class of small, noncoding RNAs that impart posttranscriptional gene regulation through several mechanisms including translational repression and mRNA degradation (24). They are important in many physiological processes such as carcinogenesis and immune system modulation. Aberrant expression of miRNAs has been linked to a variety of human pathologies including MS and other inflammatory diseases (25, 26).

Here, we report that miR-21 expression was specifically elevated in Th17 cells and that miR-21–deficient (mir21−/−) mice showed a defect in Th17 differentiation and strong resistance to EAE. miR-21 promoted Th17 differentiation by targeting SMAD-7, a negative regulator of TGF-β signaling. The defects in Th17 differentiation in Mir21−/− T cells were associated with defects in SMAD-2/3 activation and IL-2 suppression. Anti-miR-21 treatment dramatically reduced the clinical severity of EAE and decreased Th17 cell numbers. Thus, our results characterize a previously unknown T cell–intrinsic miRNA pathway that promotes Th17 differentiation and autoimmunity and identifies miR-21 as a potential therapeutic target in the amelioration of MS and other Th17-mediated autoimmune diseases.

Results

miR-21 promotes Th17 differentiation. CD4+ T cells play a major role in autoimmune disease. Increased expression of miR-21 has been observed in human autoimmune conditions including MS, systemic lupus erythematosus (SLE), and psoriasis (27–30). However, the role of miR-21 and its intrinsic requirement in Th cell differentiation and autoimmunity remains unclear. To investigate the expression of miR-21 in Th cell subsets, we activated naïve CD4+CD62LhiCD44− T cells under polarizing conditions in vitro and obtained Th1, Th2, Th17, and Treg cells with selective expression of Ifng, Il4, Il17, and Foxp3, respectively (Supplemental Figure 1A; supplemental material available online with this
β that observed in unstimulated cells. The addition of TGF-
CD28 led to a small increase in miR-21 levels compared with
imulation in T cells and found that stimulation via the TCR and
lation was required for TGF-
then investigated whether T cell antigen receptor (TCR) stimu-
ent endogenous controls (small nucleolar RNA135 [snoRNA135]
and inducible Tregs (iTregs) (Figure 1, A and B). We used 2 differ-
mir-21 in Th17 cells and relatively small amounts in Th1, Th2,
expression (Supplemental Figure 1D). Taken together, these
data indicate that miR-21 is differentially expressed in Th17 cells.

To directly assess whether miR-21 regulated the differentiation of Th17 cells, we performed an in vitro T cell differentiation assay. When naive CD4+ T cells were activated under Th17-polarizing conditions, IL-17 production in Mir21+/− T cells was significantly reduced compared with that in WT T cells (Figure 2, A and B). Consistent with this, Mir21−/− T cells differentiated under Th17-polarizing conditions expressed lower levels of Th17-associated transcription factors (Rorgt, Rora, Irf4, Ifi6, and Baf41), cytokines (Il17f, Il12i, Il22, and Gmcsf), and cell-surface receptors (Il23r and Ccr6) (Figure 2, C–E). In contrast, in vitro differentiation of Th1 and Th2 effector cells was largely independent of miR-21 (Supplemental Figure 2A). Phenotypical analysis of iTregs revealed a dispensable role of miR-21 in iTreg cell differentiation, as reflected by unaltered FOXP3 expression (Supplemental Figure 2B). Moreover, we detected no developmental defect in the generation of CD25+FOXP3+ natural Tregs (nTregs) in the absence of miR-21 (Supplemental Figure 2C).

To investigate whether deletion of miR-21 affected the homeostasis of T cells, B cells, and DC populations, we analyzed 6- to 8-week-old naive Mir21−/− mice and WT control mice. In the thymus, the frequencies of CD4+ and CD8+ single-positive as well as CD4+CD8+ double-positive T cells were unaltered (Supplemental Figure 3A). In addition, we found comparable sizes of lymphoid and myeloid cell subsets in the spleen (Supplemental Figure 3, A–D). Furthermore, the size and total cellularity of spleens and inguinal lymph nodes (LNs) were comparable between WT and Mir21−/− mice (Supplemental Figure 3, E and F). Collectively, these results provide evidence that miR-21 is not required to effectively induce Th1, Th2, or Tregs and suggest a selective role for miR-21 in Th17 cells.

miR-21 regulates EAE development. These observations led us to explore the potential role of miR-21 in Th17-mediated inflammatory immune pathogenesis in vivo. To investigate the role of miR-21 in EAE development, we first analyzed the expression of miR-21 in mice during the course of EAE. Since CD4+ T cells are the key mediators of EAE, we asked whether CD4+ T cells express miR-21 during EAE. We analyzed miR-21 expression in CD4+T cells from C57BL/6 mice immunized against EAE with MOG35–55 peptide emulsified in CFA and found that miR-21 expression was markedly increased in CD4+ T cells isolated from the spleen and CNS of EAE mice compared with that in naive mice (Figure 3A). The potential role of miR-21 in EAE pathogenesis was then tested using Mir21−/− mice. In 4 independent experiments, Mir21−/− mice were resistant to EAE induction (2 of 38 mice developed EAE), whereas WT mice developed severe EAE (35 of 38 mice developed EAE) (Figure 3B). The absence of paralytic symptoms in Mir21−/− mice was associated with a marked reduction in inflammation and demyelination (Figure 3, C and D). Although we found that EAE was associated with demyelination and infiltration of CD4+ T cells and CD11c+ cells from the peripheral lymphoid organs to the CNS in both WT and Mir21−/− mice, the total number of CD4+ T cells and CD11c+ cells was markedly lower in the CNS of Mir21−/− mice at the peak of disease (Figure 3E).

