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MicroRNA-125a suppresses intestinal mucosal inflammation through targeting ETS-1 in patients with inflammatory bowel diseases

Yadong Ge[a], Mingming Sun[a], Wei Wu[a], Caiyun Ma[a], Cui Zhang[a], Chong He[b], Junxiang Li[c], Yingzi Cong[d], Dekui Zhang[e,**], Zhanju Liu[a,*]

[a] Department of Gastroenterology, The Shanghai Tenth People's Hospital, Tongji University, Shanghai, 200072, China
[b] Department of Gastroenterology, Sichuan Provincial People's Hospital, University of Electronic Science and Technology of China, Chengdu, China
[c] Department of Gastroenterology, Dongfang Hospital, Beijing University of Chinese Medicine, Beijing, 100078, China
[d] Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX, 77555, USA
[e] Department of Gastroenterology, The Second Hospital of Lanzhou University, Lanzhou, 730030, China

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ABSTRACT

MicroRNA (miR)-125a is highly expressed in T cells and regulates the functions of Treg through the IL-6-STAT3 signaling pathway. However, the role of miR-125a in regulating immune responses in intestinal mucosa of patients with inflammatory bowel diseases (IBD) is still not understood. Here we showed that miR-125a expression was decreased in PBMC and inflamed intestinal mucosa from IBD patients compared with that in healthy controls. Transduction with LV-miR-125a into IBD CD4$^+$ T cells could significantly inhibit proinflammatory cytokine production, including IFN-γ, TNF-α and IL-17A. RNA-seq analysis of miR-125a$^{-/-}$ CD4$^+$ T cells revealed enhanced genes (e.g., *Stat1*, *Stat3*, *RORγt*, *Irf4*, *Klf13*) in T cell activation and effector pathways, while ETS-1 as its functional target promoted IBD CD4$^+$ T cell differentiation into Th1 cells. Consistently, miR-125a$^{-/-}$ mice developed more severe colitis induced by TNBS compared with WT mice. Thus, our data suggest that miR-125a protects intestinal mucosa from inflammatory injury and that ETS-1 as its target participates in the pathogenesis of IBD.

1. Introduction

Inflammatory bowel diseases (IBD), including Crohn's disease (CD) and ulcerative colitis (UC), are chronic and idiopathic inflammatory diseases of the gastrointestinal tract, which are considered to be disorders of the host immune response to microbiota in genetically susceptible individuals [1,2]. Accumulating lines of evidence have proven that a variety of factors are involved in the development of inflammatory bowel diseases (IBD), including environmental and microbiota factors, compromised epithelial barrier, genetic susceptibility, and dysregulated intestinal mucosal immune responses. Importantly, adapted immune response, especially the immunomodulatory homeostasis of CD4$^+$ T cells in intestinal mucosa, has been classically thought to play a critical role in the pathogenesis of IBD [3,4]. Although subsequent studies have demonstrated that both T helper (Th)1 and Th17 cells, characterized by production of interferon (IFN)-γ and IL-17A, respectively, are found to be associated with the pathogenesis of IBD, the underlying mechanisms whereby Th1 and Th17 cell activation and differentiation are regulated remain incompletely understood [5–7].

MicroRNAs (miRs), which are typically a group of single-stranded, short noncoding RNAs that affect the post-transcriptional regulation process by binding to the 3′-untranslated region (3′UTR) of a target message RNA (mRNA), have been recently reported to be involved in the pathogeneses of IBD [8]. Deficiency of miR-210 is found to promote Th17 cell differentiation in a CD4$^+$ T cell transfer model of chronic colitis in mice [9]. miR-155 is positively related to Th1/Th17 cell-mediated immune response [10]. In our previous studies, expression of miR-301a is increased in intestinal mucosal CD4$^+$ T cells and intestinal epithelial cells (IECs), and it could induce abnormal immune responses and epithelial damage in IBD patients [11,12]. Moreover, our another study has illustrated that miR-10a is downregulated in CD4$^+$ T cells of intestinal mucosa and suppresses mucosal immune response by blocking NOD2 and IL-12/IL-23p40 expression, as well as Th1/Th17 cell-mediated immune responses [13].

miR-125, a highly conserved miR which has three transcripts

* Corresponding author. Department of Gastroenterology, The Shanghai Tenth People's Hospital of Tongji University, Shanghai, China.
** Corresponding author. Department of Gastroenterology, The Second Hospital of Lanzhou University, Lanzhou, China.
E-mail addresses: sczdk1972@163.com (D. Zhang), liuzhanju88@126.com (Z. Liu).

