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miR-17-92 Cluster Targets Phosphatase and Tensin Homology and Ikaros Family Zinc Finger 4 to Promote T_H17-mediated Inflammation*[S]

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Background: miRNA is a key component of post-transcriptional network governing the fate of T cells.
Results: By targeting PTEN and IKZF4, miR-17-92 cluster promotes T_H17 differentiation and T_H17-related inflammation.
Conclusion: miR-19b and miR-17 within the cluster additively promote T_H17 responses through distinct regulatory networks.
Significance: Our study provides novel regulatory mechanisms and potential therapeutic candidates against autoimmunity.

The miR-17-92 cluster regulates a broad spectrum of biological processes of T cell immunity. This cluster was found to facilitate T cell proliferation, enhance antitumor activities and promote T cell-dependent antibody responses. However, little is known about the role of this miRNA cluster in the development of autoimmune diseases. Multiple sclerosis is a neuro-destructive autoimmune disease caused by the pathogenicity of T_H17 cells, whose differentiation is tightly controlled by a variety of transcriptional and post-transcriptional regulators. Our study unveils the critical role of miR-17-92 in T_H17 differentiation: T cell-specific miR-17-92 deficiency reduced T_H17 differentiation and ameliorated experimental autoimmune encephalomyelitis (EAE) symptoms. We demonstrated that miR-17 and miR-19b are the two miRNAs in this cluster responsible for promoting T_H17 responses. MiR-19b represses the expression of Phosphatase and Tensin Homology (PTEN), thereby augmenting the PI3K-AKT-mTOR axis essential for proper T_H17 differentiation. Meanwhile, miR-17 enhances T_H17 polarization by inhibiting a novel target, Ikaros Family Zinc Finger 4 (IKZF4). By establishing the miR-17-92 cluster as a key driver of T_H17 responses, our data identify this miRNA cluster as a potential therapeutic target for the clinical intervention of multiple sclerosis.

MicroRNAs (miRNAs)[2] are a class of small non-coding RNAs with versatile regulatory functions in eukaryotic cells. Mature miRNAs are mounted onto the RNA-induced silencing complex which interacts with a particular mRNA molecule. This process leads to translational repression and/or RNA decay of target mRNA (1). Dysregulated miRNA expression has been associated with a variety of immunological diseases (2), depicting miRNA's indispensable role in maintaining proper immune defense and immune homeostasis (3–5).

As a critical compartment of the adaptive immune system, $CD4^+$ T cells are well recognized by their capability of differentiating into various T cell subtypes to accomplish different tasks: T_H1 cells secrete IFNγ and are involved in anti-viral and anti-tumor immunity; T_H2 cells produce IL-4, IL-5, and IL-13 to defend against helminth infection; T_H17 cells can produce IL-17, IL-22, and IL-23 and controls fungi invasion; T follicular helper cells (T_{FH}) supports B cell affinity maturation to elicit proper humoral immunity; Tregs, on the other hand, downscale immune responses to prevent excessive inflammation (6).

Among the numerous immunoregulatory miRNA species, the miR-17-92 cluster had been suggested to play pivotal roles in the regulation of immune responses, including $CD4^+$ T cell differentiation. MiR-17-92 was the first "oncomiR" ever identified: its expression was highly elevated in lymphoma B cell from cancer patients and its overexpression accelerated tumorigenesis (7); on the other hand, in the immune system, our group demonstrated that miR-17-92 is indispensable for anti-tumor $CD4^+$ T cell responses by enhancing IFNγ-secreting T_H1 differentiation and by supporting tumor-specific T cell survival and proliferation (8). These pro-inflammatory functions of this unique cluster were complemented by recent reports that miR-17-92 is essential for eliciting optimal antibody responses by affecting T_{FH} differentiation (9, 10). Moreover, miR-17-92 also carries a mechanism that biases $CD4^+$ T cells away from immune tolerant T_{reg} induction. In the absence of miR-17-92, $CD4^+$ T cells were poised for strong iTreg differentiation (8); and, thymic-derived Tregs critically rely on miR-17-92 for their IL-10 production and immune suppressive functions (11).

Although miR-17-92's regulatory functions have been elaborated in a variety of immunological responses, its effect in autoimmunity was not well established. Some evidence suggests this cluster may be involved in multiple sclerosis (MS), as one member of miR-17-92, miR-17 was shown to be up-regulated in MS patients (12). MS is an autoimmunity-mediated neuro-demyelination disease elicited by the activation of T_H17 cells. Besides its pivotal role in anti-fungal responses, T_H17 is also a pathogenic branch of $CD4^+$ T cells contributing to the

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[2] The abbreviations used are: miRNA, microRNA; RORγt, retinoid-related orphan receptor-γ; STAT3, signal transducer and activator of transcription 3; FOXP3, Forkhead box P3; GFI1, growth factor-independent 1 transcription repressor; TGFBRII, transforming growth factor, β-receptor II; CREB1, cAMP responsive element-binding protein 1.

onset of various autoimmune diseases, including MS (13–15). In MS patients, myelin specific T_H17 cells infiltrate into the central nervous system (CNS) and produce pro-inflammatory cytokines such as GM-CSF and IL-17 (16, 17). IL-17 secreted from T_H17 cells can stimulate astrocytes and attract neutrophils to foster the inflammation (18). IL-17 was also shown to directly target glial cells, which potentially impedes myelin repair and aggravates disease progression (19). Because of the importance of T_H17-mediated pathogenesis in MS, key regulators of T_H17 differentiation have been extensively studied (20, 21). Transcriptional network mapping has uncovered the key modulators of T_H17 lineage initiation, commitment and stabilization (22, 23). However, since the role of miR-17-92 in T_H17 lineage is yet to be investigated, it is unclear whether this transcription factor network relates to the post-transcriptional regulatory network of miR-17-92.

In this study, we utilized a mouse strain in which miR-17-92 was specifically depleted in T cells. In combination with the experimental autoimmune encephalomyelitis (EAE) mouse model of MS, we showed that loss of miR-17-92 resulted in impaired T_H17 differentiation, which consequently reduced EAE pathogenesis. We further dissected this cluster and identified specific miRNAs executing the whole cluster's function. Through identification of their functionally relevant gene targets, we elucidated the molecular mechanism underlying these regulatory miRNAs.

EXPERIMENTAL PROCEDURES

Animals—miR-17-92$^{f/f}$ and Rag2$^{-/-}$ mice were purchased from the Jackson Laboratory. CD4-Cre mice were purchased from Taconic. CD4$^+$ T cell conditional miR-17-92 knock-out mice were generated by mating miR-17-92$^{f/f}$ and CD4-Cre mice and back-crossing to the C57BL/6 background. All animal-related procedures were conducted with the approval from the Duke University Animal Care and Use Committee.