miR-21 has been reported to target various tumor-suppressor genes such as Btg2, Pldcd4, Pten, and sprouty, which are known to regulate cell death and proliferation (32–39). We therefore tested...
whether miR-21 affects the cell death and proliferation of CD4+ T cells by targeting these tumor suppressors. Although we found that in vitro–stimulated Mir21−/− T cells expressed higher levels of Pten than did their WT counterparts (Supplemental Figure 4A), no significant differences in the expression of these target genes were observed between WT and Mir21−/− T cells in vivo (Supplemental Figure 4B). Consistent with the increased expression of Pten in vitro, we found that naive Mir21−/− T cells stimulated in vitro exhibited a proliferative defect (Supplemental Figure 4C). However, we found no significant differences in any of the examined proliferative parameters between MOG-immunized WT and Mir21−/− T cells in response to MOG antigen (Supplemental Figure 4D). In addition, no difference in the frequency of apoptotic T cells between WT and Mir21−/− mice was observed (Supplemental Figure 4E and F) either in vitro or in vivo. Consistent with these findings, the total spleen cellularity, frequency of activated (CD44hi) CD4+ T cells, and percentage of MOG-tetramer+ T cells were similar between WT and Mir21−/− mice during EAE induction (Supplemental Figure 4G–I). Together, these results establish the presence of MOG-specific T cells in Mir21−/− mice and demonstrate the ability of these T cells to expand in response to MOG peptide, suggesting that a general proliferative or apoptotic abnormality in Mir21−/− T cells is not a major contributor to the striking EAE resistance we observed in these mice.

We further investigated whether the EAE resistance in Mir21−/− was associated with a defect in cytokine production from T cells. At peak disease, CNS-infiltrating CD4+ T cells from Mir21−/− mice produced less IL-17, whereas IFN-γ levels were unaffected (Figure 3F). In line with a reduction in the frequency of IL-17–producing cells in the CNS, mRNA expression of other Th17-associated genes in T cells from Mir21−/− mice was also reduced in comparison with expression in T cells from WT mice (Figure 3G). Consistent with these RNA data, analysis of CNS-infiltrating Th17 cells for intracellular cytokines revealed a significant defect in expression of the Th17-related cytokines IL-17A and IL-17F in Mir21−/− mice. However, the protein levels of IL-21 and IL-22 were not altered between WT and Mir21−/− mice (Supplemental Figure 5). In addition, we found that Mir21−/− mice had substantially diminished percentages of Th17 cells in the periphery compared with those detected in WT mice at the onset and peak stages of EAE (Supplemental Figure 7).

Figure 2. miR-21 promotes Th17 differentiation. (A) Il17a expression in WT and Mir21−/− T cells cultured under Th17 conditions determined by qRT-PCR. (B) WT and Mir21−/−–derived naive CD4+ T cells cultured under Th17 conditions were restimulated with PMA plus ionomycin on day 5 and stained for IL-17 and IFN-γ. Numbers represent the frequency of CD4+ T cells. (C–E) Naive CD4+ T cells from WT and Mir21−/− mice were differentiated into Th17 cells. mRNA expression levels of Th17-related cytokines (Il17a, Il17f, Il21, Il22, and Gmcsf), transcription factors (Rorgt, Rora, Ifi4, Hif1α, and Batf), and surface receptors (Il23r and Ccr6) were analyzed by qRT-PCR. Data are representative of 3 independent experiments. Error bars represent the mean ± SEM. **P < 0.01, and ***P < 0.001 by unpaired Student’s t test.
levels of costimulatory molecules (Supplemental Figure 8A) and Th17-polarizing cytokines \( \text{Il6}, \text{Il23}, \text{Il1b}, \) and \( \text{Tnfa} \) after LPS stimulation (Supplemental Figure 8B). In addition, coculture of naive CD4+ T cells and DCs indicated that \( \text{Mir21}^{--} \) DCs were able to support Th17 development normally. However, Th17 development from \( \text{Mir21}^{--} \) naive CD4+ T cells was markedly impaired, regardless of the origin of the cocultured DCs (Supplemental Figure 8C).

IL-17–producing innate lymphoid cells have been shown to play a pathological role in certain autoimmune diseases (41). However, we did not find any difference between WT and \( \text{Mir21}^{--} \) mice in the frequency of innate lymphoid cells or the expression of IL-17 in these cells (Supplemental Figure 9). Collectively, these data together, these data demonstrate that miR-21 positively regulates Th17 differentiation both in vivo and in vitro.

DCs play an important role in the development of EAE and produce inflammatory cytokines that are required for Th17 differentiation and function in vivo (8). Among the DC-secreted cytokines, IL-23 is crucial for the functional maturation of Th17 cells. In fact, IL-23R-deficient mice were shown to be resistant to EAE, owing to a specific defect in Th17 cells (40). To investigate the possibility that impaired Th17 differentiation in \( \text{Mir21}^{--} \) mice was caused by a defect in DCs, we evaluated inflammatory cytokine expression and costimulatory molecule expression in DCs. We found that DCs from both WT and \( \text{Mir21}^{--} \) mice expressed normal levels of costimulatory molecules (Supplemental Figure 8A) and Th17-polarizing cytokines \( \text{Il6}, \text{Il23}, \text{Il1b}, \) and \( \text{Tnfa} \) after LPS stimulation (Supplemental Figure 8B). In addition, coculture of naive CD4+ T cells and DCs indicated that \( \text{Mir21}^{--} \) DCs were able to support Th17 development normally. However, Th17 development from \( \text{Mir21}^{--} \) naive CD4+ T cells was markedly impaired, regardless of the origin of the cocultured DCs (Supplemental Figure 8C). IL-17–producing innate lymphoid cells have been shown to play a pathological role in certain autoimmune diseases (41). However, we did not find any difference between WT and \( \text{Mir21}^{--} \) mice in the frequency of innate lymphoid cells or the expression of IL-17 in these cells (Supplemental Figure 9). Collectively, these data
suggest that Mir21–/– CD4+ T cells have an intrinsic defect in their ability to differentiate into Th17 cells, resulting in amelioration of EAE in Mir21–/– animals.