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Abbreviations

A-CD	Active Crohn's disease
A-UC	Active ulcerative colitis
CD	Crohn's disease
CDAI	Crohn's Disease Activity Index
DC	Dendritic cell
HC	Healthy control subject
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
LP	Lamina propria
LV	Lentivirus-expressing
miR-125a	microRNA-125a
MLN	Mesenteric lymph nodes
PBMC	Peripheral blood mononuclear cell
qRT-PCR	Quantitative RT-PCR
R-UC	Ulcerative colitis in remission
TNBS	Trinitrobenzene sulfonic acid
UC	Ulcerative colitis

containing miR-125a, miR-125b-1 and miR-125b-2, has been recently implicated to be involved in many processes of immune responses [14,15]. It is identified as an important player in the mechanisms that stabilize STAT3 activity in tolerogenic antigen presenting cells [16]. Overexpression of miR-125a downregulates LPS-dependent proinflammatory cytokine expression, including TNF-α, IL-6, and IL-12p40, in THP-1 cells, a human monocytic cell line [17]. Interestingly, it plays an important role in restraining M1 macrophage polarization and diminishing LPS-induced expression of TNF-α, IL-12 and iNOS through targeting on KLF13, which is associated with T cell-mediated inflammatory response in the pathogenesis of systemic lupus erythematosus (SLE) [18–20]. Recently, overexpression of miR-125a is also found to reduce the neutrophil recruitment by downregulation of IL-16 in pristine-induced lung inflammation [21]. Moreover, it is observed to control experimental colitis via downregulating T cell-derived IL-6 expression by targeting IRF4 [22,23], which binds to IL-17A gene promoter and induces IL-17A production [24]. It stabilizes the immunoregulatory capacity of Treg cells, thus alleviating colitis and experimental autoimmune encephalomyelitis (EAE) in mice [25]. These data indicate that miR-125a may play a protective role in the pathogenesis of several autoimmune diseases. However, the exact role of miR-125a in regulating mucosal immune responses of IBD is still elusive.

In this study, we demonstrated that the expression of miR-125a was markedly decreased in PBMC and inflamed mucosa of patients with IBD and that it was mainly expressed in $CD4^+$ T cells. Overexpression of miR-125a could inhibit the differentiation of IBD $CD4^+$ T cells into Th1 and Th17 cells, and suppress production of IFN-γ, TNF-α and IL-17A. ETS-1, an important transcription factor of IFN-γ [26], was identified as the target of miR-125a. Downregulated expression of ETS-1 could markedly decrease the expression of IFN-γ and TNF-α. miR-125a$^{-/-}$ mice exhibited more severe colitis induced by trinitrobenzene sulfonic acid (TNBS) compared with wild-type (WT) mice. Therefore, our data demonstrate that miR-125a plays an antiinflammatory role in the pathogenesis of IBD by inhibiting Th1/Th17 cell immune responses. This study may provide new insight into the targeted therapy for human IBD.

2. Materials and methods

2.1. Patients and samples

Colonoscopic biopsies were obtained from IBD patients and healthy control subjects (HC), including 53 patients with active CD (A-CD), 28 patients with CD in remission (R-CD), 59 patients with active UC (A-UC), 24 patients with UC in remission (R-UC), and 26 HC who underwent endoscopy for routine physical examinations. EDTA anticoagulated blood samples were harvested from IBD patents and HC after overnight fasting, including A-CD (n = 25), R-CD (n = 24), A-UC (n = 26), R-UC (n = 31), and HC (n = 16). All patients involved in this study were recruited from the Department of Gastroenterology, the Shanghai Tenth People's Hospital (Shanghai, China) from January 2016 to July 2018. All samples of healthy controls were obtained from our out-patient service for routine physical examinations at our hospital during the same period. The diagnosis of CD and UC was based on clinical, endoscopic examination, histological analysis, and radiological findings. Crohn's Disease Activity Index (CDAI) and Mayo score were used to evaluate the severity of CD and UC, respectively. The characteristics of IBD patients and healthy controls are described in Table S1. This study was approved by the Institutional Review Board for Clinical Research of Shanghai Tenth People's Hospital of Tongji University. Written informed consent was obtained from all subjects before the study protocol.

2.2. Th cell differentiation in vitro

$CD4^+$ T cells were isolated from peripheral blood of healthy donors using anti-human CD4 magnetic beads. They (2.5×10^5/well) were activated with plate-coated anti-CD3 mAb (5 μg/mL; eBioscience) and anti-CD28 mAb (2 mg/mL; eBioscience) in complete RPMI 1640 medium, and then induced to differentiate into Th1 cells by supplementation with IL-12 (10 ng/mL; R&D) plus *anti*-IL-4 mAb (10 μg/mL), Th2 cells with IL-4 (40 ng/mL; R&D) plus *anti*-IFN-γ mAb (10 μg/mL; eBioscience), Th17 cells with a Th17 'cocktail' containing IL-1β (10 ng/mL; R&D), IL-6 (30 ng/mL; R&D), IL-23 (20 ng/mL; R&D), TGF-β (2 ng/mL, R&D) and TNF-α (10 ng/mL; R&D) plus *anti*-IFN-γ mAb (10 μg/mL; eBioscience) and *anti*-IL-4 mAb (10 μg/mL; eBioscience), and inducible Treg cells with TGF-β (5 ng/mL, R&D), respectively. Th0 cells were just stimulated with *anti*-IFN-γ (10 μg/mL; eBioscience) and *anti*-IL-4 mAb (10 μg/mL; eBioscience). These cells were harvested to determine the levels of miR-125a using qRT-PCR 5 days later.