In Vitro Cell Culture, T_H17 Polarization and Retrovirus Infection—For *in vitro* cultures, total lymph nodes or spleen were collected from mice and single cell suspension was prepared. CD4$^+$ Cells were then purified with Dynabeads® Untouched™ Mouse CD4 Cells kit (Invitrogen). Cells were stimulated by 1 μg/ml plate-bond α-CD3 and α-CD28antibodies. 10 μg/ml α-IFNγ, 10 μg/ml α-IL-4, 50 ng/ml mouse IL-6, 4 ng/ml human TGFβ, 20 ng/ml mouse IL-1β, and 50 ng/ml IL-23 were added in the culture for T_H17 polarization. Antibodies described above were purchased from BioXCell, and cytokines from Biolegend. PIK-75 (PI3K inhibitor) was purchased from EMD Biosciences (#528116) and was applied at the concentration of 25 nM. miRNA overexpression vectors were constructed with MSCV retrovirus backbone, and retroviruses were generated by transfecting the BOSC 23 packaging cell line. Viruses were harvested from the supernatant 48 h post-transfection. Retroviruses were then used to infect activated T cells by centrifugation at 1258 $\times$ *g* at 37 °C.

RT-qPCR, Luciferase Assay and Western Blot—RNA was extracted from cells by mirVana™ miRNA Isolation kit (Invitrogen). Reverse transcription was performed using qScript™ Flex cDNA kit (Quanta Biosciences). Quantitative PCR was conducted with PerfeCTa® SYBR® Green FastMix® (Quanta). 96-well qPCR array primers were synthesized from Integrated DNA Technologies. miRNA-target binding was predicted using online algorithm TargetScan. Luciferase reporter vector was constructed by cloning target 3′-UTR downstream of firefly luciferase. This vector also contains *Renilla* luciferase transcribed from a separate promoter. By normalizing firefly luciferase to *Renilla* luciferase, the repression efficacy of miRNA was assessed. Western blot was performed using Novex® 10% Tris-Glycin Gel (Invitrogen). Primary antibody α-IKZF4 was acquired from Santa Cruz Biotechnology (sc-292209), α-Actinβ from Sigma-Aldrich. Secondary antibody α-rabbit-Alexa Fluor 680 was purchased from Invitrogen.

Flow Cytometry—Flow cytometry antibodies were purchased from Biologend. For surface staining, the cells were harvested and stained in FACS buffer (PBS with 2% FBS and 2 mM EDTA). For intracellular cytokine staining, the cells were harvested and further stimulated with 500 ng/ml Ionomycin and 1 μM PdBU (Sigma Aldrich) in the presence of Golgi block Monensin and Brefeldin A (eBioscience) for 4 h, followed by fixation in 2% paraformaldehyde-PBS and permeabilization in 0.1% Saponin-FACS buffer. For Foxp3 stanining, cells were processed and stained with Foxp3 staining buffer kit (eBioscience). For intracellular IKZF4 staining, the cells were first fixed in 2% paraformaldehyde-PBS and permeabilized in 90% methanol-PBS. Subsequently, cells were subjected to primary rabbit-α-mouse IKZF4 (Santa Cruz Biotechnology) and secondary antibody staining (Jackson Immunoresearch). Flow cytometry was conducted with BD FACSCanto™ II analyzer.

Experimental Autoimmune Encephalomyelitis Induction—125 μg of myelin oligodendrocyte glycoprotein $(MOG)_{35-55}$ peptide was emulsified in complete freund's adjuvant (CFA). The mix was injected into the mouse subcutaneously. 200 ng/mouse of pertussis toxin was injected intravenously on day 0 and day 2 postimmunization. Clinical score was assessed on a daily basis according to the extent of paralysis: no clinical symptoms (0); limp tail (1); weak paralysis of two hind limbs (2); complete paralysis of two hind limbs (3); paralysis of both hind and fore limbs (4); death (5).

Colitis Model—Mouse CD4$^+$CD25$^-$CD45RB^{Hi} naïve T cells were sorted from donor mice and 0.5 million cells were injected into recipient mice intraperitoneally. Mouse body weights were monitored on a daily basis. At the experiment end-point, mice were sacrificed and the colon weight/length ratios were measured. Mesenteric lymph nodes and spleens were collected, and T cells were analyzed for intracellular expression of IL-17A and IFNγ expression by flow cytometry.

Statistics—Two-tailed Student's t tests were used to determine whether the means from two experimental groups were significantly different. p values of <0.05 was indicative of significant difference. In certain time-course studies, the difference between two groups were accessed via testing whether the regression slope were significantly different. p value of <0.05 indicates the samples of interest do not derive from a single population.

RESULTS

miR-17-92 Deficiency in CD4$^+$ T Cells Leads to Impaired T_H17 Differentiation in Vitro and Mitigates EAE Progression—To investigate the role of miR-17-92 cluster in the differentia-