To establish that the resistance to EAE in Mir21–/– mice resulted from a T cell–intrinsic defect, we transferred WT CD4+ T cells into WT or Mir21–/– mice before MOG immunization. Both WT and Mir21–/– mice receiving CD4+ T cells from WT mice developed EAE accompanied by CNS-infiltrating IL-17–producing CD4+ T cells, suggesting that mir21–sufficient T cells from WT mice can overcome the EAE resistance in Mir21–/– mice (Figure 3, I and J). We further examined whether the EAE resistance of Mir21–/– mice might be due to their nonfunctional T cells. To test this hypothesis, MOG-specific Th17 and Th1 cells of Mir21–/– and WT origin were injected into syngeneic WT recipients, and the animals were monitored for signs of EAE. While the recipients of Th17 cells from WT mice developed severe EAE, Th17 cells from Mir21–/– mice were not able to induce disease (Figure 3K). Interestingly, we found that injection of MOG-specific WT Th1 cells resulted in severe EAE, whereas, transfer of MOG-specific Mir21–/– Th1 cells did not elicit any signs of EAE (Supplemental Figure 10). GM-CSF is the main effector cytokine in both Th1 and Th17 cell–driven EAE (42–44). In fact, Th1 cells that lack the ability to produce GM-CSF do not transfer EAE (42). We therefore assessed GM-CSF expression in Th1 and Th17 cells infiltrating the brains of WT and Mir21–/– mice with EAE. Interestingly, CNS-infiltrating Th1 and Th17 cells from Mir21–/– mice showed a dramatic drop in GM-CSF expression, suggesting that miR-21 is critical for the encephalitogenic function of T cells in vivo (Figure 3L). It has been shown that the recruitment of myeloid cells is associated with pathogenic Th17 responses and GM-CSF secretion (45). Consistent with our observation of impaired IL-17 and GM-CSF secretion by CNS-infiltrating Mir21–/– Th17 cells, we observed a significant reduction in the number of CNS-infiltrating DCs and macrophages in Mir21–/– mice (Figure 3E). It has also been shown that Th17 cells initially access the CNS and create a microenvironment conducive to the subsequent entry of Th1 cells (31). The defective Th17 differentiation, coupled with a reduction of GM-CSF secretion by both Th1 and Th17 cells, could explain the highly resistant EAE phenotype observed in Mir21–/– mice. Together, these observations demonstrate that EAE resistance in Mir21–/– mice is caused by T cell–intrinsic defects.

miR-21 promotes Th17 differentiation through SMAD-7 inhibition. We then investigated the mechanism by which miR-21 regulates Th17 development. It is possible that miR-21 could control Th17 development by regulating IL-6 and TGF-β signaling. A critical mechanism of Th17 differentiation is IL-6–induced STAT-3 activation (46). However, we did not find any difference in phosphorylated STAT-3 (p–STAT-3) levels between WT and Mir21–/– CD4+ T cells stimulated with IL-6 (Supplemental Figure 11A). In addition, there was no difference in IL-6 receptor (IL-6R) expression between the 2 genotypes, suggesting that Mir21–/– does not play a role in the modulation of signaling events downstream of the IL-6R (Supplemental Figure 11B). TGF-β initiates its cellular function by binding to TGF-βRII, which then phosphorylates TGF-βRI. TGF-βRII propagates the signal by inducing SMAD-2 and SMAD-3 phosphorylation, which subsequently leads to nuclear translocation, SMAD-2 and SMAD-3 DNA binding, and the transcriptional activation of TGF-β-responsive genes (18). Although we did not find any defect in TGF-β or TGF-βRI expression in Mir21–/– CD4+ T cells (Supplemental Figure 12), stimulation of Mir21–/– CD4+ T cells with TGF-β resulted in reduced phosphorylation of both SMAD-2 and SMAD-3 when compared with that in WT CD4+ T cells (Figure 4A). Strikingly, stimulation of Mir21–/– CD4+ T cells with TGF-β resulted in higher SMAD-7 expression (Figure 4B). Contrary to the function of SMAD-2/3, TGF-β–induced SMAD-7 participates in a negative feedback loop to control excessive TGF-β signaling. We thus applied the Web-based target prediction software program TargetScan (www.targetscan.org) to identify potential miR-21 targets and found miR-21 to be a potential upstream regulator of SMAD-7 (Figure 4C). In addition, recent data from other disease models suggest that miR-21 is an upstream regulator of SMAD-7 (47, 48). To determine whether SMAD-7 is a target of miR-21 in Th17 differentiation, we performed luciferase assays using the WT and mutant Smad7 3′ UTR. We found that miR-21 inhibited the luciferase activity of a reporter containing the WT Smad7 3′ UTR, but not that of a reporter with a mutated 3′ UTR unable to bind to miR-21 (Figure 4, D and E). The effect of miR-21 on SMAD-7 expression was then tested by using a chemically modified locked nucleic acid (LNA) inhibitor. We found that inhibition of miR-21 led to an increase in SMAD-7 expression in CD4+ T cells treated with TGF-β (Figure 4F). Consistent with the increased levels of SMAD-7, inhibition of miR-21 also led to decreased phosphorylation of SMAD-2 and SMAD-3 in CD4+ T cells (Figure 4F). On the other hand, miR-21 overexpression substantially decreased the levels of SMAD-7 while increasing the expression of SMAD-2 and SMAD-3 (Figure 4G). In addition to the SMAD-dependent signaling pathway, TGF-β is also known to signal via a SMAD-independent pathway (18). However, we did not find any difference between WT and Mir21–/– T cells in the activation levels of molecules associated with non-SMAD TGF-β signaling pathways including p38 MAPK, ERK-1/2, and stress-activated protein kinase (SAPK)/Jun amino-terminal kinase (JNK) (Supplemental Figure 13). Taken together, our results suggest that by targeting SMAD-7, miR-21 promotes SMAD-mediated TGF-β signaling and that its deletion results in reduced sensitivity to the effects of TGF-β in T cells.