2.3. Lentivirus-mediated $CD4^+$ T cell transduction

PB-$CD4^+$ T cells were obtained using anti-human CD4 particles (BD Biosciences; San Diego, CA, USA), and preactivated *in vitro* with immobilized anti-human CD3 (5 μg/mL) and anti-human CD28 (2 μg/mL) mAbs for 48 h. These cells (1×10^5/well) were then transduced with lentivirus (multiplicity of infection, MOI = 180) encoding miR-125a (LV-miR125a), LV-sh-miR-125a which could downregulate miR-125a expression, LV-sh-ETS-1 which could downregulate ETS-1 expression, and empty control (LV-NC), respectively, according to the manufacturer's protocols (Biolink; Shanghai, China). After centrifugation for 2 h, PB-$CD4^+$ T cells were incubated with the lentivirus for another 5 h in complete RPMI 1640 medium containing protamine (10 μg/mL) in 24-well plates. After 3 washes with RPMI 1640 medium, these transduced cells were resuspended in complete RPMI 1640 medium and stimulated with immobilized anti-CD3 (5 μg/mL) and anti-CD28 (2 μg/mL) mAbs for 5 days. Cells were then collected, and total RNA was extracted to determine the levels of IFN-γ, T-bet, TNF-α, IL-17A, RORC, and IL-10 mRNA by quantitative RT-PCR (qRT-PCR). Supernatants were also harvested to analyze TNF-α, IFN-γ, IL-17A and IL-10 production by ELISA.

2.4. Mice

Female C57BL/6 mice were purchased from the Nanjing Biomedical Research Institution of Nanjing University (Nanjing, China) and maintained under specific pathogen-free (SPF) conditions at the animal facility of the Shanghai Tenth People's Hospital of Tongji University. All mice were raised in microisolator cages with sterile water, autoclaved food and filtered air. Eight-to 10-week-old mice were used for all experiments. All animal experiments were approved by the Institutional Animal Care and Use Committee of Tongji University.

2.5. Generation of miR-125a$^{-/-}$ mice

C57BL/6 miR-125a$^{-/-}$ mice were generated at the Institute of Biomedical Sciences and School of Life Sciences, East China Normal University (Shanghai, China), using the CRISPR/Cas9 system according to miR-125a gene sequence (NCBI gene ID: 387235), as described previously [27]. One sequence was identified to design miR-125a sgRNA: 5′-CCCTTTAACCTGTGAGGACGTCC-3'. mMessage mMachine Sp6 Kit (Life Technologies, cat. No. AM1340) was used to transcribe linearized DNA. Superovulated C57BL/6 female mice (SLAC; Shanghai, China) were mated with male mice of the same strain, and zygotes were then obtained. Cas9 mRNA was microinjected into the cytoplasm embryos at 1-cell-stage. The injected zygotes were then immediately transferred to infundibulum of pseudopregnant ICR female mice to generate founder mice. The founder mice were genotyped by PCR with appropriate primers (forward, 5′-ACACCATTGCCCAAGAGTT-3'; reverse, 5′-CTAAGGAAATAAGACCCATAAC-3′). PCR products (594 bp) were sent for sequencing. The mutated offsprings were verified by means of alignment. Mate founder mutants to C57BL/6 mice to obtain heterozygous F1 progeny. Confirmed F1 heterozygous mutants were intercrossed to obtain homozygous miR-125a$^{-/-}$ mice, which were used for further study.

2.6. TNBS-induced colitis in mice

An experimental colitis model in mice was established using a method as described previously [11]. Briefly, WT and miR-125a$^{-/-}$ mice (12 mice per group) were fasted for 24 h with sterile drinking water alone in order to remove as much the stool as possible. Mice were then anesthetized with 1.25% pentobarbital sodium intraperitoneally. After anesthetized, mice were disposed with 0.15 μL of a 2.5% (w/v) TNBS solution in 50% ethanol intrarectally using a polyethylene catheter (2 mm in outer diameter) which was inserted about 3.5–5 cm from the anus, and then kept vertically in a headfirst position for at least 2 min. Another 2 groups of WT and miR-125a$^{-/-}$ mice (12 mice per group) were administrated with 50% ethanol alone intrarectally as controls. Characteristics of acute colitis were recorded daily, including body weight, diarrhea, and bloody stools. All mice were sacrificed on day 6. Once the colonic tissues were obtained, they were fixed in 10% paraformaldehyde solution, embedded in paraffin, and sectioned. Hematoxylin and eosin (H&E) staining was performed to evaluate the histological grading of colonic inflammation, as described previously [28].

2.7. Isolation of lamina propria mononuclear cells (LPMC)

LPMC were prepared using a method as described previously [5]. Briefly, colons from the sacrificed mice were sliced into 0.5 cm pieces, and washed with cold PBS to remove fecal contents. Tissues were shaken gently in PBS supplemented with 1 mmol/L EDTA and 5% FBS at 37 °C for 2 × 20 min to remove epithelial cells, and then digested in 10 mL of 5% FBS-RPMI-collagenase A (1 mg/mL; Roche, Mannheim, Germany) and 10 mg/mL DNase at 37 °C for 30 min. Cells were collected and purified further via density gradient centrifugation with 40% and 75% percoll-RPMI solution. LPMC was collected from the interface and supplemented with 5% FBS-RPMI medium, and the cells were prepared for following experiments.

2.8. Complete RPMI 1640 medium

Cells were cultured in complete RPMI 1640 medium supplemented with 10% fatal bovine serum (FBS), penicillin (100 U/mL), streptomycin (100 mg/mL), HEPES (10 mmol/L), sodium pyruvate (1 mmol/L), and 2-ME (50 mmol/L).