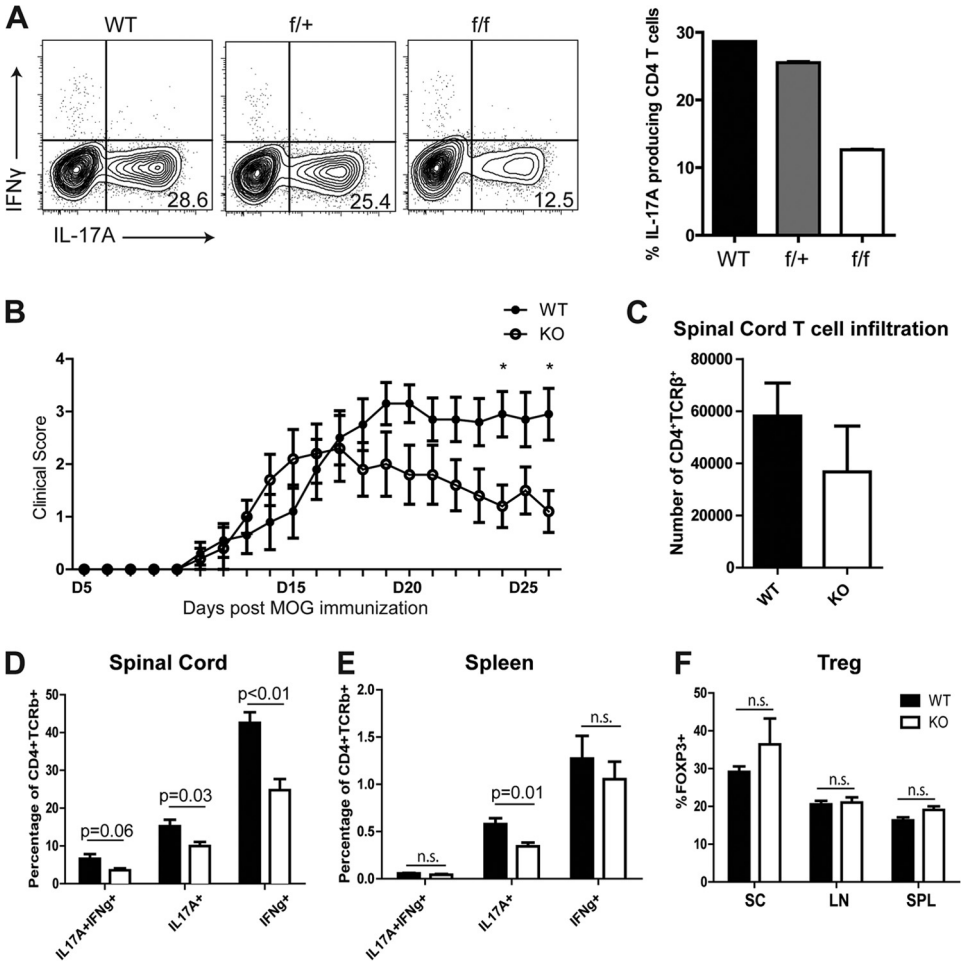


FIGURE 1. **MiR-17-92 deficiency in $CD4^+$ T cells impairs T_H17 differentiation and mitigates EAE progression.** *A*, $CD4^+$ T cells were isolated from wild type (WT), miR-17-$92^{f/+}$-CD4-Cre (f/+), or miR-17-$92^{f/f}$-CD4-Cre mice (f/f) mice and stimulated by anti-CD3ϵ and anti-CD28 for 5 days in T_H17 skewing conditions, followed by intracellular staining for IL-17A and IFNγ and flow cytometry analysis. *B*, wild type (WT, $n = 10$) and miR-17-$92^{f/f}$-CD4-Cre (KO, $n = 5$) mice were immunized with MOG peptide and injected with pertussis toxin to elicit EAE, and the clinical score is measured daily according to "Experimental Procedures." *C–F*, cells from EAE day 26 mice were collected from spinal cord (*SC*), spleen (*SPL*), and lymph nodes (*LN*) and analyzed for T cell number (*C*), IL-17A, IFNγ expression (*D*, *E*), and Foxp3 expression (*F*). Statistics were done by unpaired Student's *t* test with error bars indicating S.E.

tion of T_H17 cells, we employed the miR-17-$92^{f/f}$-CD4-Cre conditional knock-out mouse as previously described (8). This mouse line features two loxp sites flanking the whole miRNA cluster (24), together with Cre expression under the control of CD4 promoter to ensure T cell-specific miR-17-92 depletion. With these mice, we first tested whether miR-17-92 cluster affects T_H17 differentiation *in vitro*. Using plate-bound anti-CD3 and anti-CD28 antibodies, $CD4^+$ T cells from total lymph nodes of either wild type (WT), miR-17-92 heterozygotes (f/+) or miR-17-92 knock-out (f/f) mouse were stimulated through their antigen receptors. The cells were cultured under T_H17 polarization conditions to assess their differentiation capacity. T_H17 differentiation of miR-17-92-deficient T cells was markedly decreased *in vitro*, as identified by reduced IL-17A production (Fig. 1*A*).

We next studied the impact of this impaired T_H17 differentiation *in vivo* using the EAE mouse model of MS. Since T_H17 cells are known to be the critical mediators of MS in human patients, we induced EAE in either wild-type (WT) mice or miR-17-$92^{f/f}$-CD4-Cre (KO) mice. In KO mice, we observed alleviation in EAE symptoms, as indicated by reduced clinical scores (Fig. 1*B*). We found no significant differences in the numbers of pathological $CD4^+$ T cells infiltrating the central nervous system (Fig. 1*C*). However, a closer examination at the effector function of these $CD4^+$ T cells revealed that KO cells collected from the spinal cords and spleens of EAE mice had significantly diminished IL-17 expression (Fig. 1, *D* and *E*), together, the IL-17 producing cell number was also significantly decreased (supplemental Fig. S1). Consistent with our previous findings that miR-17-92 facilitates IFNγ production (8), we also observed a significant decrease in IFNγ producing T cells in the spinal cord. However, although the loss of miR-17-92 enhanced iT_{reg} induction *in vitro* and within the tumor microenvironment (8), under our EAE inducing condition, we were unable to identify differences in Treg percentages between WT and KO mice, in both the spinal cords and spleens (Fig. 1*F*). Taken together, our data suggest that miR-17-92 is important for T_H17 differentiation *in vitro* and T_H17 responses *in vivo* and the ablation of this cluster mitigates EAE progression.

miR-17-92-deficient $CD4^+$ T Cells Are Impaired in Colitis Induction—We next validated the function of miR-17-92 in T_H17 induction using the colitis model. In lymphopenic $Rag2^{-/-}$

