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DDIT4 and Associated lncDDIT4 Modulate Th17 Differentiation through the DDIT4/TSC/mTOR Pathway

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Inflammation that complicates many autoimmune diseases, such as multiple sclerosis (MS), has been correlated to abnormal differentiation of Th17 cells. However, the reasons that promote Th17 cell–driven autoimmunity are yet to be discovered. In this study, we sought evidence that DNA-damage-inducible transcript 4 (DDIT4) and its associated long noncoding RNA DDIT4 (lncDDIT4) inhibit Th17 cell differentiation. We recruited 36 patients. Six MS patients and five healthy volunteers (controls) contributed PBMCs as material for microarray analysis. Microarray assays of lncDDIT4 and DDIT4 RNA expression identified outstanding differences between MS and control subjects, which were verified with real-time quantitative PCR. We then interrupted the expression of lncDDIT4 and DDIT4 mRNA in MS patients' naive $CD4^+$ T cells and observed the resulting changes in Th17 cells. The expression of lncDDIT4 and DDIT4 mRNA were higher both in PBMCs and $CD4^+$ T cells of MS patients than in healthy controls. DDIT4 (2.79-fold upregulation) was then recognized as a candidate for the *cis*-regulated target of lncDDIT4 (4.32-fold upregulation). Isolation of naive $CD4^+$ T cells revealed enhanced levels of lncDDIT4 and DDIT4 after stimulated with Th17-inducing cytokines, but not after Th1, Th2, or T regulatory cell induction. Overexpression of lncDDIT4 in naive $CD4^+$ T cells inhibited IL-17 transcription through increased DDIT4 expression and decreased activation of the DDIT4/mTOR pathway. Consistently, silencing lncDDIT4 in naive $CD4^+$ T cells enhanced Th17 differentiation through increased activation of the DDIT4/mTOR pathway. However, these results vanished when DDIT4 was silenced. This outcome suggests that lncDDIT4 regulates Th17 cell differentiation by directly targeting DDIT4. ***The Journal of Immunology*****, 2018, 200: 1618–1626.**

T helper 17 cells produce IL-17A and IL-17F, which enhance local chemokine production, thereby recruiting lymphocytes to sites of inflammation (1). Th17 cells play an important role in the development of many autoimmune diseases, including multiple sclerosis (MS), systemic lupus erythematosus, and psoriasis (2–6). Accordingly, altered generation of Th17 is crucial for the development of disease. MS is a demyelinating disease of the human CNS mediated by autoreactive $CD4^+$ T cells (7). In MS, Th1 and Th17 cells have been considered to participate in the inflammatory response of the CNS (8).

DNA-damage-inducible transcript 4 (DDIT4) is a cytoplasmic protein originally characterized by its transcriptional upregulation in the setting of DNA damage. Since then, the upregulation of DDIT4 has been attributed to multiple forms of cellular stress, including oxidative stress (9), endoplasmic reticulum stress (10), hypoxia (11), and starvation (12). DDIT4 is known to inhibit mTORC1 activity, although the precise mechanism is still unknown. The enhancement of mTORC1 increases activation of downstream targets by phosphorylation, including P70S6K and 4EBP1, which are believed to be involved in regulating translation and cell proliferation (12). The mTOR pathway has emerged as an important regulator of immune responses (13), including regulating T cell activity (14). Molitoris et al. (15) showed that DDIT4 also functions as an inhibitor of the mTOR pathway in thymocytes, leading to the induction of autophagy as a survival mechanism after dexamethasone treatment. DDIT4 was also found to be essential for optimal T cell proliferation and survival (16).

Long noncoding RNAs (lncRNAs), which play important roles in development, cellular differentiation, proliferation, cell cycle control, and cell death (17), have been implicated in a variety of human autoimmune diseases (18, 19), including MS (20). In our microarray data, we found that lncRNA DDIT4 (lncDDIT4) and DDIT4 were upregulated in PBMCs of MS patients, as verified through real-time PCR. DDIT4, which is upstream of lncDDIT4 (2.87-fold upregulation), is a candidate for the *cis*-regulated target of lncDDIT4. Although many bioinformatics methods support the correlation between lncDDIT4 and DDIT4, their biological roles and molecular mechanisms underlying MS initiation and progression have not been reported.

Our findings suggest that the DDIT4/mTOR axis is involved in the differentiation of Th17 cells. lncDDIT4 as a new member of the family of lncRNAs could directly regulate Th17 cell differentiation through target DDIT4.

Materials and Methods

Human subjects

During an open enrollment, we recruited a total of 36 patients at the acute stage of relapsing-remitting MS according to the McDonald Criteria of MS as revised

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J.H. and G.L. conceived and designed the study; F.Z., C.W., and D.L. acquired and analyzed data; J.H. and F.Z. drafted the manuscript or figures.

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The online version of this article contains supplemental material.

Abbreviations used in this article: Ad, adenovirus; Ad-lncDDIT4, Ad carrying lncDDIT4; Ad-shDDIT4, Ad carrying short hairpin RNA against DDIT4; Ad-sh lncDDIT4, Ad carrying short hairpin RNA against lncDDIT4; CST, Cell Signaling Technology; DDIT4, DNA-damage-inducible transcript 4; lncDDIT4, lncRNA DDIT4; lncRNA, long noncoding RNA; MS, multiple sclerosis; RT-qPCR, real-time quantitative PCR; Treg, T regulatory cell.

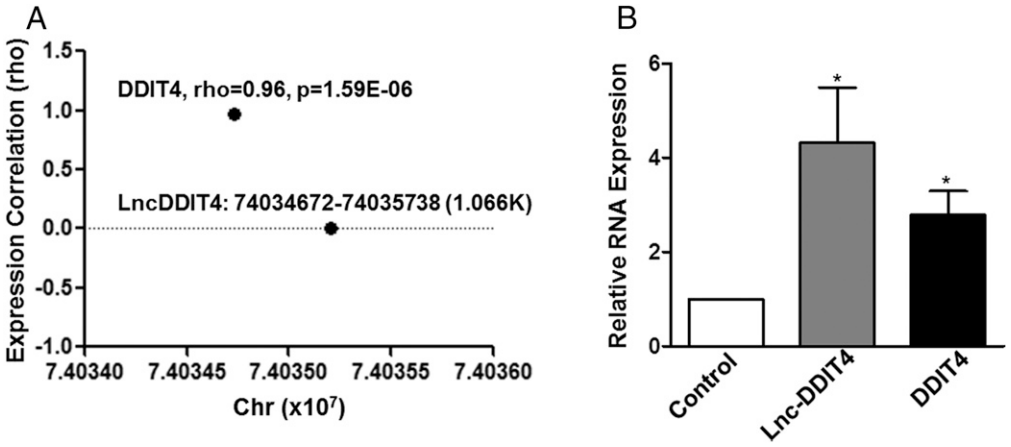

FIGURE 1. (**A**) lncDDIT4 and the positions of its "cis" gene DDIT4 in the chromosome. The *x* abscissa represents the chromosome genome position, and the *y*-coordinate represents the correlation coefficient of lncDDIT4 and DDIT4, with a greater correlation coefficient corresponding to a higher position. Rho values for the encoding genes and lncRNA expression correlation coefficients between the values, as well as the *p* values for the correlation coefficients, are shown. (**B**) Validation of lncDDIT4 and DDIT4 expression levels of $CD4^+$ T cells from MS patients by real-time quantitative PCR (RT-qPCR). Mean ± SEM, $n = 30$ MS versus 26 control. $*p < 0.05$ versus control.

in 2010 (Table I). The site and timing, respectively, were Tianjin Medical University General Hospital and Tianjin Huan Hu Hospital from May 2014 to June 2016. All of these patients presented with numerous disseminations of the disease in space and time. We also tested oligoclonal bands in the cerebrospinal fluid of MS patients. Exclusion criteria were the following: 1) presence of other diseases of the CNS in addition to MS, 2) tumor(s), 3) systemic hematologic diseases, 4) recent infection, and/or 5) concomitant use of antineoplastic or immune-modulating therapies prior to blood sampling. The ethical committees of Tianjin Medical University General Hospital and Tianjin Huan Hu Hospital approved the use of PBMCs from MS patients for research purpose, and informed consent was obtained from each subject. We also recruited 26 healthy volunteers for validation and mechanistic study.