Next, we tested the functional role of miR-21 in regulating the effects of TGF-β on Th17 differentiation and the role of SMAD-7 in regulating these effects. CD4+ T cells were transfected with lentiviral shRNA against SMAD-7 and cultured under Th17 conditions. Knockdown of SMAD-7 in CD4+ T cells resulted in increased production of IL-17 and other Th17-related cytokines with concomitant downregulation of IL-2 (Figure 4, H–J). Consistent with the downregulation of IL-2, SMAD-7 inhibition resulted in enhanced SMAD-2 and SMAD-3 phosphorylation (Figure 4K). Similarly, we found an increase in IL-17 levels when SMAD-7 was knocked down in CD4+ T cells using SMAD-7–conditional–KO mice (Figure 4L). To determine whether reducing SMAD-7 levels in Mir21–/– mice would restore Th17 differentiation in Mir21–/– CD4+ T cells, we blocked SMAD-7 expression using lentiviral shRNAs and found that blocking SMAD-7 activity restored IL-17 levels in Mir21–/– Th17 cells, which was associated with a reduction in IL-2 levels (Figure 4M). It has been demonstrated that TGF-β inhibits IL-2 production of T cells in a SMAD-3–dependent manner (22, 47). In addition, a SMAD-binding element
Figure 4. miR-21 promotes Th17 differentiation by targeting SMAD-7. (A and B) Representative immunoblots of phosphorylated and total SMAD-2/3/7 proteins in CD4+ T cells from WT and Mir21−/− mice stimulated with TGF-β (2 ng/ml) for the indicated times. (C) miR-21 aligned with the highly conserved 3′ UTR of Smad7 mRNA. (D and E) Luciferase activity of a reporter carrying a mutant or WT Smad7 3′ UTR cotransfected into HEK-293 T cells with miR-21 or with its control. (F) Western blot analysis of SMAD-7, p-SMAD-2, and p-SMAD-3 in CD4+ T cells from WT mice treated with miR-21 inhibitors or with its control. (G) Western blot analysis of SMAD-7, p-SMAD-2, and p-SMAD-3 in CD4+ T cells from WT mice treated with miR-21 precursors or with its control. (H and I) SMAD-7 knockdown increased IL-17 and expression of other Th17-related cytokines in CD4+ T cells. Numbers represent the frequency of CD4+ cells. (J) ELISA of IL-2 in Th17 cells transduced with control or SMAD-7–specific shRNA. (K) SMAD-7 knockdown increased TGF-β–induced SMAD-2/3 phosphorylation, while downregulating SMAD-7 levels. (L) IL17 expression in WT and Smad7−/− T cells cultured under Th17 conditions. (M) SMAD-7 knockdown increased IL-17, while downregulating IL-2 in Mir21−/− Th17 cells. (N) ELISA of IL-2 in Th17 cells from WT and Mir21−/− mice. (O) Neutralization of IL-2 restored IL-17 levels in Mir21−/− Th17 cells. Data are representative of 2 to 3 independent experiments. Error bars represent the mean ± SEM. **P < 0.01 and ***P < 0.001 by unpaired Student’s t test.
(SBE) is located upstream of the Il2 promoter, which is important for SMAD-mediated transcriptional suppression of IL-2 (49, 50). Furthermore, IL-2 has been shown to inhibit Th17 differentiation both in vitro and in vivo (23, 51, 52). In order to elucidate the connection between impaired TGF-β-mediated SMAD signaling and reduced Th17 responses in the absence of miR-21, we investigated IL-2 expression. Substantially elevated levels of IL-2 were observed in Th17 cells from Mir21−/− T cells compared with the levels detected in Th17 cells from WT T cells, which correlated with less production of IL-17 by Mir21−/− T cells (Figure 4N). To determine whether the increased production of IL-2 in Mir21−/− mice inhibited the generation of Th17 cells, we blocked IL-2 activity in Th17 cultures using a combination of neutralizing anti-IL-2 and blocking anti-CD25 antibodies and found that blocking IL-2 activity restored IL-17 levels in Mir21−/− Th17 cells (Figure 4O). Together, these observations demonstrate that miR-21 limits the production of IL-2 to promote Th17 differentiation. Furthermore, enhanced IL-2 levels can offset the effect of TGF-β in Mir21−/− T cells and might explain the normal differentiation of Tregs observed in these mice.