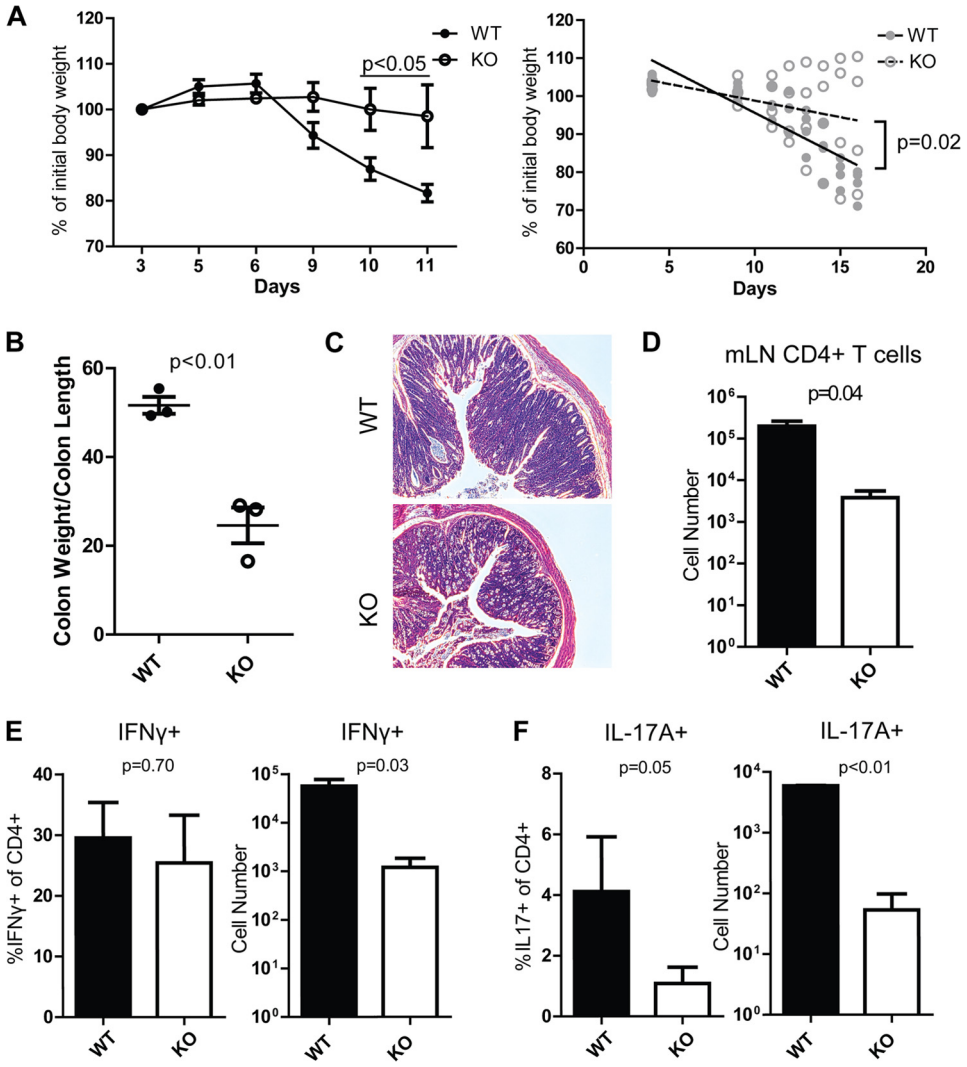


FIGURE 2. **miR-17-92deficient CD4+ T cells are impaired in colitis induction.** *A*, $CD4^+CD25^-CD45RB^{Hi}$ naïve T cells were sorted from wild type (WT) and or miR-17-$92^{f/f}$-CD4-Cre mice (*KO*) mice and adoptively transferred into $Rag2^{-/-}$ recipients. Body weights of WT and KO recipients were measured daily after T cell transfer, and were normalized to the initial body weight of each mouse. The *left* and *right panels* represent two independent experiments. *B*, colons were harvested from mice 11 days post WT or KO T cell transfer, and colon weight/length ratio (an indicator of colitis severity) was calculated. *C*, H&E staining of the colon sections of mice with WT or KO T cell transfer. Note the differences between the two groups, in terms of mononuclear cell infiltration (density of blue staining), numbers of goblet cells (cells with white vacuoles) and villus integrity. *D*—*F*, quantification of absolute $CD4^+$ T cell numbers, IFNγ^+ and IL-$17A^+$ T cell percentages and cell numbers in the mesenteric lymph nodes of WT and KO groups. Statistics were calculated by unpaired Student's *t* test; error bars indicates S.E. Specifically, the *p* value of *right panel* in *A* was determined by testing whether there is a significant difference between the regression slopes of WT and KO group.

mouse, the adoptively transferred naïve $CD4^+$ T cells become activated and differentiate into T_H1 and T_H17 lineages to induce colitis (25). We adoptively transferred $CD4^+CD25^-CD45RB^{Hi}$ naïve WT or KO T cells into the $Rag2^{-/-}$ hosts, and monitored their body weights daily. Compared with WT T cells, we found that KO T cells were ineffectual at eliciting colitis-induced weight loss: in recipients of KO T cells, weight loss was significantly retarded (Fig. 2*A*). In addition, the colon weight/length ratio, an indicator of colon inflammation severity, was significantly reduced in mice receiving KO T cells (Fig. 2*B*). Accordingly, H&E staining of the colon sections revealed stark differences between the two groups of recipient mice: in the colons of mice receiving WT T cells, there was drastic loss of villus integrity, together with substantial infiltration of inflammatory mononuclear cells into the villi, and, severe depletion of goblet cells; In the colons of mice receiving KO T cells, while mononuclear infiltration was present, the gross architecture of both villi and goblet cells remained intact (Fig. 2*C*). Further analysis revealed that miR-17-92 deletion results in significantly reduced accumulation of $CD4^+$ cells in the mesenteric lymph nodes (Fig. 2*D*), which is likely a consequence of reduced proliferation and increased activation-induced apoptosis associated with miR-17-92 deletion (8). More importantly, while IFNγ production was comparable between two groups in this disease model (Fig. 2*E*), we observed suppressed IL-17A expression in transferred KO T cells (Fig. 2*F*). Notably, in our colitis model, iTreg differentiation was negligible in either WT or KO $CD4^+$ T cell transfer (supplemental Fig. S2). Our data imply that miR-17-92 deficiency hinders the $CD4^+$ cells' ability to induce colitis, potentially by reducing T_H17 responses.

miR-17 and miR-19b Are Core Components in the Cluster that Promote T_H17 Differentiation—To better understand the mechanism by which miR-17-92 cluster regulates T_H17 differentiation, we dissected the contribution from individual

miRNAs in this cluster. By utilizing retroviral vectors to overexpress individual miRNAs in miR-17-92 knock-out T cells, we were able to achieve miRNA expression above the wild type level (supplemental Fig. S3). We then found that two miRNAs, miR-17 and miR-19b, were most potent in rescuing the T_H17 differentiation defect (Fig. 3, *A* and *B*). We then asked if miR-17 and miR-19b promotes T_H17 differentiation in an additive or synergistic manner. To examine their combinatory effects, we designed a retroviral vector that expresses a miR-17-92 mutant, miR-1719b. miR-1719b harbors deletions of the miR-19a and miR-20a guide strands, and contains mutations covering the whole seed regions of miR-18 and miR-92. This allows us to overexpress miR-17 and miR-19b simultaneously in the context of their original cluster (Fig. 3*C*). Combined miR-17 and miR-19b expression demonstrated an additive effect in driving T_H17 differentiation of KO $CD4^+$ T cells in comparison to those cells expressing miR-17 or miR-19b alone (Fig. 3*D*). Accordingly, the transcription of *il17a*, *il17f*, and master regulator *rorc* were also highly induced by concurrent expression of miR-17 and miR-19b (Fig. 3*D*). Collectively, by breaking down the miR-17-92 cluster into individual miRNAs, we demonstrated that miR-17 and miR-19b are potentially the major players in promoting T_H17 differentiation.