Isolation of PBMCs from peripheral blood

Peripheral blood was obtained during the acute phase of disease from all of these MS patients and healthy volunteers. PBMCs were isolated from peripheral blood by Ficoll-Hypaque density-gradient centrifugation.

T cell differentiation

Human naive $CD4^+$ T cells in culture were purified to >95% by negative selection with magnetic beads (Miltenyi) and were stimulated with Dynabeads Human T-Activator CD3/CD28 (Life Technologies). Th1 polarization was initiated with 10 ng/ml IL-12 and Th2-neutralizing Ab anti–IL-4 (2 μg/ml). Th2 polarization was induced by activation with PHA (4 μg/ml) in the presence of IL-4 (10 ng/ml), neutralizing anti–IFN-γ (2 μg/ml), and anti–IL-12 (2 μg/ml) (21). All remaining reagents listed in this paragraph came from R&D Systems: IL-6 (100 ng/ml), TGF-β1 (3 ng/ml), IL-1β (10 μg/ml), TNF-α (10 μg/ml), IL-23 (10 μg/ml), anti–IL-4 (2 μg/ml; MAB3007), and anti–IFN-γ (2 μg/ml; MAB 285) were used for the generation of Th17 cells. IL-2 (20 ng/ml; 202-IL), TGF-β1 (3 ng/ml), anti–IL-4 (2 μg/ml; MAB3007), and anti–IFN-γ (2 μg/ml; MAB 285) were used for the generation of T regulatory cells (Tregs).

Transfection of adenovirus into naive $CD4^+$ T cells

FITC (3 μl), GFP-labeled, adenovirus (Ad)-targeting lncDDIT4 and DDIT4 or GFP-labeled negative controls were transfected into activated $CD4^+$ naive T cells (Hanbio Biotechnology).

Intracellular cytokine staining

Cells were stimulated for 4 h with PMA, ionomycin, and BFA. Data were acquired using a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) and analyzed with FlowJo software (Tree Star, Ashland, OR).

RNA isolation and real-time quantitative PCR

Total mRNA was isolated using TRIzol (Invitrogen) following the manufacturer's instructions. Expression was normalized to GAPDH. Gene expression was analyzed by comparative cycle threshold.

ELISA

We stimulated naive $CD4^+$ T cells first. Then cytokines were measured in supernatants after 72 h.

Western blotting

Proteins were incubated with anti-DDIT4 (Cell Signaling Technology [CST]), anti–p-STAT3 (CST), anti-mTOR (CST), anti–p-mTOR (CST), anti-P70S6K (CST), anti–p-P70S6K (CST), anti-4EBP1 (CST), anti–p-4EBP1 (CST), and anti-GAPDH (Sigma-Aldrich). The protein-specific signals were detected using Bio-Rad 721BR08844. Bands were analyzed with ImageJ software.

Statistical analysis

SPSS for Windows version 17.0 software (SPSS, Chicago, IL) was used for the analyses. For continuous variables, such as lncDDIT4/DDIT4 expression level, descriptive statistics were calculated and reported

Table I. Baseline characteristics

	Control ($n = 26$)	MS ($n = 36$)	*p* Value
Gender, M/F	8/18	9/27	0.77
Age at onset, median (range), y		32 (19–54)	
Disease duration, median (range), y		5.0 (1–24)	
Annual relapse rate, median (range)		0.7 (0.1–2.4)	
OCBs positive/tested (%)		24/36 (67)	
Brain MRI abnormalities (%)		36/36 (100)	
Spinal MRI abnormalities (%)		24/36 (67)	
EDSS score, median (range)		3 (1–8)	
Poor neurologic outcome (%)		11/36 (31)	

EDSS, Expanded Disability Status Scale; MRI, magnetic resonance imaging; OCB, oligoclonal band.

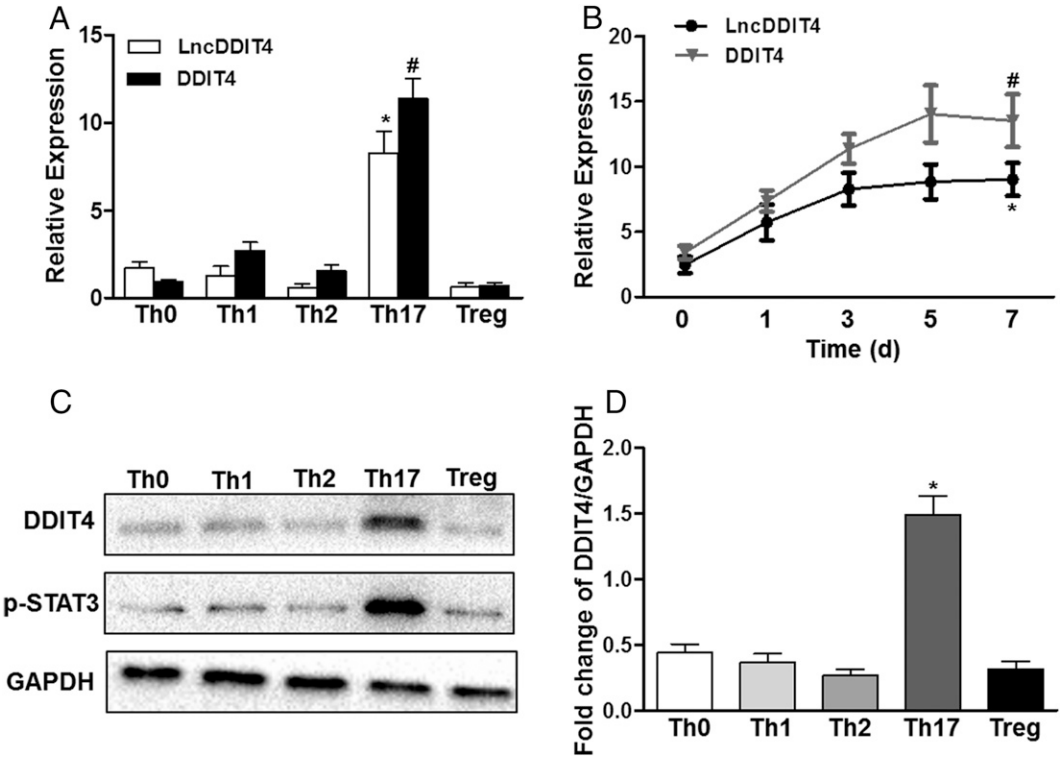

FIGURE 2. lncDDIT4 and DDIT4 expression are induced preferentially during Th17 differentiation in MS patients. (**A**) RT-qPCR analysis of lncDDIT4 and DDIT4 mRNA in naive $CD4^+$ T cells from MS patients. The cells were stimulated for 72 h under Th0-, Th1-, Th2-, Th17-, or Treg-inducing conditions. Results were normalized to GAPDH. Mean ± SEM. $^*p < 0.05$ versus Th0 (lncDDIT4), $^{\#}p < 0.05$ versus Th0 (DDIT4). (**B**) Expression of lncDDIT4 and DDIT4 mRNA in naive $CD4^+$ T cells at several time points during Th17 differentiation. Data are representative of three independent experiments. Mean ± SEM; $n = 30$. $^*p < 0.05$ versus lncDDIT4 (0 d); $^{\#}p < 0.05$ versus DDIT4 (0 d). (**C**) Western blot analysis of DDIT4 and p-STAT3 in cells from MS patients stimulated under Th0, Th1, Th2, Th17, and Treg conditions. (**D**) Cumulative data of densitometry are also shown. Mean ± SEM; $n = 30$. $^*p < 0.05$ versus Th0.

as mean ± SEM. Nonnormally distributed or discontinuous variables were reported as median (range) and compared as groups using a Mann–Whitney *U* test. All continuous variables were compared for the Ad-lncDDIT4 (Ad carrying lncDDIT4)/Ad-sh lncDDIT4 (Ad carrying short hairpin RNA against lncDDIT4) group versus the control Ad group using the *t* test. Categorical variables were compared for the age of MS patients versus controls using the χ^2 test. Statistical significance is defined as $p < 0.05$.