2.9. Flow cytometry

For surface staining, cells were first blocked by Fc blocking and incubated with surface marker mAbs for 30 min at 4 °C. T cell subpopulations were gated as CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cells, and B cells were gated as B220$^+$ cells. For intracellular cytokine staining, CD4$^+$ T cells were obtained after *in vitro* culture or from spleen, MLN and LPMC of mice, and incubated under stimulation with phorbol 12-myristate 13-acetate (PMA) (50 ng/mL; Sigma) and ionomycin (1 μg/mL; Sigma) for 5 h, and brefeldin A (3 μg/mL; eBioscience) was added into cultures for the last 3 h in 10% FBS-RPMI medium at 37 °C. Surface staining was performed as described above, and cells were then permeabilized using eBioscience Foxp3/Transcription Factor Staining Buffer Set and stained according to the manufacturer's protocol. The data were acquired by FACSCanto II (BD Biosciences; San Diego, CA, USA) and analyzed using Flowjo software.

2.10. Immunohistochemistry

To examine ETS-1 expression, colonic biopsies from patients with active IBD and healthy controls were fixed and embedded as described previously. After incubation with Envision flex peroxidase-blocking reagent for 10 min, the sections were incubated with rabbit anti-human ETS-1 mAb (Abcam, dilution 1:200) at 4 °C overnight. After washing in PBS for 3 times, the sections were incubated with HRP-conjugated goat *anti*-IgG (dilution 1:400) at room temperature for 1 h. The color reaction was developed with 3,3′-diaminobenzidine and the sections were counterstained with hematoxylin. As negative controls, sections were treated with PBS instead of primary antibody. ETS-1 expression was observed under a light microscopy.

2.11. qRT-PCR

Total RNA was extracted from the cells and tissues, and the complementary DNA (cDNA) was synthesized with 5 × All-in-one RT Mastermix (Abcam) according to the manufacturer's instructions. The synthesized cDNA was stored at −20 °C. qRT-PCR was performed using SYBR green methodology according to the following conditions: 95 °C for 1 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 30 s with 40 cycles. The qPCR results were calculated using the $2^{-\triangle\triangle Ct}$ method.

2.12. Reagents

Anti-human CD3, anti-human CD28, anti-mouse CD3, and anti-mouse CD28 mAbs were purchased from BioLegend (San Diego, CA, USA). Anti-human CD4, anti-mouse CD4, anti-human CD8, anti-human CD14, and anti-human CD19 magnetic particles were purchased from BD Biosciences (San Diego, CA, USA). The paired antibodies for ELISA including human IFN-γ, IL-17A, TNF-α, and IL-10 were all purchased from BioLegend. Fluorochrome-conjugated anti-mouse IFN-γ and anti-mouse IL-17A mAbs were purchased from BD Biosciences. Anti-human ETS-1 mAb was purchased from Abcam (Cambridge, UK). Ionomycin, phorbol 12-myristat 13-acetate (PMA), and trinitrobenzene sulfonic acid (TNBS) were purchased from Sigma-Aldrich (St Louis, MO, USA). HEPES, sodium pyruvate, penicillin, streptomycin, 2-mercaptoethanol (2-ME), and eBioscience Foxp3/Transcription Factor Staining Buffer Set

were purchased from Life Technologies (Carlsbad, CA, USA).

2.13. Statistical analysis

Data were expressed as mean ± SEM and analyzed using GraphPad Prism 6 software (GraphPad Software Inc.; San Diego, CA, USA). Unpaired 2-tailed Student's *t*-test was used to evaluate the significance. Pearson's correlation was performed to analyze the correlation between miR-125a expression in intestinal mucosa and CDAI and Mayo score, respectively. Differences were considered to be statistically significant when *p < .05, **p < .01, and ***p < .001.

3. Results

3.1. miR-125a expression is decreased in inflamed mucosa and PBMC of IBD patients

Several lines of evidence have shown that miR-125a participates in the pathogenesis of several autoimmune diseases, including multiple sclerosis (MS), rheumatic diseases and SLE [15,25,29]. In this study, we investigated the association between miR-125a expression and disease severity of IBD. qRT-PCR analysis revealed that miR-125a expression was significantly decreased in inflamed mucosa from both patients with active CD and active UC compared with HC (Fig. 1A). The levels of miR-125a expression in patients with CD or UC in remission were also decreased compared with those in HC. Biopsies of the inflamed and unaffected mucosa from the same patients with CD or UC were obtained to compare miR-125a expression. As shown in Fig. 1B and C, the levels of miR-125a expression were lower in inflamed mucosa than those in unaffected intestinal mucosa. Moreover, the expression of miR-125a was also significantly decreased in PBMC from both patients with active CD or UC than in HC (Fig. 1C). Since Crohn's Disease Activity Index (CDAI) and Mayo score are used as the evaluative criteria of the disease severity of CD and UC, respectively, the correlation of miR-125a expression in inflamed mucosa with CDAI and Mayo score was then analyzed. Consequently, we found that miR-125a expression in inflamed mucosa from patients with CD and UC was negatively correlated with CDAI and Mayo score, respectively (Figs. S1A and B).

We next sought to determine which subtypes of immune cells were the main resources of miR-125a. B cells, neutrophils, monocytes, $CD4^+$ and $CD8^+$ T cells were isolated from peripheral blood of 6 healthy donors, and we found that $CD4^+$ T cells expressed a comparatively high level of miR-125a (Fig. 1E). IEC, $CD4^+$, $CD8^+$ T cells, B cells and dendritic cells (DC) were also isolated from normal intestinal mucosa from 6 patients who underwent colectomy for colon cancer, and we observed that miR-125a was also mainly expressed in mucosal $CD4^+$ T cells (Fig. 1F).