miR-19b Enhances T_H17 Differentiation by Targeting PTEN—We then examined how miR-19b functions to foster T_H17 differentiation. We and others have validated phosphatase and tensin homology (PTEN) as a target of miR-19b in tumor cells, B cells, and $CD4^+$ T cells (8, 26). By dephosphorylating PIP_3, PTEN is well recognized as a negative regulator of the PI3K-AKT-mTOR signaling pathway (27). Importantly, recent studies imply that activation of the PI3K-AKT-mTOR pathway is essential for the induction of T_H17 differentiation *in vitro* and *in vivo* (28). Therefore, we speculated that miR-19b promotes T_H17 response through the inhibition of PTEN to enhance the PI3K-AKT-mTOR pathway. We first confirmed that miR-19b overexpression is capable of down-regulating PTEN during the differentiation of T_H17 cells (Fig. 4*A*). One direct consequence of PTEN dysregulation is the alteration of AKT activation, marked by the phosphorylation of two critical residues on AKT: threonine 308 (T308) and serine 473 (S473) (29). When we activated WT and KO $CD4^+$ T cells via TCR stimulation under the T_H17-differentiating conditions, we observed that both T308 and S473 phosphorylation were significantly retarded in KO cells (Fig. 4*B*). We then examined whether this miR-19b-mediated PTEN down-regulation is sufficient to influence T_H17 differentiation. With a low dose of the selective inhibitor PIK-75, we specifically blocked the activity of p110α subunit of PI_3 kinase to assess the contribution of the PI3K-AKT-mTOR pathway for T_H17 differentiation in miR-17-92 KO $CD4^+$ T cells (30). We reasoned that the loss of miR-19b should already have accumulated sufficient PTEN in these KO cells to increase the PI3K-AKT-mTOR signaling threshold required for T_H17 differentiation. Therefore, KO cells would be less susceptible to further pharmaceutical inhibition of PI3K. By differentiating CD4 T cells *in vitro* in the presence or absence of PIK-75, we could determine the inhibition index of T_H17 differentiation ($1\text{-IL-}17_{\text{PIK-75}}/\text{IL-}17_{\text{DMSO}}$). While PIK-75 is still capable of reducing IL-17 production in KO $CD4^+$ T cells (Fig. 4, *C* and *D*), the extent of reduction is significantly lower in these miR-17-92-deficient T cells (Fig. 4*E*). In addition, in the absence of PI3K inhibition, both miR-17 and miR-19b rescued KO cells' defect in Th17 differentiation (Fig. 4, *C* and *D*). However, in the presence of PI3K inhibition, the rescue by miR-17 and miR-19b differs significantly: rescue delivered by miR-19b depends on PI3K activation (Fig. 4, *C* and *D*) and is highly sensitive to PIK-75 treatment; rescue delivered by miR-17, which functions through a PTEN-independent mechanism (Fig. 4*A*), is more resistant to PI3K inhibition (Fig. 4*E*). Taken together, we concluded that miR-19b enhances T_H17 differentiation by targeting PTEN.

miR-17 Promotes T_H17 Differentiation by Repressing Novel Target IKZF4—Previously, we identified that miR-17 protects $CD4^+$ T cells from excessive activation induced cell death and inhibits iTreg differentiation by targeting the upstream signaling molecule TGFBRII and the transcription factor CREB1 (8). Although they are implicated in T cell differentiation (31–33), we failed to detect any significant change in the expression of these two proteins in T_H17-biased $CD4^+$ T cells (supplemental Fig. S4). To identify the direct target(s) of miR-17 during T_H17 differentiation, we conducted a qPCR array on 93 core mediators controlling various stages of T_H17 differentiation. These genes were selected based on recent transcription factor network analysis of differentiated T_H17 cells and dynamic regulatory network analysis of T_H17 cells during the differentiation process (22, 23). Our list of core mediators (supplemental Table S1) includes validated T_H17 regulators (RORγt, STAT3, BATF, etc.) as well as new and predicted key modulators (MINA, FOSL2, SP1, etc.). We collected RNA samples from WT and KO cells transduced with control, miR-17 or miR-19b vectors and performed 5 rounds of qPCR array (Fig. 5*A*, data as in supplemental Table S1). Among the significantly changed genes, we focused on genes that are up-regulated in miR-17-deficient KO $CD4^+$ T cells, and, down-regulated when miR-17 was re-introduced (Fig. 5*A*). With these filtering criteria, we identified two genes, EBI3 and IKZF4, which fit this pattern and are also known to be negative regulators in T_H17 differentiation. IKZF4 belongs to the Ikaros zinc finger transcription factor family and was recently shown to be a composite of the negative module in T_H17 differentiation network (23). With computational algorisms, we failed to identify any conserved miR-17 binding sites in EBI3 mRNA; but, we predicted two potential miR-17 binding sites in the 3′-untranslated region (3′-UTR) of murine IKZF4 (Fig. 5*B*), with position 2394 being highly conserved among mammals. We cloned the IKZF4 3′-UTR containing these two miR-17 binding sites into a luciferase reporters and transfected the construct into 3T3 cells stably transduced with control or miR-17 retrovirus. As expected, miR-17 significantly repressed the expression of IKZF4 3′-UTR conjugated reporter (Fig. 5*C*). Importantly, during T_H17 differentiation, protein levels of IKZF4 was also increased in the KO cells but reduced when miR-17 was reintroduced (Fig. 5*D*). Therefore, we concluded that IKZF4 is a direct target of miR-17.

To further validate whether miR-17- mediated IKZF4 suppression directly promotes T_H17 differentiation, we constructed a rescue vector with concomitant expression of