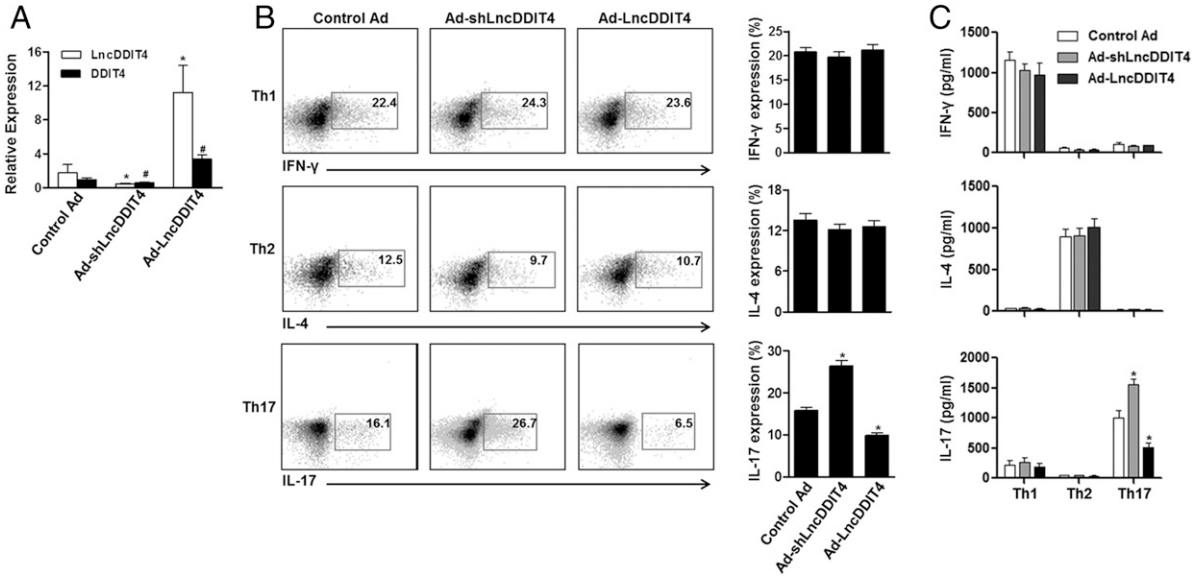

FIGURE 3. lncDDIT4 and DDIT4 inhibit Th17 differentiation in MS patients. (**A**) RT-qPCR analysis of lncDDIT4 and DDIT4 expression in activated naive $CD4^+$ T cells (in the absence of polarizing cytokines) 72 h after transfection with control Ad, Ad-lncDDIT4, or Ad-sh lncDDIT4. Mean ± SEM. (**B**) Naive $CD4^+$ T cells from MS patients differentiated for 72 h under Th1, Th2, or Th17 conditions after transfection with GFP^+ control Ad, Ad-lncDDIT4, or GFP^+ Ad-sh lncDDIT4 for 24 h. The cells were gated for GFP^+ and stained for the intracellular expression of IFN-γ, IL-4, and IL-17A. A profile representative of 30 MS patients per group is shown. Mean ± SEM. (**C**) ELISA of IFN-γ, IL-4, and IL-17 in supernatants of naive $CD4^+$ T cells from MS patients differentiated for 72 h in Th1, Th2, or Th17 conditions in the presence of control Ad, Ad-lncDDIT4, or Ad-sh lncDDIT4. Mean ± SEM; $n = 30$. $^*p < 0.05$, $^{\#}p < 0.05$ versus control Ad.

Results

Microarray analysis demonstrates upregulation of lncDDIT4 and DDIT4 transcripts in PBMCs of MS patients

In our previous study, we found that the mTOR pathway is among the top 15 most enrichment pathways tested in 3739 MS patients (22). In our microarray data (http://www.ebi.ac.uk/arrayexpress/experiments/E-MTAB-6193), we found that the mTOR pathway is among the top 12 most enriched pathways of MS patients tested by using KEGG analysis (PBMCs of six MS patients versus five controls). We also found that DDIT4, one of the most important targets of the mTOR pathway, is highly expressed in MS patients when compared with healthy controls (20). lncDDIT4 and DDIT4 upregulation in $CD4^+$ T cells of MS patients was verified through real-time quantitative PCR (RT-qPCR). DDIT4 (2.79-fold upregulation), which is upstream of lncDDIT4 (4.32-fold upregulation), is a candidate for the *cis*-regulated target of lncDDIT4 (Fig. 1, Table I). We also tested the expression level of lncDDIT4/DDIT4 in monocytes, $CD8^+$ T cells, and B cells. We found that there is no difference between groups when we compared lncDDIT4 and DDIT4 expression levels (Supplemental Fig. 1).

lncDDIT4 and DDIT4 expressions are induced preferentially during Th17 differentiation

lncDDIT4 and DDIT4 expressions are increased in PBMCs and $CD4^+$ T cells from MS patients. To investigate the role that such enhancement of lncDDIT4 and DDIT4 plays in T cell differentiation, we isolated naive $CD4^+$ T cells from MS patients and were stimulated to generate either Th1, Th2, Th17, or Tregs. As shown in Fig. 2A, lncDDIT4 and DDIT4 expressions were much stronger in Th17 cells than in the other CD4 subsets. To characterize this trend, we stimulated naive $CD4^+$ T cells under Th0- or Th17-inducing conditions, then lncDDIT4 and DDIT4 expression levels were tested by RT-qPCR at several time points. lncDDIT4 and DDIT4 expressions were higher during Th17 differentiation (Fig. 2B). In turn, Th17 cell stimulation increased DDIT4 at the protein level (Fig. 2C, 2D). These results indicate that lncDDIT4 and DDIT4 are preferentially induced during Th17 polarization in MS patients.