3.2. TNF-α downregulates miR-125a expression in $CD4^+$ T cells

To further figure out which subsets of helper T cells were the major resources of miR-125a, PB-$CD4^+$ T cells were isolated from 3 healthy donors, and T cell differentiation experiments were performed under different skewing conditions for 5 days *in vitro*. Interestingly, induced Treg cells (iTreg) were observed to be the major subset expressing miR-125a, while Th1, Th2 and Th17 subsets expressed at relatively low levels, implying that miR-125a is downregulated in inflamed mucosa of IBD patients presumably due to compromised functional activities of Treg during intestinal mucosal inflammation (Fig. 2A).

Considering that increased expression of proinflammatory cytokines (e.g., IL-1β, IL-6 and TNF-α) is present in inflamed mucosa of IBD and that they function as an intrinsic factor contributing to abnormal adaptive immune response during intestinal inflammation, we then investigated whether these cytokines were capable of regulating miR-125a expression in human PB-$CD4^+$ T cells. For this purpose, we isolated PB-$CD4^+$ T cells from 3 healthy donors and stimulated with anti-

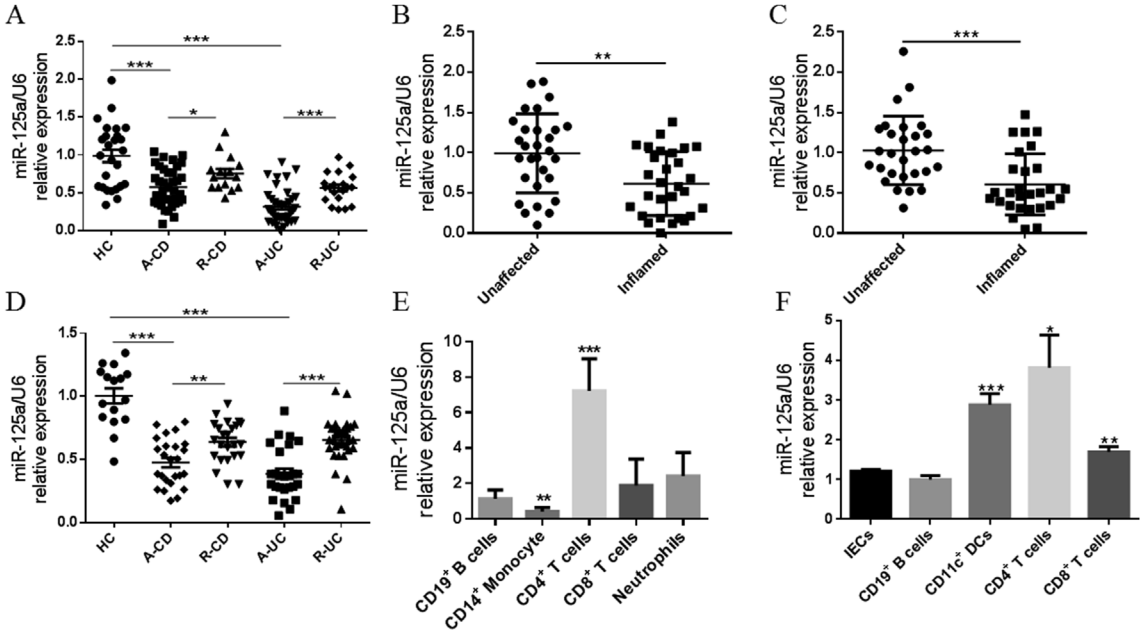

Fig. 1. miR-125a expression is downregulated in IBD patients and mainly expressed in $CD4^+$ T cells. (A) Colonoscopic biopsies were collected from 37 patients with A-CD, 15 patients with R-CD, 39 patients with A-UC, 20 patients with R-UC, and 26 HCs. miR-125a expression in colon biopsies was analyzed by qRT-PCR. (B and C) miR-125a expression in inflamed and unaffected intestinal mucosa from the same patients with A-CD (B, n = 29) or A-UC (C, n = 28) was examined using qRT-PCR. (D) PBMC were isolated from patients with A-CD (n = 25), patients with R-CD (n = 24), patients with A-UC (n = 26), patients with R-UC (n = 31), and HC (n = 16). miR-125a expression in PBMC was determined by qRT-PCR. (E) miR-125a expression in different immune cells. $CD19^+$ B cells, $CD14^+$ monocytes, neutrophils, $CD4^+$ and $CD8^+$ T cells (1×10^6 for each subset) were isolated from peripheral blood of 6 healthy donors, and miR-125a expression was determined using qRT-PCR. (F) IEC, $CD19^+$ B cells, $CD11c^+$ dendritic cells (DC), $CD4^+$ and $CD8^+$ T cells (1×10^6 for each subset) were isolated from normal intestinal mucosa from 6 patients who underwent colectomy for colon cancer, and miR-125a expression was determined using qRT-PCR. Gene expression was normalized to U6 in each sample. *p < .05, **p < .01, ***p < .001. Data are representative of 3 independent experiments.