Silencing miR-21 ameliorates the clinical severity of EAE. The effect of miR-21 on IL-17 expression was then tested using the miRNA inhibitor LNA-anti–miR-21. We found that silencing miR-21 in vitro led to decreased levels of miR-21 and Il17 transcripts in T cells cultured under Th17-polarizing conditions (Figure 5, A and B). Furthermore, miR-21 silencing significantly decreased the expression of other cytokines that are related to the Th17 phenotype (Figure 5C). We next investigated whether silencing miR-21 could inhibit the encephalitogenicity of Th17 cells in vivo. For this, we stimulated MOG-specific 2D2 T cells under Th17-polarizing conditions in the presence of control or anti–miR-21 inhibitors and transferred the resulting T cells into syngeneic WT mice. Transplanted mice were injected with pertussis toxin (PT) on the day of T cell transfer and 48 hours later and monitored for the development of EAE. We found that mice that received Th17 cells differentiated in the presence of control
inhibitor developed EAE. However, anti–miR-21 treatment suppressed EAE induced by adoptive transfer of T cells differentiated under Th17-polarizing conditions (Figure 5D). We and others have demonstrated the therapeutic efficacy of LNA-modified oligonucleotide (LNA-anti-miR-21) in EAE and other disease models (53–55). We therefore investigated whether systemic administration of anti–miR-21 in vivo affects the course of EAE. Administration of LNA-modified anti–miR-21 during the preclinical stage of EAE (beginning on day 5 after immunization) substantially ameliorated clinical disease (Figure 5E). To investigate whether anti–miR-21 treatment affected Ag-specific IL-17 recall responses, we isolated spleens from MOG-immunized mice treated with scrambled control or anti–miR-21 and stimulated them in vitro with MOG peptide. We found that splenocytes from anti–miR-21–treated mice had diminished MOG-specific IL-17 expression (Figure 5F). In addition, consistent with our data and with the inhibitory effect of anti–miR-21 on clinical EAE symptoms, we found that anti–miR-21 treatment prevented upregulation of IL17 transcripts in CD4+ T cells isolated from the CNS of anti–miR-21–treated mice (Figure 5G).

Discussion

Th17 cells play an important role in inflammation and autoimmunity. Although the differentiation of Th17 cells is known to be regulated by specific transcription factors and cytokines, the role of miRNA pathways that intrinsically control Th17 differentiation remains elusive. Here, we report that miR-21 expression is specifically elevated in Th17 cells. CD4+ T cells lacking miR-21 exhibit a specific Th17 cell defect in vitro and during neuroinflammatory disease in vivo. Resistance to EAE induction in Mir21−/− mice and the substantial reduction of IL-17+ CNS cell infiltrates in these mice demonstrate that miR-21 is crucial for the control of Th17 development. Critically, we found that in vivo silencing of miR-21 using LNA-modified anti-miR-21 reduced the clinical severity of EAE and was associated with a decrease in Th17 cells.

TGF-β signaling plays an essential role in the generation of Th17 cells (6–8), yet little is known about the miRNA pathways that intrinsically control these pathways. Our results suggest that miR-21 promotes Th17 differentiation by targeting SMAD-7, a negative regulator of TGF-β signaling. SMAD-7 inhibits TGF-β-induced transcriptional responses by blocking activation of SMAD-2/3 and their complex formation with SMAD-4 (18). Most importantly, SMAD-7 has been shown to bind to SMAD-2 and SMAD-3, which are thought to competitively bind to TGF-βRII, and prevents their activation upon TGF-β stimulation. Mechanistically, we have shown that miR-21 deficiency renders T cells less sensitive to TGF-β-induced SMAD-2/3 activation due to enhanced SMAD-7. Overexpression of miR-21 reduced SMAD-7 expression while upregulating SMAD-2/3 levels, and miR-21 knockdown reversed this TGF-β-induced SMAD-7 expression. Furthermore, silencing SMAD-7 resulted in enhanced IL-17 production, which correlated with increased SMAD-2/3 activation in T cells. Consistent with our data, a recent study demonstrated that the generation of Th17 cells is associated with increased TGF-β-induced SMAD-2/3 activation (21). As a functional miR-21 target, SMAD-7 therefore represents a distinct regulator of Th17 differentiation.

TGF-β signaling through SMAD-dependent and SMAD-independent pathways plays a role in Treg differentiation and function (56–61). Although TGF-β signaling through the SMAD-2/3 pathway has been demonstrated to partially regulate iTreg differentiation, it has also been shown that a combination of SMAD-2 and SMAD-3 deficiency does not alter FOXP3 expression or the suppressive activity of iTregs in vivo (58). In fact, the development, homeostasis, and function of Tregs remained intact in SMAD-2 and SMAD-3 double-deficient mice, suggesting a role for a SMAD-independent pathway in Treg differentiation and function (60). Among the SMAD-independent TGF-β signaling pathways, p38 MAPK signaling has been shown to be required for the conversion of naive CD4+ T cells into iTregs (61). Furthermore, IL-2 has been shown to stabilize TGF-β-induced FOXP3 expression and compensate for the loss of defective SMAD-dependent TGF-β signaling in iTreg differentiation (21). Our observation of enhanced IL-2 levels and the unaltered SMAD-independent TGF-β signaling pathway in Mir21−/− T cells seems to be able to offset the effect of TGF-β in these cells and might explain the normal differentiation of Tregs observed in Mir21−/− mice.