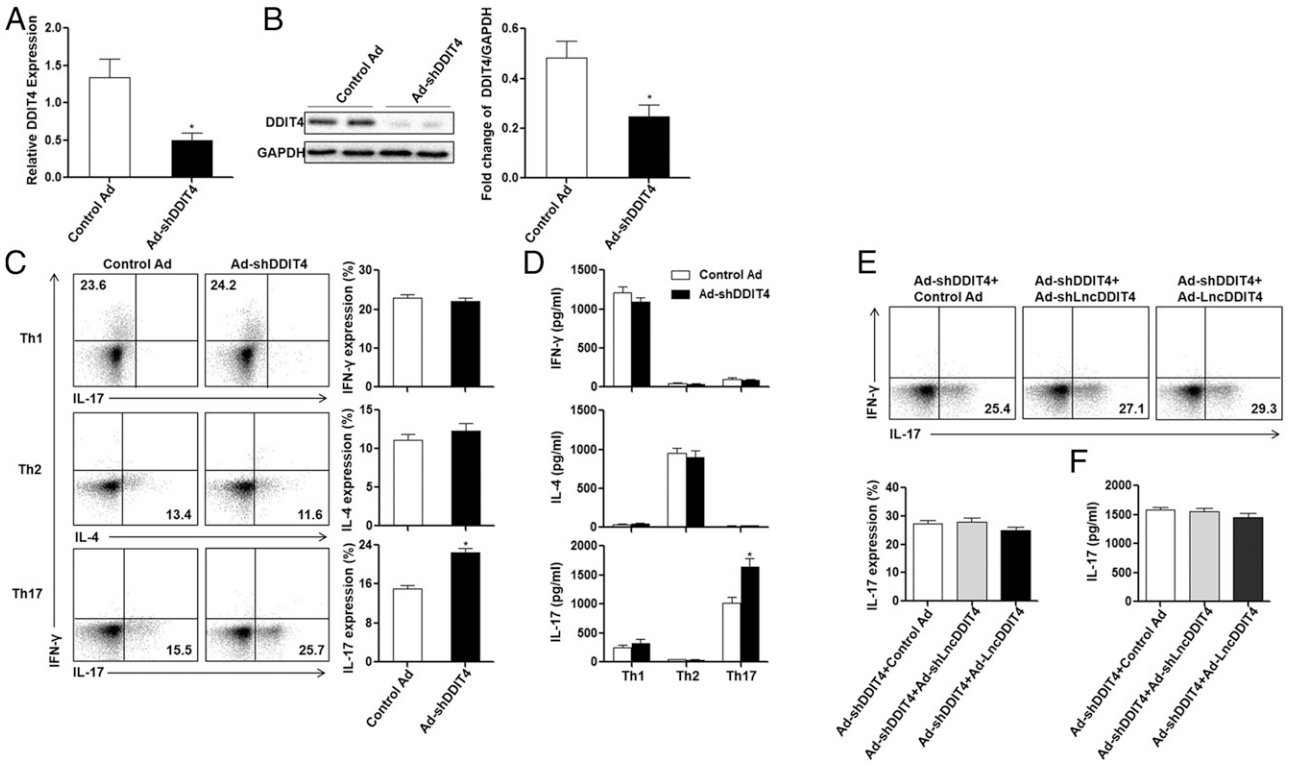

FIGURE 4. lncDDIT4 inhibits differentiation of Th17 cells by targeting DDIT4 in MS patients. (**A**) RT-qPCR analysis of the expression of DDIT4 in activated naive $CD4^+$ T cells 72 h after transfection with control Ad or Ad-shDDIT4. (**B**) Western blot analysis of DDIT4 in activated naive $CD4^+$ T cells from MS patients 72 h after transfection with control Ad or Ad-shDDIT4. (**C**) Naive $CD4^+$ T cells from MS patients differentiated for 72 h under Th1, Th2, or Th17 conditions after transfection with GFP^+ control Ad or GFP^+ Ad-shDDIT4 for 24 h. The cells were gated for GFP^+ and stained for the intracellular expression of IFN-γ, IL-4, and IL-17A. (**D**) ELISA of IFN-γ, IL-4, and IL-17 in supernatants of naive $CD4^+$ T cells from MS patients differentiated for 72 h in Th1, Th2, or Th17 conditions in the presence of control Ad or Ad-shDDIT4 ($*p < 0.05$ versus control Ad; mean ± SEM). (**E**) Naive $CD4^+$ T cells from MS patients differentiated for 72 h under Th17 conditions after transfection with control Ad+Ad-shDDIT4, Ad-sh lncDDIT4+Ad-shDDIT4, or Ad-lncDDIT4+Ad-shDDIT4, respectively, for 24 h. The cells were gated for GFP^+ and stained for the intracellular expression of IFN-γ and IL-17A. (**F**) ELISA of IL-17 in supernatants of naive $CD4^+$ T cells from MS patients differentiated for 72 h in Th17 conditions in the presence of control Ad+Ad-shDDIT4 or Ad-sh lncDDIT4+Ad-shDDIT4 or Ad-lncDDIT4+Ad-shDDIT4 for 24 h. A profile representative of 30 MS patients per group is shown. Mean ± SEM. $*p < 0.05$ versus control Ad.

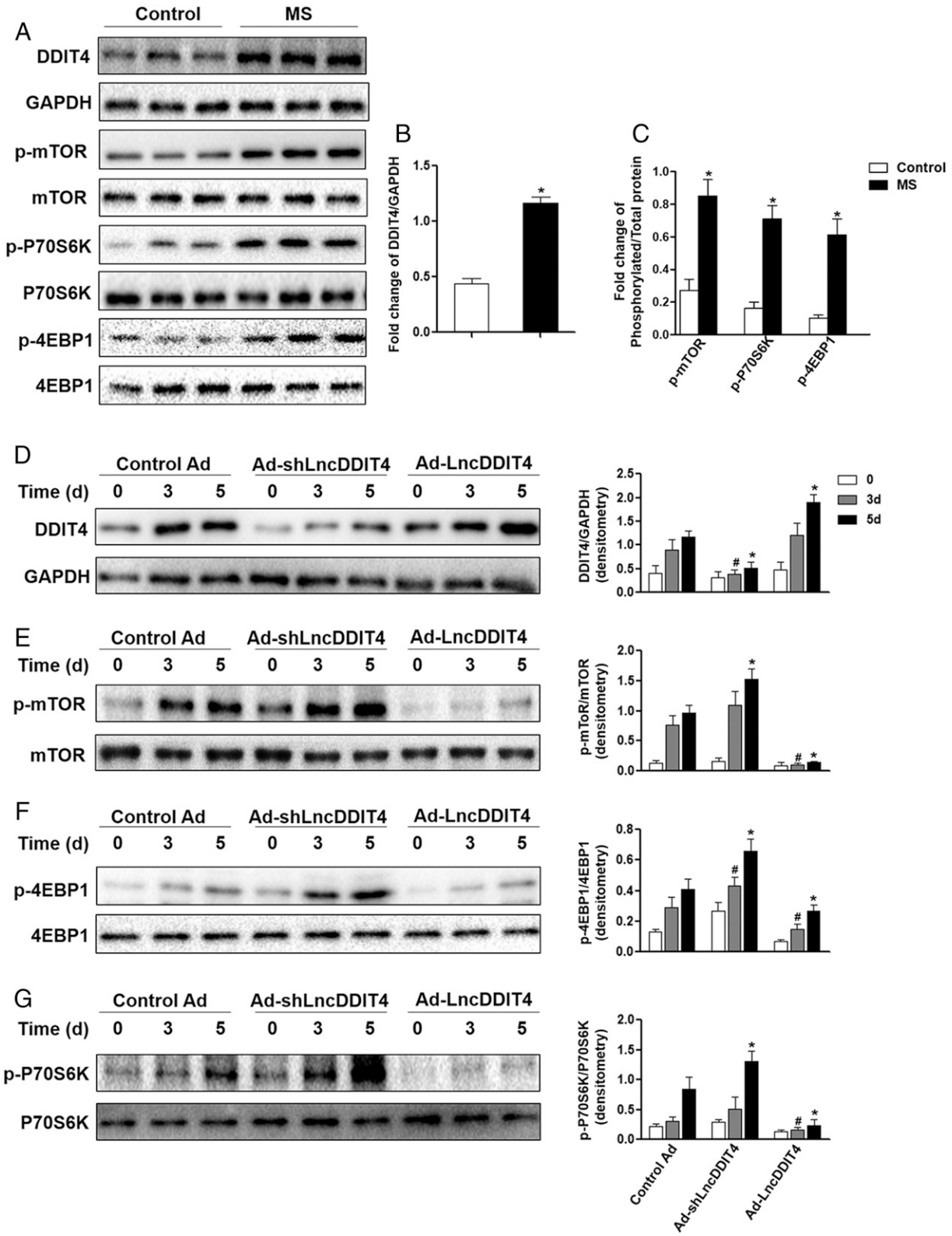

FIGURE 5. lncDDIT4 inhibits DDIT4/mTOR signaling in MS patients. (**A**) Western blotting analysis of DDIT4, p-mTOR, mTOR, p-P70S6K, P70S6K, p-4EBP1, and 4EBP1 in $CD4^+$ T cells of patients with acute MS. The graphs in (**B**) and (**C**) show cumulative data of densitometry (*$p < 0.05$ versus control). (**D**) DDIT4, (**E**) p-mTOR, (**F**) p-4EBP1, and (**G**) p-P70S6K in naive $CD4^+$ T cells from MS patients in Th17 conditions for the indicated times after transfection with control Ad, Ad-sh lncDDIT4, or Ad-lncDDIT4 for 24 h ($^{\#}p < 0.05$ versus control Ad, 3 d; *$p < 0.05$ versus control Ad, 5 d).