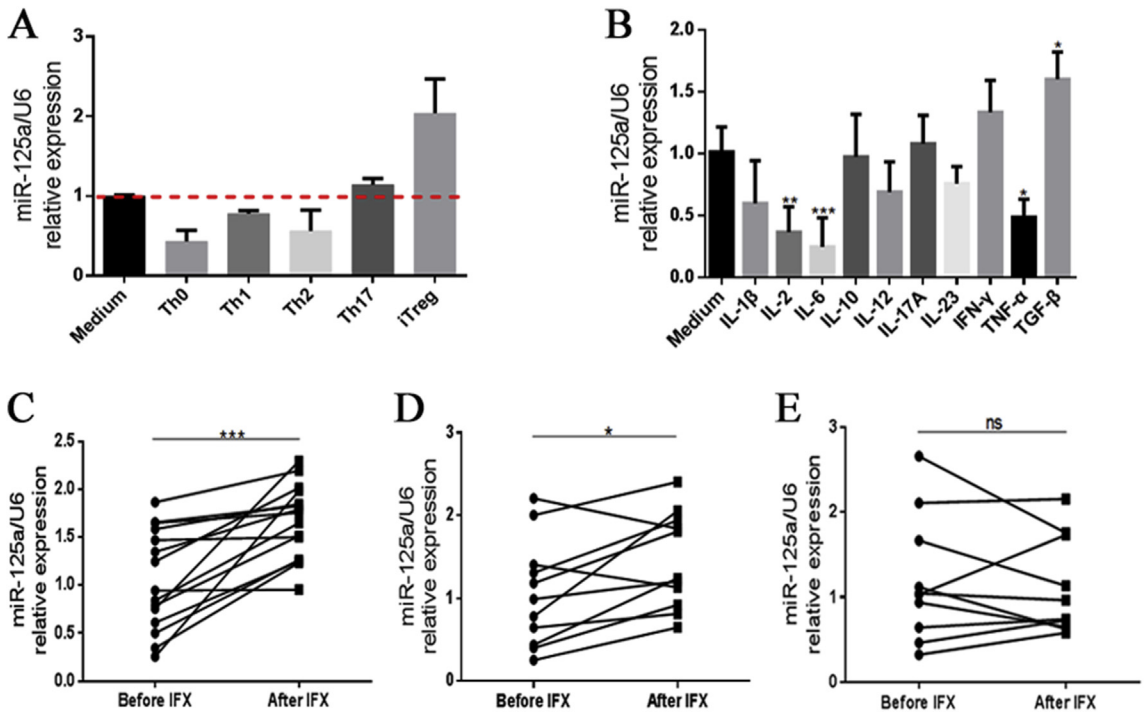

Fig. 2. TNF-α downregulates miR-125a expression. (A) PB-CD4$^+$ T cells (2.5×10^5/well) isolated from 3 healthy donors were stimulated with immobilized anti-CD3 (5 μg/mL) and anti-CD28 (2 μg/mL) mAbs under Th0, Th1, Th2, Th17, or regulatory T (Treg) cell skewing conditions *in vitro* for 5 days. miR-125a expression was performed by qRT-PCR analysis, and normalized to U6 in each sample. (B) PB-CD4$^+$ T cells (5×10^5/well) isolated from 3 healthy donors were stimulated with immobilized anti-CD3 (5 μg/mL) and anti-CD28 (2 μg/mL) mAbs in the presence of different cytokines (20 ng/mL) for 48 h, and miR-125a expression was performed by qRT-PCR, and normalized to U6 in each sample. *p < .05, **p < .01, ***p < .001 versus controls. (C–E) 35 patients with active CD were receiving IFX treatment, and intestinal mucosal biopsies were collected from these patients. 15 patients achieved clinical remission (C), 11 patients were responsive to IFX therapy but not in clinical remission (D), and 9 patients failed to response to IFX therapy (E) before and 12 weeks after the first infusion. miR-125a expression was determined by qRT-PCR, and normalized to U6 in each group. *p < .05 versus data before IFX therapy.

CD3 and anti-CD28 mAbs in the presence of IL-1β, IL-2, IL-6, IL-10, IL-17A, IFN-γ, TNF-α, or TGF-β, respectively, for 48 h. We found that TNF-α, IL-2, and IL-6 were powerful in suppressing miR-125a expression in CD4$^+$ T cells (Fig. 2B).

To further investigate the correlation between the increased TNF-α expression and the levels of miR-125a in inflamed mucosa of IBD patients, miR-125a expression was assessed in intestinal mucosa from patients with active CD before and after *anti*-TNF-α mAb (i.e., infliximab, IFX) treatment. To this end, patients diagnosed as active CD received IFX treatment at weeks 0, 2 and 6 as indicated previously [13]. We recruited 15 patients who achieved clinical remission (CDAI ≤ 150, termed as Remission group), 11 patients who reached to standard clinical response (a decrease of CDAI ≥ 70, but still ≥ 150, termed Response group), and 9 patients who were defined as failure to IFX (a decrease of CDAI ≤ 70 and CDAI ≥ 150 or an increase of CDAI from the baseline, termed as Failure group). Compared with the Remission and Response groups before IFX treatment, miR-125a was found to be significantly increased in inflamed mucosa of patients with CD after IFX therapy, but it did not alter in the Failure group (Fig. 2C–E). Taken together, these data indicate that miR-125a expression can be downregulated by TNF-α *in vitro* and that blockage of TNF-α therapy (i.e., IFX) could upregulate miR-125a expression in intestinal mucosa.