IL-2 has been shown to negatively regulate Th17 differentiation both in vitro and in vivo (23, 49, 52, 62). For example, the uptake of extracellular IL-2 by Tregs promotes Th17 differentiation (52). In addition to Tregs, the consumption of IL-2 by effector Th cells may limit the availability of IL-2 in the cellular microenvironment and prevent the paracrine effect of IL-2 on Th17 differentiation. In fact, IL-2 has been shown to promote clonal expansion and effector development of T cells in vivo and has been the growth-supporting cytokine in classical T cell clone–transferred EAE studies (63–65). Within Th17 cells, downregulation of IL-2 expression by TGF-β occurs by a SMAD-mediated inhibition of gene transcription (20–23). We found that during Th17 differentiation, miR-21 plays a significant role in the downregulation of IL-2 expression and that the antibody against IL-2 abrogated the observed defect in Th17 differentiation induced by Mir21−/− T cells. Therefore, our results suggest that miR-21 promotes the SMAD-mediated transcriptional downregulation of IL-2, acting as a positive regulator of Th17 differentiation. In the absence of miR-21, SMAD-7 levels are elevated, which in turn results in impaired TGF-β signaling, increased IL-2 expression, and the observed inhibition of Th17 differentiation (Figure 6). Thus, in addition to the T cell–extrinsic mechanism of IL-2 modulation, our results identify a T cell–intrinsic mechanism that limits the production of IL-2 to promote Th17 differentiation.

miR-21 was one of the earliest identified “oncomirs,” and therefore much of the research involving this miRNA has focused on its role in tumor promotion (66); however, our data build upon emerging reports establishing a crucial role for miR-21 in Th17-mediated autoimmunity. Specifically, our study corroborates the previous observation that miR-21 is overexpressed in the CNS-infiltrating T cells of animals with EAE (67) and is consistent with miR-21 involvement in other autoimmune disease models. For example, silencing miR-21 in vivo has led to a significant reduction of splenomegaly in lupus mice (68), targeted ablation of miR-21 has led to reduced lung eosinophilia after allergen challenge in mice (69), and most recently, Mir21−/− mice have been shown to be resistant to dextran sulfate sodium–induced (DSS-induced) colitis (70). Supporting these animal models, increased expression...
of miR-21 has been observed in peripheral blood mononuclear cells from patients with MS (27). Increased miR-21 levels have also been reported in T cells from patients with SLE and psoriasis (28–30), and these levels correlated with disease activity. Given the ameliorating effect of anti–miR-21 we observed in EAE, the implication of miR-21 in multiple autoimmune disease models, and the expression profile of miR-21 in patients with MS and other autoimmune diseases, silencing miR-21 may be an effective therapeutic approach in the treatment of MS and other Th17-mediated inflammatory diseases. In conclusion, we have identified a T cell–intrinsic miRNA pathway that enhances TGF-β signaling, limits the autocrine inhibitory effects of IL-2, and thereby promotes Th17 differentiation and autoimmunity.

Methods

Mice. C57BL/6, C57BL/6×SV129 (F1), Smad7−/−, and Cd4-Cre mice were obtained from The Jackson Laboratory. Mir21−/− C57BL/6×SV129 (F1) mice were provided by Eric N. Olson (University of Texas Southwestern Medical Center, Dallas, Texas, USA).

Induction and evaluation of EAE. Mice were injected s.c. into both flanks with 100 μg MOG35–55 peptide (MEVGWYRSPFSRVVHLNYRNGK) dissolved in PBS emulsified in an equal volume of CFA (Difco) supplemented with 5 mg/ml Mycobacterium tuberculosis H37Ra. They were also injected twice i.p. with 200 ng PT (List Biological Laboratories) administered on the day of immunization and 48 hours later. Clinical assessment of EAE was performed daily after disease induction according to the following criteria: 0, no disease; 1, tail paralysis; 2, hind limb weakness or partial paralysis; 3, complete hind limb paralysis; 4, forelimb and hind limb paralysis; 5, moribund state. Mean clinical scores on separate days were calculated by adding the scores of individual mice and dividing by the total number of mice in each group, including mice that did not develop signs of EAE. For histopathological studies, spinal cords were dissected from female mice (n = 5/group), fixed in 10% formalin in PBS, and embedded in a single paraffin block. Sections (6–10 μm thick) were stained with H&E and Luxol fast blue. Stained sections were evaluated for immune cell infiltration and demyelination.

Proliferation assay. CellTrace Violet–labeled naive CD4+ T cells were cultured in flat-bottom 96-well plates and stimulated with plate-bound anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml). Cell proliferation was assessed by flow cytometry after 72 hours of culture using the Proliferation Platform analysis tool in FlowJo (version 9) software. We examined the following parameters, as defined by FlowJo software: Percent Divided, the fraction of original cell populations that have undergone at least 1 cell division; Division Index, average number of cell divisions that cells in the original cell population have undergone (includes undivided cells/peak); Proliferation Index, total number of cell divisions divided by the number of proliferating cells (excludes undivided cells/peak); Expansion Index, the fold-change expansion of the overall cultures (includes undivided cells/peak); and Replication Index, the fold-change expansion of only the proliferating cells (excludes undivided cells/peak). Alternatively, splenocyte suspensions were generated from MOG35–55 immunized WT and Mir21−/− mice. Splenocytes from individual mice depleted of rbc were labeled with CellTrace Violet and plated (5 × 10⁵ cells/well) in 200 μl Iscove’s modified Dulbecco’s medium (IMDM) with and without 25 μg/ml MOG35–55 peptide. After 72 hours, proliferation of MOG35–55 specific T cells was analyzed by flow cytometry.