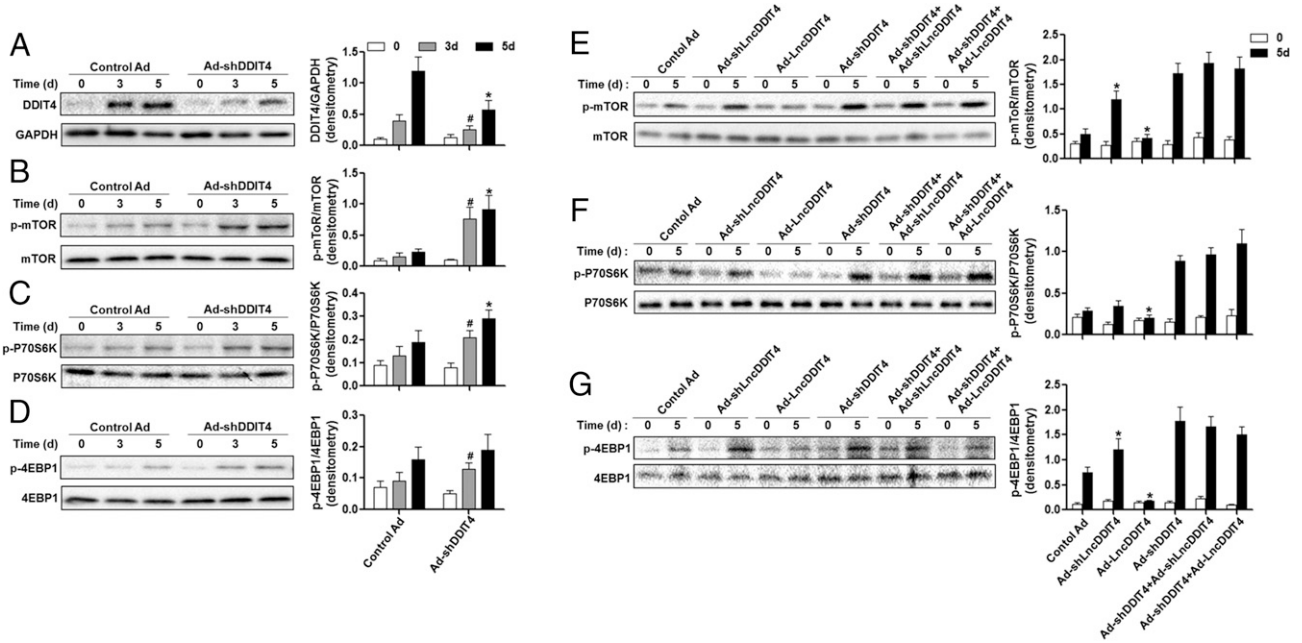

FIGURE 6. lncDDIT4 inhibits DDIT4/mTOR signaling by targeting DDIT4 in MS patients. (**A**) DDIT4, (**B**) p-mTOR, (**C**) p-P70S6K, and (**D**) p-4EBP1 in naive CD4$^+$ T cells from MS patients under Th17 conditions for the indicated times after transfection with control Ad or Ad-shDDIT4 for 24 h. (**E**) p-mTOR, (**F**) p-P70S6K, and (**G**) p-4EBP1 in naive CD4$^+$ T cells from MS patients under Th17 conditions for the indicated times after transfection with either control Ad, Ad-sh lncDDIT4, Ad-lncDDIT4, Ad-shDDIT4, Ad-shDDIT4+Ad-sh lncDDIT4, or Ad-shDDIT4+Ad-lncDDIT4 for 24 h. A profile representative of 30 MS patients per group is shown. $^{\#}p < 0.05$ versus control Ad, 3 d; $^{*}p < 0.05$ versus control Ad, 5 d.

lncDDIT4 and DDIT4 inhibited Th17 differentiation in vitro

Notably, knockdown of lncDDIT4 in activated naive CD4$^+$ T cells led to decreased DDIT4 expression; in sharp contrast, overexpression of lncDDIT4 increased DDIT4 expression (Fig. 3A). The foregoing results suggest direct involvement of lncDDIT4 in the regulation of DDIT4 transcription. To determine whether lncDDIT4 and DDIT4 participate in Th17 cell differentiation, we transfected naive CD4$^+$ T cells with either an empty vector, lncDDIT4 knockdown, or overexpression Ad. Cells were then stimulated under Th1-, Th2-, or Th17-inducing conditions, and Th1, Th2, and Th17 cells were tested by flow cytometry. As shown in Fig. 3B, the absence of lncDDIT4 increased the proportion of Th17 cells. As expected, lncDDIT4 overexpression caused reduced numbers of Th17 cells. Consistent with these observations, amounts of IL-17 also increased in the absence of lncDDIT4. However, its roles on Th1 and Th2 differentiation were negligible (Fig. 3C). These data demonstrate that lncDDIT4 plays an important role in Th17 cell differentiation.

lncDDIT4 inhibits differentiation of Th17 cells through targeting DDIT4 in MS patients

Ad-targeting DDIT4 was transfected in activated CD4$^+$ naive T cells, and DDIT4 transcript levels were tested by RT-qPCR at 72 h posttransfection and activation. Upon knockdown of DDIT4 in activated CD4$^+$ naive T cells from MS patients, DDIT4 expression decreased both at the mRNA and protein levels (Fig. 4A, 4B). As Fig. 4C and 4D illustrate, the absence of DDIT4 induced increases in the percentage of Th17 cells and Th17 cell–associated cytokines (IL-17). Naive CD4$^+$ T cells from MS patients underwent differentiation for 72 h under Th17 conditions after transfection with control Ad + Ad-shDDIT4 (Ad carrying short hairpin RNA against DDIT4), Ad-sh lncDDIT4 + Ad-shDDIT4, or Ad-lncDDIT4 + Ad-shDDIT4 for 24 h. The cells were gated for GFP positivity and stained for the intracellular expression of IFN-γ and IL-17A. However, because these groups did not express notable differences (Fig. 4E, 4F), lncDDIT4 presumably regulates Th17 cell differentiation directly through target DDIT4.

lncDDIT4 inhibits DDIT4/mTOR signaling

To clarify the effect of mTOR signaling in MS patients, we quantified DDIT4 and the presence of phosphorylation in mTOR, p70S6k, and 4EBP1 (Fig. 5A). We found that the p-mTOR, p-p70S6k, and p-4EBP1 were highly expressed in MS patients (Fig. 5C). This outcome signified that the mTOR signaling pathway is activated in acute stage of MS. We also found that DDIT4 was strongly expressed in patients undergoing the acute stage of MS (Fig. 5B). The activation of the mTOR pathway increases Th17 cell differentiation (23), and the disruption of mTORC1 impairs Th17 differentiation (24). Therefore, lncDDIT4 might inhibit Th17 production by inhibiting the DDIT4/mTOR pathway. lncDDIT4 inhibited mTOR phosphorylation (Fig. 5E). lncDDIT4 also inhibited the phosphorylation of p70S6k and 4EBP1, substrates of mTOR (Fig. 5F, 5G).

lncDDIT4 inhibits DDIT4/mTOR signaling by targeting DDIT4

As shown in Fig. 6B–D, phosphorylation of mTOR, 4EBP1, and p70S6k clearly increased in the absence of DDIT4. To confirm these observations, we transfected naive CD4$^+$ T cells of MS patients with Ad targeting lncDDIT4 or DDIT4 and quantified mTOR, 4EBP1, and p70S6k phosphorylation by Western blot. Subsequently, lncDDIT4 decreased mTOR, 4EBP1, and p70S6k phosphorylation in naive CD4$^+$ T cells in Th17-polarizing conditions in the presence of DDIT4. However, this effect disappeared when DDIT4 was absent. In concordance, knockdown of DDIT4 in naive CD4$^+$ T cells in Th17-polarizing conditions induced increases of