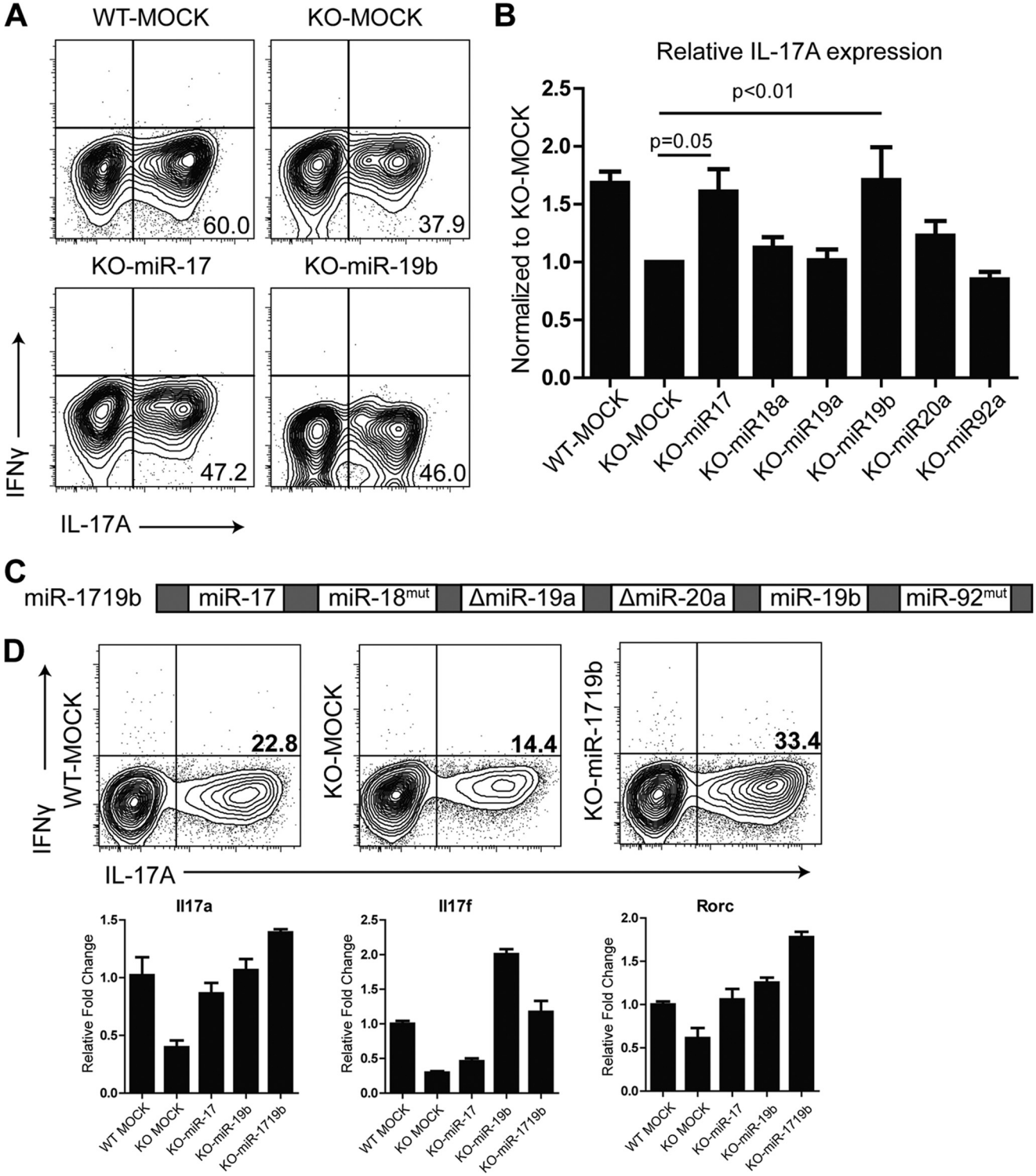
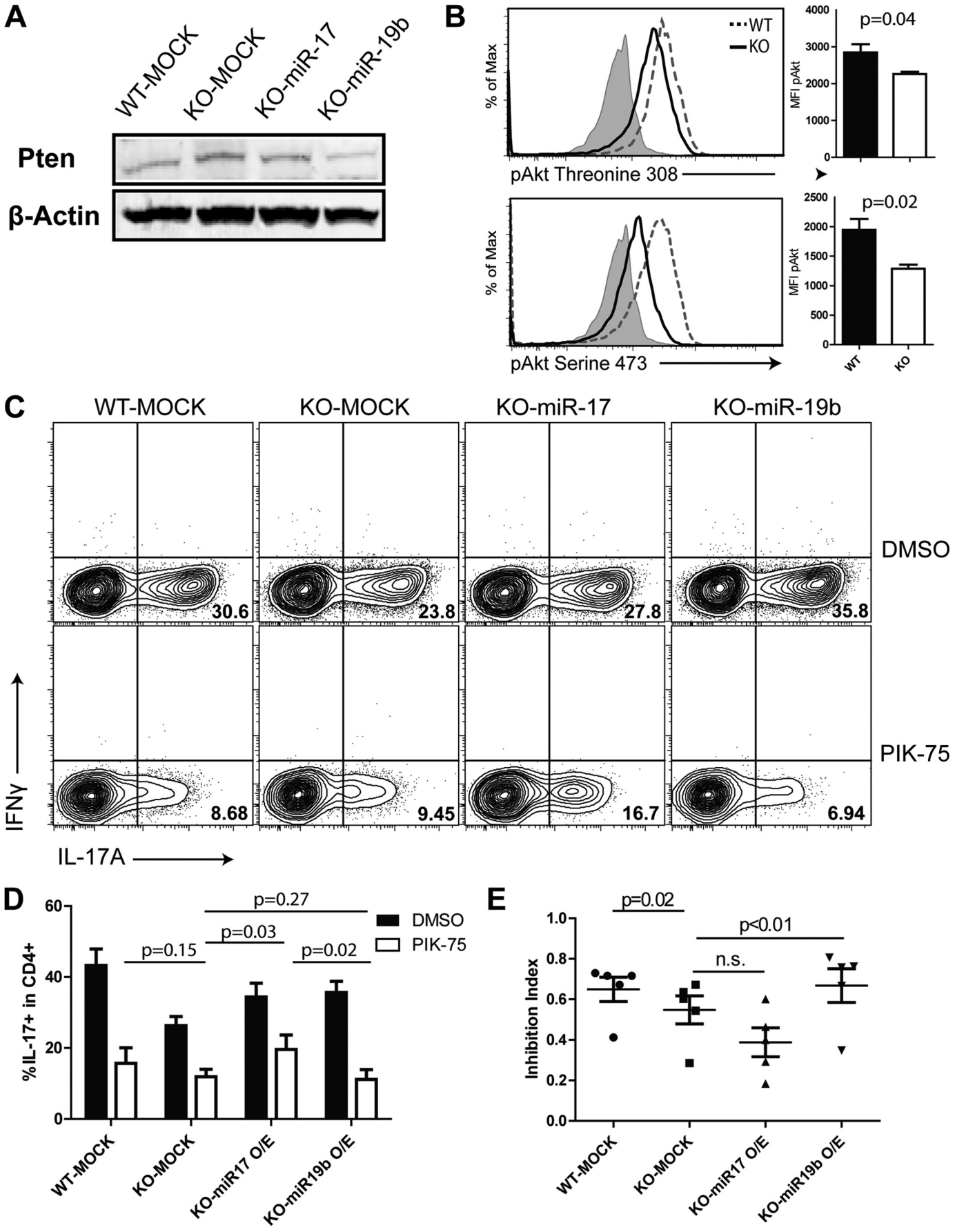


FIGURE 3. **Dissection of miR-17-92 cluster shows miR-17 and miR-19b are core components in the cluster to promote T_H17 differentiation.** *A* and *B*, WT and KO cells ($n = 5$) were stimulated by anti-CD3ϵ and anti-CD28, and subsequently infected with retrovirus expressing empty vector (WT-MOCK and KO-MOCK) or overexpressing each miRNA (KO-miR). Representative of IL-17A and IFNγ staining were shown in *A*, and IL-17A$^+$ percentage is normalized to KO-MOCK group and quantified in *B*. *C*, schematics of miR-1719b retrovirus construct. Seed region of miR-18 and miR-92 was mutated, miR-19a and miR-20a were removed. *D*, WT and KO T cells were stimulated and infected with MOCK or miR-1719b retrovirus as in *A* and *B*. mRNA level of IL-17A, IL-17F, and RORγt was quantified by RT-qPCR. Statistics was calculated by paired *t* test for *B*, with error bars indicating S.E.

miR-17 and the IKZF4 coding sequence concordantly. We found that while miR-17 increases T_H17 differentiation in WT cells, this increase is significantly tuned down when exogenous IKZF4, which can bypass miR-17 targeting, was introduced (Fig. 5*E*). Therefore, we concluded that transcription suppressor IKZF4 is a novel target of miR-17, and this targeting is crucial for miR-17's effect in promoting T_H17 differentiation.