IL-17 secretion assay. IL-17–secreting CD4+ T cells were isolated using the Miltenyi Biotec Mouse IL-17 Secretion Assay – Cell Enrichment and Detection Kit. In brief, total CD4+ T cells from MOG35–55 peptide–immunized WT mice were stimulated with PMA plus ionomycin for 4 hours. Subsequently, an IL-17–specific catch reagent was attached to the cell surface of T cells. The cells were then incubated for 45 minutes at 37°C to allow cytokine secretion. The secreted IL-17 binds to the IL-17 catch reagent on the positive IL-17–secreting cells. These cells were subsequently labeled with a second IL-17–specific detection antibody, and IL-17+ and IL-17– CD4+ T cells were sorted by flow cytometry.

Analysis of miR-21 expression. For analysis of miR-21 expression, real-time reverse transcription PCR (RT-PCR) was performed using TaqMan MicroRNA Assays (Applied Biosystems, Thermo Fisher Scientific). Relative expression was calculated using the Ct method and
normalized to uniformly expressed U6 snRNA or snoRNA135 (Applied Biosystems, Thermo Fisher Scientific). miR-21 values were expressed relative to the expression of U6 snRNA or snoRNA135. The levels of miR-21 in CNS-infiltrating T cells during peak EAE were normalized to T cells isolated from spleens of naïve mice.

RNA isolation, cDNA synthesis, and real-time RT-PCR. Total RNA was isolated from cell pellets using the RNeasy Micro Kit (QIAGEN). RNA was stored at -80°C. First-strand cDNA synthesis was performed for each RNA sample from 0.5 to 1 μg of total RNA using TaqMan reverse transcription reagents. cDNA was amplified using sequence-specific primers. The probes used were identified by the following Applied Biosystems assay numbers: Ifng, Mm01168134_m1; Il17a, Mm99999062_m1; Rorgt, Mm01261019-m1; Rora, Mm00443103_m1; Irf4, Mm00516431_m1; Batf, Mm00479410_m1; Hifla, Mm01283760_m1; Il17f, Mm00521423_m1; Foxp3, Mm00475151_m1; Il4, Mm00445259_m1; Il23r, Mm00519942_m1; Il25, Mm01160011_m1; Il6, Mm99999064_m1; Il1b, Mm01336189_m1; Tnfa, Mm00443258_m1; Ccr6, Mm01323931_m1; Il6r, Mm00439653_m1; Tgfbr1, Mm00439674_m1; Tgfbr2, Mm00436977_m1; Il2, Mm00432562_m1; Btg2, Mm00476162_m1; Pdcd4, Mm00476162_m1; Pten, Mm00427708_m1; Spry1, Mm01285700_m1; Spry2, Mm00444234_m1; and Real-Time PCR Mix (Applied Biosystems, Thermo Fisher Scientific) on the ABI7500 cycler. The Gapdh gene was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. All values were expressed relative to Gapdh expression.

Anti-miR-21 treatment. An antisense oligonucleotide modified by LNA was synthesized to inhibit miR-21 (Exiqon). For in vivo anti-miR-21 treatment, anti-miR-21 and scrambled controls (30 μg/mouse) were administered i.v. to MOG-immunized mice on days 5, 7, 9, 11, and 13 after immunization.

Th differentiation. For in vitro Th17 cell differentiation, naïve CD4+CD62L-CD44+ cells from WT and Mir21−/− mice were sorted by flow cytometry and activated with plate-bound anti-CD3 (2 μg/ml) and anti-CD28 (2 μg/ml) in the presence of TGF-β (2 ng/ml) and IL-6 (30 ng/ml). IL-2 activity in Th17 cell cultures was blocked with a combination of neutralizing anti-IL-2 (10 μg/ml) and blocking anti-CD25 (10 μg/ml) antibodies (BD Biosciences). For Th1 polarizations, naïve CD4+ T cells were activated with IL-12 (20 ng/ml) in the presence of anti-IL-4 (20 μg/ml) antibody (BD Biosciences). For Th2 polarizations, naïve CD4+ T cells were activated with IL-4 (20 ng/ml) in the presence of anti-IFN-γ (20 μg/ml) antibody (BD Biosciences). For iTreg differentiations, naïve CD4+ T cells were activated with TGF-β (5 ng/ml) in the presence of IL-2 (50 U/ml). Twenty-four hours after culture, transcription factor expression was analyzed by real-time RT-PCR. Five days after activation, cells were restimulated with PMA plus ionomycin for 4 hours for intracellular cytokine analysis by flow cytometry.

Generation and isolation of DCs. DCs were derived from BM progenitor cells. In brief, femoral and tibial cells were harvested in DC culture medium (RPMI 1640 medium, 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 20 ng/ml GM-CSF, and 10 ng/ml IL-4) and seeded in 24-well plates at a density of 1 × 106 cells/well. Culture medium was replaced with fresh medium after 3 days. On day 6, dislodged cells were used as BM-derived DCs. Splenic DCs were isolated from spleens using magnetic CD11c beads (Miltenyi Biotec).

Lentiviral shRNA transfection. CD4+ T cells were cultured in the presence of lentiviral particles and polybrene (8 μg/ml) (Sigma-Aldrich).

Cells were centrifuged at 900 g for 30 minutes at room temperature and incubated overnight. Cells were then centrifuged to remove the viral particles and cultured in fresh T cell differentiation medium. After 4 days, cells were analyzed for IL-17 expression by flow cytometry.