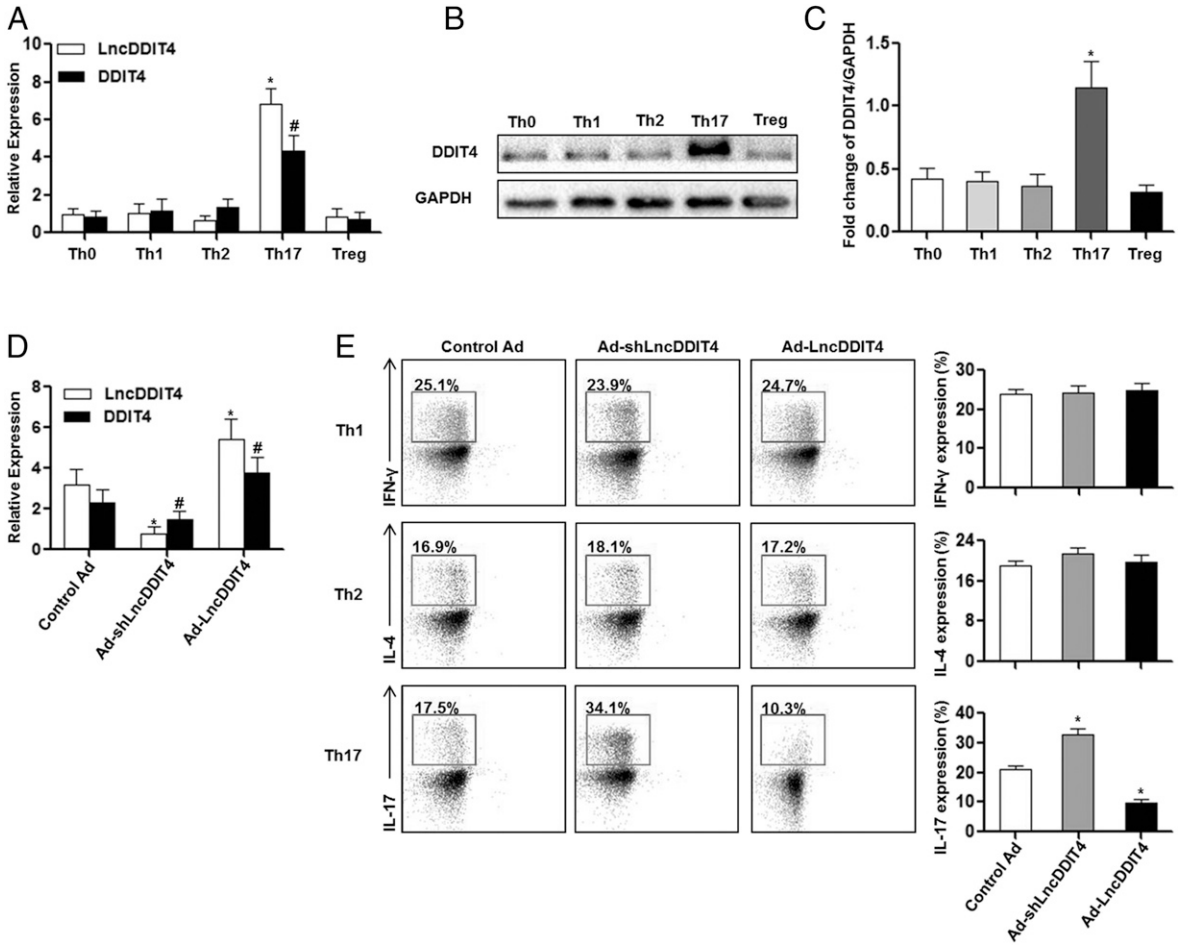

FIGURE 7. lncDDIT4 and DDIT4 expression are induced preferentially during Th17 differentiation and inhibit Th17 differentiation in healthy volunteers. (**A**) RT-qPCR analysis of lncDDIT4 and DDIT4 mRNA in naive $CD4^+$ T cells from healthy controls. The cells were stimulated for 72 h under Th0-, Th1-, Th2-, Th17-, or Treg-polarizing conditions. Results were normalized to GAPDH. Mean ± SEM. $*p < 0.05$ versus Th0 [lncDDIT4]; $^{\#}p < 0.05$ versus Th0 [DDIT4]. (**B**) Western blot analysis of DDIT4 in cells from healthy controls stimulated under Th0, Th1, Th2, Th17, and Treg conditions. (**C**) Cumulative data from densitometry are also shown. Mean ± SEM; $n = 20$. $*p < 0.05$ versus Th0. (**D**) RT-qPCR analysis of lncDDIT4 and DDIT4 expression in activated naive $CD4^+$ T cells (in the absence of polarizing cytokines) 72 h after transfection with control Ad, Ad-lncDDIT4, or Ad-sh lncDDIT4. Mean ± SEM. $*p < 0.05$ versus control Ad; $^{\#}p < 0.05$ versus control Ad. (**E**) Naive $CD4^+$ T cells from healthy controls differentiated for 72 h under Th1, Th2, or Th17 conditions after transfection with GFP^+ control Ad, GFP^+ Ad-lncDDIT4, or GFP^+ Ad-sh lncDDIT4 for 24 h. The cells were gated for GFP^+ and stained for the intracellular expression of IFN-γ, IL-4, and IL-17A. A profile representative of 20 healthy controls per group is shown. Mean ± SEM. $*p < 0.05$ versus control Ad.

phosphorylation in mTOR, 4EBP1, and p70S6k (Fig. 6E–G). Together, these data reveal that lncDDIT4 facilitates DDIT4/mTOR signaling by directly targeting DDIT4.

lncDDIT4 and DDIT4 expressions are induced preferentially during Th17 differentiation, and lncDDIT4 and DDIT4 inhibited Th17 differentiation also in healthy controls

To elucidate the role lncDDIT4 and DDIT4 play in Th17 cell function of healthy volunteers, we isolated naive $CD4^+$ T cells from healthy volunteers and stimulated them to generate either Th1, Th2, Th17, or Tregs. As shown in Fig. 7A, lncDDIT4 and DDIT4 induction was much stronger in Th17 cells. Th17 stimulation increased protein expression of DDIT4 (Fig. 7B, 7C). These results indicate that lncDDIT4 and DDIT4 are preferentially induced during Th17 polarization in healthy volunteer materials.

Knockdown of lncDDIT4 in activated naive $CD4^+$ T cells led to decreased DDIT4 expression; in contrast, overexpression of lncDDIT4 increased DDIT4 expression (Fig. 7D). To determine whether lncDDIT4 and DDIT4 participate in Th17 cell differentiation, we then isolated naive $CD4^+$ T cells from PBMCs of healthy volunteers and transfected the cells with either an empty vector, lncDDIT4 knockdown, or overexpression Ad. Cells were then stimulated under Th1, Th2, or Th17-inducing conditions, and Th1, Th2, and Th17 cells were tested by flow cytometry. As shown in Fig. 7E, the absence of lncDDIT4 caused an increase in Th17 cells. As expected, lncDDIT4 overexpression resulted in decreased Th17 cells.

lncDDIT4 inhibits differentiation of Th17 cells through targeting DDIT4 in healthy controls

Upon knockdown of DDIT4 in activated $CD4^+$ naive T cells from healthy controls, DDIT4 expression decreased (Fig. 8A). As shown in Fig. 8B, phosphorylation of mTOR, 4EBP1, and p70S6k clearly increased in the absence of DDIT4. Subsequently, lncDDIT4 decreased mTOR, 4EBP1, and p70S6k phosphorylation in naive $CD4^+$ T cells in Th17-polarizing conditions in the presence of DDIT4. However, this effect disappeared when DDIT4 was absent. In concordance, knockdown of DDIT4 in naive $CD4^+$ T cells in Th17-polarizing conditions induced increases of phosphorylation in mTOR, 4EBP1, and p70S6k. As illustrated in Fig. 8C and 8D, the absence of DDIT4 resulted in increases of Th17 cells and Th17