3.3. miR-125a inhibits Th1/Th17 cell differentiation and TNF-α production in IBD

Since previous study has demonstrated that miR-125a facilitates Treg cell differentiation and reduces inflammation in murine EAE model, we then investigated whether miR-125a could regulate CD4$^+$ T cell activation in IBD. To this end, we isolated PB-CD4$^+$ T cells from IBD patients and healthy donors, and then transduced these cells with LV-miR-125a, LV-sh-miR-125a, and empty vectors (LV-NC), respectively. Transduction efficacy of miR-125a was confirmed by qRT-PCR (Fig. S3), showing that miR-125a expression was markedly increased in LV-miR-125a-transuced cells but markedly decreased in LV-miR-125a-sh-treated cells.

After stimulated with immobilized anti-CD3 plus anti-CD28 mAbs for 5 days, these transduced cells were collected for gene expression analysis by qRT-PCR, including IL-17A, RORC, IFN-γ, T-bet, TNF-α and IL-10. As shown in Fig. 3A–E, the transcriptional levels of IL-17A, RORC, IFN-γ, T-bet and TNF-α were significantly downregulated in LV-miR-125a-transducd cells, but upregulated in LV-miR-125a-sh-transduced cells compared with controls (Fig. 3). Consistently, the protein levels of IL-17A, IFN-γ, and TNF-α in the supernatants were also markedly decreased in LV-miR-125a-transuced cells, but significantly increased in LV-sh-miR-125a-transduced cells compared with controls (Fig. 3G–I). However, no differences in IL-4, GATA3, and IL-10 were observed between these groups (Fig. 3F and S3A-C). Taken together, these data indicate that miR-125a inhibits IBD CD4$^+$ T cell differentiation into Th1 and Th17 cells.

3.4. miR-125a targets ETS-1

In order to clarify the general functions of miR-125a, we isolated CD4$^+$ T cells from the spleen of 6- to 8-week-old miR-125a$^{-/-}$ mice and their littermate WT mice for RNA sequencing (RNA-seq) analysis. Heatmap reveals an increased expression of effector T cell genes including several upregulated inflammation-related transcription factors (e.g., *Stat1*, *RORγt*, *Stat3*, *Irf4*, and *Klf13*) and inflammation-associated genes (e.g., *IL-17ra*, *IL-17rb*, *Ccr4*) in miR-125a$^{-/-}$CD4$^+$ T cells compared with controls (Fig. 3A). Conversely, Treg cell-related gene Tgfb1 decreased in miR-125a$^{-/-}$ CD4$^+$ T cells than in WT controls. These