A
WT-MOCK
KO-MOCK
KO-miR-17
KO-miR-19b
Pten
β-Actin
B
WT
KO
% of Max
pAkt Threonine 308
MFI pAkt
p=0.04
pAkt Serine 473
p=0.02
C
WT-MOCK
KO-MOCK
KO-miR-17
KO-miR-19b
DMSO
PIK-75
30.6
23.8
27.8
35.8
8.68
9.45
16.7
6.94
IFNγ
IL-17A
D
% IL-17+ in CD4+
DMSO
PIK-75
p=0.27
p=0.03
p=0.15
p=0.02
WT-MOCK
KO-MOCK
KO-miR17 O/E
KO-miR19b O/E
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Inhibition Index
p=0.02
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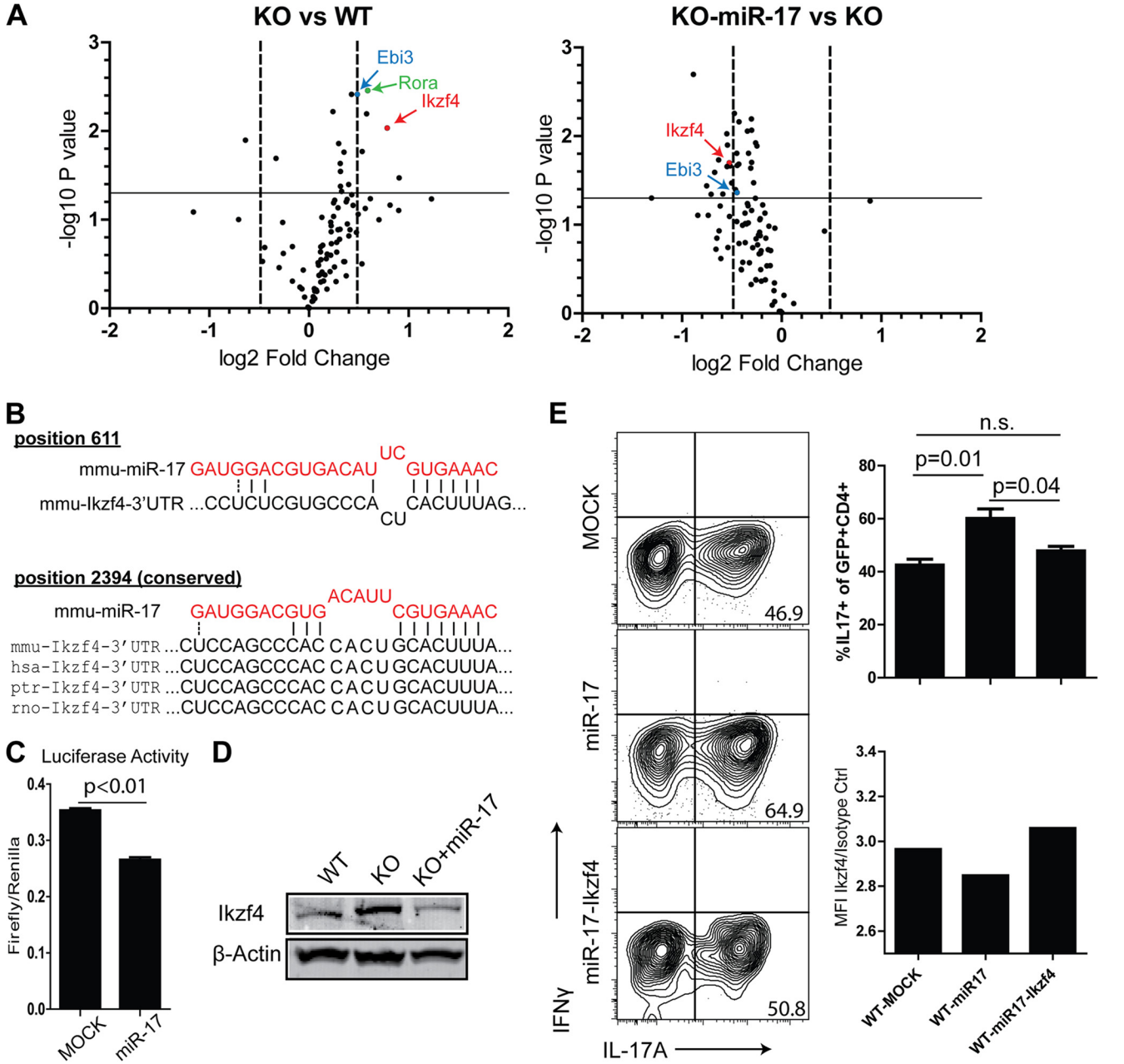

FIGURE 5. **miR-17 promotes T_H17 differentiation by repressing novel target IKZF4.** *A*, volcano graph of qPCR array of 93 key T_H17 regulators normalized to 3 endogenous controls. x axis indicates log2 value of relative fold changes, and y axis indicates log10 *p* value. y interception equals $p = 0.05$. *Left panel* compares KO-MOCK and WT-MOCK group ($n = 5$), and *right panel* compares KO-miR-17 and KO-MOCK group ($n = 5$). *B*, schematics of the two predicted miR-17 binding sites in the 3′UTR of IKZF4. *C*, luciferase assay of IKZF4 3′-UTR constructed into the firefly luciferase reporter normalized to *Renilla* luciferase control. The construct was transfected into 3T3 cells overexpressing MOCK or miR-17 ($n = 5$). *D*, Western blot of IKZF4 and β-actin in WT-MOCK, KO-MOCK, and KO-miR-17 cells cultured in T_H17 conditions. *E*, WT $CD4^+$ T cells were purified and infected with MOCK, miR-17 and miR-17-IKZF4 retrovirus and cultured in T_H17 conditions for 5 days ($n = 3$). IL-17A, IFNγ, and IKZF4 expression was measured by intracellular staining and flow cytometry. Statistics were calculated by paired *t* test for *A* and unpaired *t* test for *D* and *E*.

DISCUSSION

In this study, we demonstrated miR-17-92 cluster as a novel regulator of T_H17 differentiation and its effector pathogenicity. In combination with our previous data that miR-17-92 facilitates cell proliferation, inhibits apoptosis, promotes T_H1 differentiation, and impairs iTreg differentiation, our findings accentuate the notion that this cluster comprehensively controls inflammation elicited by antigen-specific $CD4^+$ T cell activa-

FIGURE 4. **miR-19b enhances T_H17 differentiation by targeting PTEN.** *A*, $CD4^+$ T cells were isolated from WT or miR-17-92$^{f/f}$-CD4-Cre (*KO*) mice and stimulated *in vitro* in T_H17 conditions and infected with MOCK, miR-17 or miR-19b overexpressing retrovirus for 5 days. Cell lysate was collected for Western blotting PTEN and β-actin. *B*, WT and KO $CD4^+$ T cells were purified and starved for 2h in serum-free media, and subsequently stimulated with anti-CD3ε and anti-CD28 antibodies for 30min under T_H17 conditions. Phosphorylation of AKT T308 and S473 in WT and KO $CD4^+$ T cells were measured by flow cytometry. *C*, representative of WT-MOCK, KO-MOCK, KO-miR-17, and KO-miR-19b cell culture in T_H17 skewing conditions and treated with DMSO or PIK-75. *D*, quantification of *C*, $n = 4$. *E*, inhibition index of PIK-75's inhibition efficiency on T_H17 differentiation as calculated by 1-IL-$17_{PIK\text{-}75}$/IL-17_{DMSO}. Statistics were calculated with paired *t* test, with error bars showing S.E.