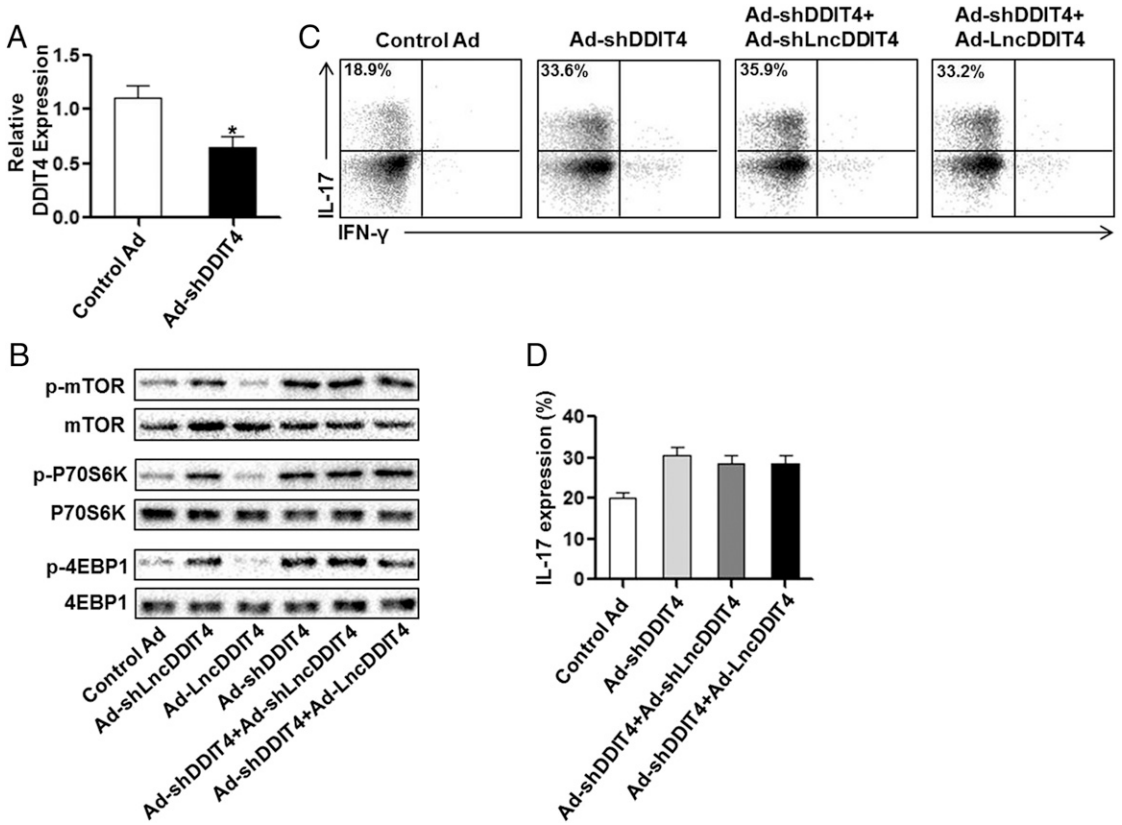

FIGURE 8. lncDDIT4 inhibits differentiation of Th17 cells by targeting DDIT4 in healthy volunteers. (**A**) RT-qPCR analysis of DDIT4 expression in activated naive $CD4^+$ T cells 72 h after transfection with control Ad or Ad-shDDIT4. Mean ± SEM. $*p < 0.05$ versus control Ad. (**B**) p-mTOR, p-P70S6K, and p-4EBP1 in naive $CD4^+$ T cells from healthy volunteers under Th17 conditions for the indicated times after transfection with either control Ad, Ad-sh lncDDIT4, Ad-lncDDIT4, Ad-shDDIT4, Ad-shDDIT4+Ad-sh lncDDIT4, or Ad-shDDIT4+Ad-lncDDIT4 for 24 h. (**C** and **D**) Naive $CD4^+$ T cells from healthy controls differentiated for 72 h under Th17 conditions after transfection with GFP^+ control Ad, Ad-shDDIT4, Ad-sh lncDDIT4+Ad-shDDIT4, or Ad-lncDDIT4+Ad-shDDIT4 for 24 h. The cells were gated for GFP^+ and stained for the intracellular expression of IFN-γ and IL-17A. A profile representative of 20 healthy controls per group is shown.

cell–associated cytokines (IL-17). However, because these groups did not express notable differences when DDIT4 was low expression, lncDDIT4 presumably regulates Th17 cell differentiation directly through target DDIT4 (Fig. 9). Together, these results imply that lncDDIT4 facilitates DDIT4/mTOR signaling by directly targeting DDIT4 also in healthy volunteer materials.

Discussion

In this article, we provide multiple facets of evidence supporting the importance of lncDDIT4 and DDIT4 in the differentiation of Th17 cells. In fact, lncDDIT4 inhibited Th17 cell differentiation directly by targeting DDIT4. Because lncDDIT4 and DDIT4 appeared to suppress the DDIT4/mTOR pathway, thereby inhibiting Th17 differentiation and subsequent production of IL-17, our observations imply that the DDIT4/mTOR axis is involved in the differentiation of Th17 cells. The likely effect is an ultimate opportunity for MS to develop and thrive.

Patients with MS display increased IL-17 expression, expansion of Th17 cells in the blood, and infiltration of Th17 cells into the CNS (25). Further, the aggravated induction of IL-17A in MS correlates with disease activity (26, 27). Accordingly, considerable evidence suggests that the abnormal expression of Th17 represents a key pathogenic player.

The mTOR pathway is critical in many normal cellular functions, including cellular proliferation, growth, survival, and mobility (28). The PI3K-AKT-mTORC1-S6K axis was reported as a positive regulator of Th17 differentiation by increasing the RORγt nuclear translocation (23). In addition, reduction of CaMK4 inhibited IL-17 transcription by lessening activation of the AKT/mTOR pathway (29). DDIT4 inhibits mTORC1 activity through activation of the TSC1/2 complex. In turn, inhibition of mTORC1 decreases activation of downstream targets by phosphorylation, including of P70S6K and 4EBP1, which are believed to be involved in regulating translation and cell proliferation (12). In our microarray data, we found that both lncDDIT4 and DDIT4 were upregulated (2.87-fold and 2.79-fold, respectively) in PBMCs of MS patients, as verified by RT-qPCR. DDIT4, which is upstream of lncRNA lncDDIT4, is the *cis*-regulated target of lncDDIT4. Although an abundance of bioinformatics analyses supports the correlation between lncDDIT4 and DDIT4, their biological role and molecular mechanisms underlying MS initiation and progression have not been reported until now. Accordingly, we assessed those presumptions and found that lncDDIT4 can activate DDIT4, which mediates regulating of IL-17. Consistent with this concept, we used Western blots, which indicated that lncDDIT4 inhibited mTOR pathway by targeting DDIT4 directly. Arguably, then, the DDIT4-mTOR axis is active in MS and may be essential to its development.

In conclusion, this specific involvement of lncDDIT4 in Th17 cell differentiation was unexpected. This newfound outcome urges the use of a pharmacologic strategy to inhibit

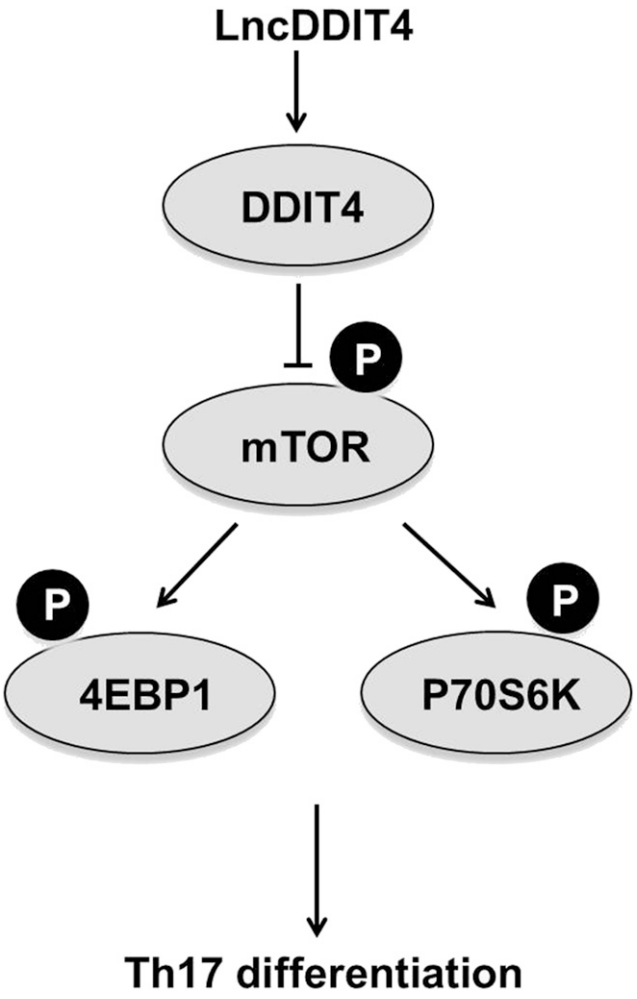

FIGURE 9. Model depicting the molecular mechanisms of DDIT4 and associated lncDDIT4 modulation of Th17 cell differentiation through the DDIT4/TSC/mTOR pathway.

lncDDIT4 in the treatment of Th17-related autoimmune diseases, most notably MS.