CD4+ T cell transfer. CD4+ T cells were prepared from the spleens and inguinal LNs of WT and Mir21−/− mice using the CD4+ T Cell Isolation Kit (Miltenyi Biotec) (purity was >95%). CD4+ T cells (2 × 107 cells/mouse) were injected i.v. into WT or Mir21−/− mice. Five days later, the recipient mice were subjected to EAE induction. For Th1- and Th17-mediated EAE, WT and Mir21−/− mice were immunized with MOG35–55 Peptide emulsified in CFA. Ten days after immunization, mice were sacrificed, and CD4+ T cells from spleens and inguinal LNs were cultured for 5 days in the presence of 50 μg/ml MOG peptide and 20 ng/ml IL-23 to generate Th17 cells, or with MOG peptide and 20 ng/ml IL-12 to generate Th1 cells. Naïve CD2+CD4+ T cell–polarized Th17 cells in the presence or absence of miR-21 inhibitors (2 × 107 cells per mouse) were transferred into naïve mice. Transplanted mice were injected with PT on the day of T cell transfer and again 48 hours later. EAE progression was monitored as described above.

Preparation and evaluation of CNS cells. Animals were perfused with cold PBS. Brains and spinal cords were dissected and incubated in 2.5 μg/ml collagenase D for 30 minutes at 37°C. Single-cell suspensions were prepared by passing them through a 70-μm strainer. Cells were washed in RPMI 1640 medium, and mononuclear cells were isolated using a discontinuous Percoll gradient (Pharmacia). Cells were washed twice, and CD4+ T cells were isolated from this suspension by magnetic separation using microbeads (Miltenyi Biotec).

 Luciferase assay. Semiconfluent (70%–80%) HEK-293 cells were cotransfected with pRL-GAPDH (gift of Matthias Brock, Center of Experimental Rheumatology, University Hospital Zurich, Switzerland) and SMAD-7 WT or mutant luciferase constructs using DharmaFECT Duo Transfection Reagent (GE Healthcare) according to the manufacturer’s instructions. Washed 6 hours after transfection and incubated for 24 hours. Cells were then lysed by passive lysis buffer, and luminescence was measured using the Dual-Glo Luciferase Assay System (Promega) on a Veritas Luminometer (Turner BioSystems). SMAD-7 luciferase readings (firefly luciferase) were normalized to GAPDH luciferase (Renilla luciferase) readings, and fold changes were calculated as compared with those in control cells.

MOG tetramer staining. Splenocytes derived from WT and Mir21−/− mice immunized with MOG35–55 peptide were harvested at disease onset and stained with MOG43–49-PE tetramer (1:100) or control tetramer–PE (NH Tetramer Facility) for 2 hours at room temperature in IMDM and analyzed by flow cytometry.

Immunoblotting. Naïve CD4+ T cells were stimulated for 24 hours with plate-bound anti-CD3 and anti-CD28. After a 16-hour resting period, cells were stimulated with TGF-β (2 ng/ml) at the indicated time points (Figure 4, A and B). Cells were homogenized in RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% NP40), 1X protease inhibitor cocktail (Roche Applied Science), and 1X phosphatase inhibitor cocktail (Sigma-Aldrich), and equal amounts of protein (25 μg) were resolved by polyacrylamide gel electrophoresis. Proteins were transferred onto a nitrocellulose membrane, and immunoblotting was performed with mouse monoclonal anti-SMAD-7 (R&D Systems), GAPDH and α-tubulin (Sigma-Aldrich), rabbit polyclonal p-SMAD-2, p-SMAD-2, SMAD-3, p-p38MAPK, p-ERK, p-JNK, p38MAPK, ERK, and JNK antibodies (Cell Signaling Technology).
SMAD-7 HRP-conjugated secondary antibodies against mouse or rabbit were purchased from Jackson ImmunoResearch. Images were captured using the Bio-Rad ChemiDoc MP imaging system.

Statistics. Statistical analysis was performed using the unpaired 2-tailed Student’s t test. A P value of less than 0.05 was considered statistically significant. Data are presented as the mean ± SEM. For EAE, groups were compared using linear regression analysis.

Study approval. Animals were maintained in specific pathogen-free conditions in the animal facility of Harvard Institutes of Medicine. All mice were 6–10 weeks of age at the beginning of the experiments. All Experiments were reviewed and approved by the IACUC of Harvard Medical School.

Acknowledgments
We thank E.N. Olson (University of Texas Southwestern Medical Center, Dallas, Texas, USA) for the Mir21−/− mice; M. Brock (Center of Experimental Rheumatology, University Hospital Zurich) for pRL-GAPDH, and G. Liu (University of Alabama at Birmingham, Birmingham, Alabama, USA) for the SMAD-7 WT and mutant luciferase constructs. This work was supported by grants from the NIH (AI435801 and NS38037, to H.L. Weiner); the Nancy Davis Foundation for MS (to G. Murugaiyan); the National Multiple Sclerosis Society (RG 4904A2/1, to G. Murugaiyan); and the Swiss National Science Foundation (PP00P3_150663, to N. Joller). The Vaidya laboratory is supported by an NIH Outstanding New Environmental Scientist Award (ES017543).

Address correspondence to: Gopal Murugaiyan or Howard L. Weiner, Ann Romney Center for Neurologic Diseases, Brigham and Women’s Hospital and Harvard Medical School, HIM 720, 77 Avenue Louis Pasteur, Boston, Massachusetts 02115, USA. Phone: 617.849.0912; E-mail: mgopal@rics.bwh.harvard.edu (G. Murugaiyan). Phone: 617.525.5300; E-mail: hweiner@rics.bwh.harvard.edu (H.L. Weiner).