tion. Evolutionarily, miR-17-92 is highly conserved in mammals (34), and by promoting $CD4^+$ T cell mediated immunity, this cluster may confer survival benefits against non-self intrusions. However, overactivation of T cells also leads to severe consequences such as autoimmune diseases (35, 36). Our animal models indicate this miRNA cluster may be involved in the pathogenesis of MS in humans. By dissecting the individual miRNAs in the context of T_H17 differentiation, we revealed different behaviors of the miRNAs in the cluster, with miR-17 and miR-19b exerting the most potential in driving T_H17 differentiation and may contribute mostly to the onset of MS. This finding also suggests that MS therapies can be designed by targeting miRNAs. The safety and efficacy of oligonucleotide therapy against miRNAs have not only been tested in primates, but have also passed phase 2 human trials (36). Since miR-19b was also found to promote T_H1-mediated inflammation (8), and oligonucleotides against miR-19b may not only benefit MS patients, but also a wide array of inflammatory diseases.

The finding that miR-19b affects PI3K-AKT-mTOR axis in T_H17 differentiation is well consistent with our previous report, in which miR-19b represses PTEN to facilitate $CD4^+$ T cell's anti-tumor response (8). Indeed, PI3K-AKT-mTOR is a central regulatory axis in T_H17 differentiation: the activation of this pathway phosphorylates STAT3 and enhances the transcription of RORγt, the master transcription factor for T_H17 lineage (37); moreover, PI3K-AKT-mTOR was found to facilitate RORγt nuclear translocation, while suppressing the T_H17-negative regulator GFI1 (38). Additionally, PI3K-AKT-mTOR activation is also indispensable for efficient T_H1 differentiation (37). As an antagonist of the PI3K-AKT-mTOR axis, PTEN acts as an important anti-inflammatory mediator by reducing both T_H1 and T_H17 mediated pathogenesis. Therefore, by repressing PTEN in T cells, miR-19b is able to extensively drive immune activation and inflammation.

We previously demonstrated that miR-17 can inhibit iTreg differentiation by targeting TGFBRII (8), and the dominant negative form of TGFBRII was found to impede T_H17 differentiation (31). In view of this, there is a seemingly inverse correlation between miR-17 expression and the T_H17 pathway. However, in our study, we did not detect changes of TGFBRII when miR-17 is overexpressed in T_H17 cells (supplemental Fig. S4). This is probably due to differences in miR-17 targeting between the T_H17 and iTreg contexts.

Instead, we identified IKZF4 as a target of miR-17 in controlling T_H17 differentiation. IKZF4 is a member of the Ikaros family transcription factors. The Ikaros family was originally designated to function in hematopoiesis (39), but it is only until recently that studies begin to show the relevance of this family in T helper cell differentiation. IKZF3 was preferentially expressed in T_H17 cells and governs T_H17 differentiation by silencing IL-2 expression (40). By investigating the dynamics of the T_H17 regulatory network, IKZF4 was proposed by Yosef *et al.* as a composite in the negative regulatory module for T_H17. However, the function of this protein remained elusive. In our report, we provide the first evidence that miR-17 reinforces T_H17 differentiation by repressing IKZF4.

T_H17 and iTreg differentiation processes are mutually antagonistic; however, the plasticities of both lineages remain preserved in differentiated cells (41, 42). Although cytokine signaling plays vital roles in directing the T_H17 and iTreg differentiation paradigms (43), a more fundamental diversion should occur at the epigenetic level. Pan *et al.* demonstrated that IKZF4 has the potential to function at the epigenetic level. By recruiting CtBP1 to epigenetically silence IL-2 and IFNγ, IKZF4 reinforces the suppressive functionality of thymic-derived nTregs (44). In addition, Munn *et al.* demonstrated that IKZF4 is critical for maintaining the lineage specificity of nTregs (45). We speculate that a similar mechanism could be at play during iTreg differentiation, and, IKZF4 may function as an intrinsic modulator in T cells that decides between the T_H17 and iTreg lineage choices. This lineage choice is also impacted by miR-17: miR-17 biases T cells toward the T_H17 lineage, while inhibiting iTreg differentiation (8). Therefore, we propose that by modulating IKZF4 expression, miR-17 is a post-transcriptional mechanism controlling T_H17/iTreg plasticity.

Our molecular dissection indicated that miR-17 and miR-19b are united in many ways to promote the effector function of $CD4^+$ T cells: protecting cells from activation-induced death, inhibiting iTreg differentiation, and, supporting T_H17 differentiation. It is tempting to speculate that signaling pathways modulated by miR-17 and miR-19b may cross-talk with each other. Intriguingly, it has been shown that, in pro-B cells, the activation of PI3K is essential in controlling IKZF1 expression and therefore successful VDJ recombination (46). However, under both T_H1 and T_H17 conditions, manipulation of miR-17 did not cause any measurable impact on PTEN expression (Fig. 4); manipulation of miR-19b could not alter CREB1, TGFBRII (8), or IKZF4 expression (supplemental Fig. S5). Therefore, we concluded that miR-17 and miR-19b most likely modulate two independent signaling networks and their effects on $CD4^+$ T cells are additive.

Recently, other groups reported that miR-17-92 promotes T_{FH} differentiation by repressing genes involved in T_H17 differentiation (9). However, it should be noted that, in this study, the experimental setup does not recapitulate the physiological conditions required for optimal T_H17 induction: LCMV-specific $CD4^+$ T cells carrying miR-17-92 deficiency were transferred into recipient mice followed by LCMV inoculation. In fact, LCMV infection elicits robust T_H1, rather than T_H17 responses. Under these circumstances, the T_{FH} population generated might possess characteristics of T_H1 cells (47), which could fundamentally differ from the *bona fide* T_H17 cells generated in the EAE model. Given the context-dependent nature of miRNA targeting, they do not necessarily contradict miR-17-92's positive effect in T_H17 differentiation in the EAE and colitis settings. Interestingly, RORA was reported to be a target of miR-17-92 in their particular system, and we also detected an increase in RORA at the transcription level in miR-17-92-deficient cells by our qPCR array (Fig. 5*A*). However, in miR-17-92-deficient $CD4^+$ T_H17 cells, this increase in RORA was overpowered by the elevation of PTEN and IKZF4 and their consequent suppressive effects. Therefore, we concluded that the overall role of miR-17-92 supports the T_H17 differentiation program. Moreover, since it has been shown that T_{FH} cells are highly plastic, and certain Peyer's patch T_{FH} cells may even require preliminary T_H17 differentiation (48, 49), it may be

inappropriate to consider T_{FH} and T_H17 as strictly antagonistic lineages.

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