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Disclosures

The authors have no financial conflicts of interest.

References

1. Korn, T., E. Bettelli, M. Oukka, and V. K. Kuchroo. 2009. IL-17 and Th17 cells. *Annu. Rev. Immunol.* 27: 485–517.
2. Di Cesare, A., P. Di Meglio, and F. O. Nestle. 2009. The IL-23/Th17 axis in the immunopathogenesis of psoriasis. *J. Invest. Dermatol.* 129: 1339–1350.
3. Crispín, J. C., and G. C. Tsokos. 2010. Interleukin-17-producing T cells in lupus. *Curr. Opin. Rheumatol.* 22: 499–503.
4. Montes, M., X. Zhang, L. Berthelot, D. A. Laplaud, S. Brouard, J. Jin, S. Rogan, D. Armao, V. Jewells, J. P. Soulillou, and S. Markovic-Plese. 2009. Oligoclonal myelin-reactive T-cell infiltrates derived from multiple sclerosis lesions are enriched in Th17 cells. *Clin. Immunol.* 130: 133–144.
5. TsokosG. C. 2011. Systemic lupus erythematosus. *N. Engl. J. Med.* 365: 2110–2121.
6. Nistala, K., H. Moncrieffe, K. R. Newton, H. Varsani, P. Hunter, and L. R. Wedderburn. 2008. Interleukin-17-producing T cells are enriched in the joints of children with arthritis, but have a reciprocal relationship to regulatory T cell numbers. *Arthritis Rheum.* 58: 875–887.
7. Frohman, E. M., M. K. Racke, and C. S. Raine. 2006. Multiple sclerosis—the plaque and its pathogenesis. *N. Engl. J. Med.* 354: 942–955.
8. Langrish, C. L., Y. Chen, W. M. Blumenschein, J. Mattson, B. Basham, J. D. Sedgwick, T. McClanahan, R. A. Kastelein, and D. J. Cua. 2005. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. *J. Exp. Med.* 201: 233–240.
9. Ellisen, L. W., K. D. Ramsayer, C. M. Johannessen, A. Yang, H. Beppu, K. Minda, J. D. Oliner, F. McKeon, and D. A. Haber. 2002. REDD1, a developmentally regulated transcriptional target of p63 and p53, links p63 to regulation of reactive oxygen species. *Mol. Cell* 10: 995–1005.
10. Whitney, M. L., L. S. Jefferson, and S. R. Kimball. 2009. ATF4 is necessary and sufficient for ER stress-induced upregulation of REDD1 expression. *Biochem. Biophys. Res. Commun.* 379: 451–455.
11. Brugarolas, J., K. Lei, R. L. Hurley, B. D. Manning, J. H. Reiling, E. Hafen, L. A. Witters, L. W. Ellisen, and W. G. Kaelin, Jr. 2004. Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. *Genes Dev.* 18: 2893–2904.
12. Wullschleger, S., R. Loewith, and M. N. Hall. 2006. TOR signaling in growth and metabolism. *Cell* 124: 471–484.
13. Powell, J. D., K. N. Pollizzi, E. B. Heikamp, and M. R. Horton. 2012. Regulation of immune responses by mTOR. *Annu. Rev. Immunol.* 30: 39–68.
14. Zheng, Y., S. L. Collins, M. A. Lutz, A. N. Allen, T. P. Kole, P. E. Zarek, and J. D. Powell. 2007. A role for mammalian target of rapamycin in regulating T cell activation versus anergy. *J. Immunol.* 178: 2163–2170.
15. Molitoris, J. K., K. S. McColl, S. Swerdlow, M. Matsuyama, M. Lam, T. H. Finkel, S. Matsuyama, and C. W. Distelhorst. 2011. Glucocorticoid elevation of dexamethasone-induced gene 2 (Dig2/RTP801/REDD1) protein mediates autophagy in lymphocytes. *J. Biol. Chem.* 286: 30181–30189.
16. Reuschel, E. L., J. Wang, D. K. Shivers, K. Muthumani, D. B. Weiner, Z. Ma, and T. H. Finkel. 2015. REDD1 is essential for optimal T cell proliferation and survival. *PLoS One* 10: e0136323.
17. Bonasio, R., and R. Shiekhattar. 2014. Regulation of transcription by long noncoding RNAs. *Annu. Rev. Genet.* 48: 433–455.
18. Xu, J., F. Zhang, C. Gao, X. Ma, X. Peng, D. Kong, and J. Hao. 2016. Microarray analysis of lncRNA and mRNA expression profiles in patients with neuromyelitis optica. *Mol. Neurobiol.* 54: 2201–2208. https://link.springer.com/article/10.1007%2Fs12035-016-9754-0.
19. Xu, J., C. Gao, F. Zhang, X. Ma, X. Peng, R. Zhang, D. Kong, A. R. Simard, and J. Hao. 2016. Differentially expressed lncRNAs and mRNAs identified by microarray analysis in GBS patients vs healthy controls. *Sci. Rep.* 6: 21819.
20. Zhang, F., C. Gao, X. F. Ma, X. L. Peng, R. X. Zhang, D. X. Kong, A. R. Simard, and J. W. Hao. 2016. Expression profile of long noncoding RNAs in peripheral blood mononuclear cells from multiple sclerosis patients. *CNS Neurosci. Ther.* 22: 298–305.
21. Ranzani, V., and G. Rossetti. 2015. The long intergenic noncoding RNA landscape of human lymphocytes highlights the regulation of T cell differentiation by linc-MAF-4. *Nat. Immunol.* 16: 318–325.
22. Liu, G., F. Zhang, Y. Jiang, Y. Hu, Z. Gong, S. Liu, X. Chen, Q. Jiang, and J. Hao. 2016. Integrating genome-wide association studies and gene expression data highlights dysregulated multiple sclerosis risk pathways. *Mult. Scler.* 23: 205–212.
23. Kurebayashi, Y., S. Nagai, A. Ikejiri, M. Ohtani, K. Ichiyama, Y. Baba, T. Yamada, S. Egami, T. Hoshii, A. Hirao, et al. 2012. PI3K-Akt-mTORC1-S6K1/2 axis controls Th17 differentiation by regulating Gfi1 expression and nuclear translocation of RORγ. *Cell Reports* 1: 360–373.
24. Delgoffe, G. M., K. N. Pollizzi, A. T. Waickman, E. Heikamp, D. J. Meyers, M. R. Horton, B. Xiao, P. F. Worley, and J. D. Powell. 2011. The kinase mTOR regulates the differentiation of helper T cells through the selective activation of signaling by mTORC1 and mTORC2. *Nat. Immunol.* 12: 295–303.
25. Hemmer, B., M. Kerschensteiner, and T. Korn. 2015. Role of the innate and adaptive immune responses in the course of multiple sclerosis. *Lancet Neurol.* 14: 406–419.
26. Lock, C., G. Hermans, R. Pedotti, A. Brendolan, E. Schadt, H. Garren, A. Langer-Gould, S. Strober, B. Cannella, J. Allard, et al. 2002. Gene-microarray analysis of multiple sclerosis lesions yields new targets validated in autoimmune encephalomyelitis. *Nat. Med.* 8: 500–508.
27. Matusevicius, D., P. Kivisäkk, B. He, N. Kostulas, V. Ozenci, S. Fredrikson, and H. Link. 1999. Interleukin-17 mRNA expression in blood and CSF mononuclear cells is augmented in multiple sclerosis. *Mult. Scler.* 5: 101–104.
28. Laplante, M., and D. M. Sabatini. 2012. mTOR signaling in growth control and disease. *Cell* 149: 274–293.
29. Koga, T., C. M. Hedrich, M. Mizui, N. Yoshida, K. Otomo, L. A. Lieberman, T. Rauen, J. C. Crispín, and G. C. Tsokos. 2014. CaMK4-dependent activation of AKT/mTOR and CREM-α underlies autoimmunity-associated Th17 imbalance. *J. Clin. Invest.* 124: 2234–2245.