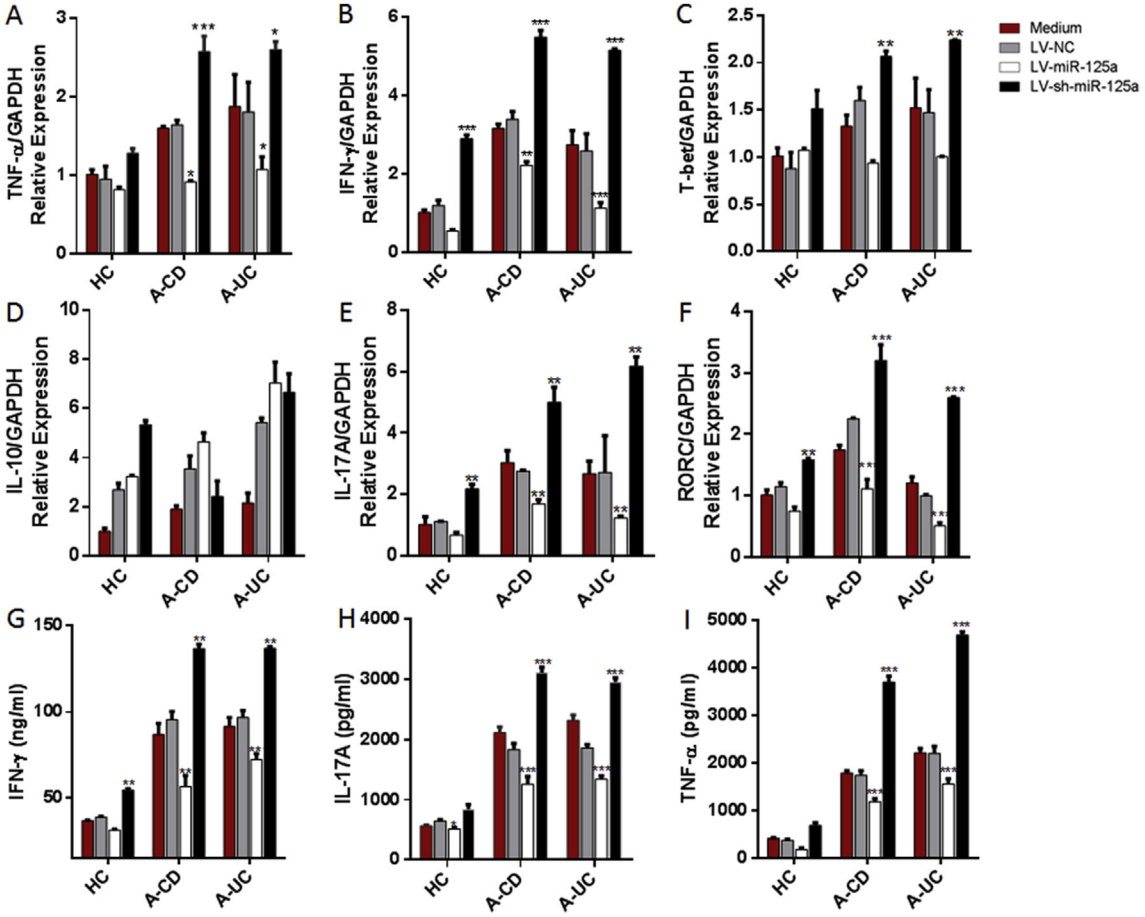

Fig. 3. miR-125a inhibits Th1/Th17 cell immune response and TNF-α production in IBD. (A–F) PB-CD4$^+$ T cells (1×10^5/well) were isolated from patients with A-CD (n = 18), A-UC (n = 16), or HC (n = 12), and cultured with immobilized anti-CD3 (5 μg/mL) and anti-CD28 (2 μg/mL) mAbs *in vitro* for 48 h, and transduced with LV-miR-125a, LV-miR-125a-sh, or LV-NC, respectively, for 5 h. After transduction, cells were stimulated with immobilized anti-CD3 (5 μg/mL) and anti-CD28 (2 μg/mL) mAbs *in vitro* for a further 5 days. Transduced cells were then collected to determine mRNA expressions of TNF-α (A), IFN-γ (B), T-bet (C), IL-10 (D), IL-17A (E) and RORC (F) by means of qRT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was served as a housekeeping gene. *p < .05, **p < .01, ***p < .001 versus medium alone. (G–I) Supernatants were collected, and protein levels of IFN-γ (G), IL-17A (H) and TNF-α (I) were determined by ELISA. **p < .01, ***p < .001 versus medium alone. Representative results from 3 independent experiments are shown.

results were in conformity with the lentivirus-mediated transduction experiments as described in Fig. 3, indicating that the downregulation of miR-125a in CD4$^+$ cells may facilitate the activation of effector T cells.

The TargetScan website (http://www.targetscan.org/vert_72/) was used to predict the potential targets of miR-125a. We overlapped the RNA-seq data and the predicted target genes (Fig. 4A), and discovered with great interest ETS-1 (E26 transformation-specific 1) as an important target in that it plays an essential role in Th1 cell differentiation [26]. To verify whether ETS-1 is an indeed target of miR-125a in IBD, TargetScan was used to predict the potential binding sites of miR-125a in the 3′UTRs of ETS-1 (Fig. 4B), and luciferase assays were performed. The 3′UTR of ETS-1 was cloned into luciferase reporter constructs and the luciferase reporter activity was markedly repressed by miR-125a (Fig. 4C). To further confirm that ETS-1 was the direct target of miR-125a in mice, we detected the mRNA levels of Ets-1 in colon tissues from miR-125a$^{-/-}$ mice and WT littermates. Fig. 4D shows that Ets-1 expression was upregulated in miR-125a$^{-/-}$ mice. Therefore, these data indicate that miR-125a directly represses Ets-1 expression to suppress CD4$^+$ T cell differentiation into Th1 cells.

3.5. Knockdown of ETS-1 inhibits Th1 cell differentiation and TNF-α production

We next investigated expression of ETS-1 in intestinal mucosa of IBD. As shown in Fig. 5A, qRT-PCR analysis revealed the increased expression of ETS-1 in inflamed mucosa of patients with A-CD and A-UC compared with that in HC. We also verified the upregulated expression of ETS-1 by immunohistochemical staining in the inflamed mucosa of patients with A-CD and A-UC compared with that in HC (Fig. 5B). To dissect which subsets of immune cells were the main resources of ETS-1, IEC, CD4$^+$, CD8$^+$ T cells, CD11c$^+$ DC and CD19$^+$ B cells were isolated from the LP of normal intestinal mucosa from 6 colon cancer patients who underwent colectomy, as well as from peripheral blood of 6 healthy donors, and ETS-1 expression was analyzed by qRT-PCR. As shown in Fig. 5C and D, we found that ETS-1 was mainly expressed in CD4$^+$ and CD8$^+$ T cells compared with other cells.

To investigate how ETS-1 affected CD4$^+$ T cell differentiation, PB-CD4$^+$ T cells were isolated from patients with IBD and healthy donors, and transduced with LV-sh-ETS-1 which could downregulate ETS-1 expression or empty vectors (LV-NC). The transduction efficacy of ETS-1 was confirmed by fluorescence imaging and qRT-PCR (Fig. S4). Total RNA was extracted from transduced cells, and the levels of IL-17A, RORC, IFN-γ, T-bet, TNF-α and IL-10 mRNA were analyzed by qRT-PCR. Interestingly, we found that the levels of T-bet, IFN-γ and TNF-α mRNA were markedly downregulated in LV-sh-ETS-1-transduced cells compared with negative controls (Fig. 5E–G). The same pattern of TNF-α and IFN-γ expression was also observed in supernatants (Fig. 5L and M). However, there were no obvious changes in genes including IL-4, GATA3, IL-17A, RORC, and IL-10 (Fig. 5H–K, and S5). Therefore, these

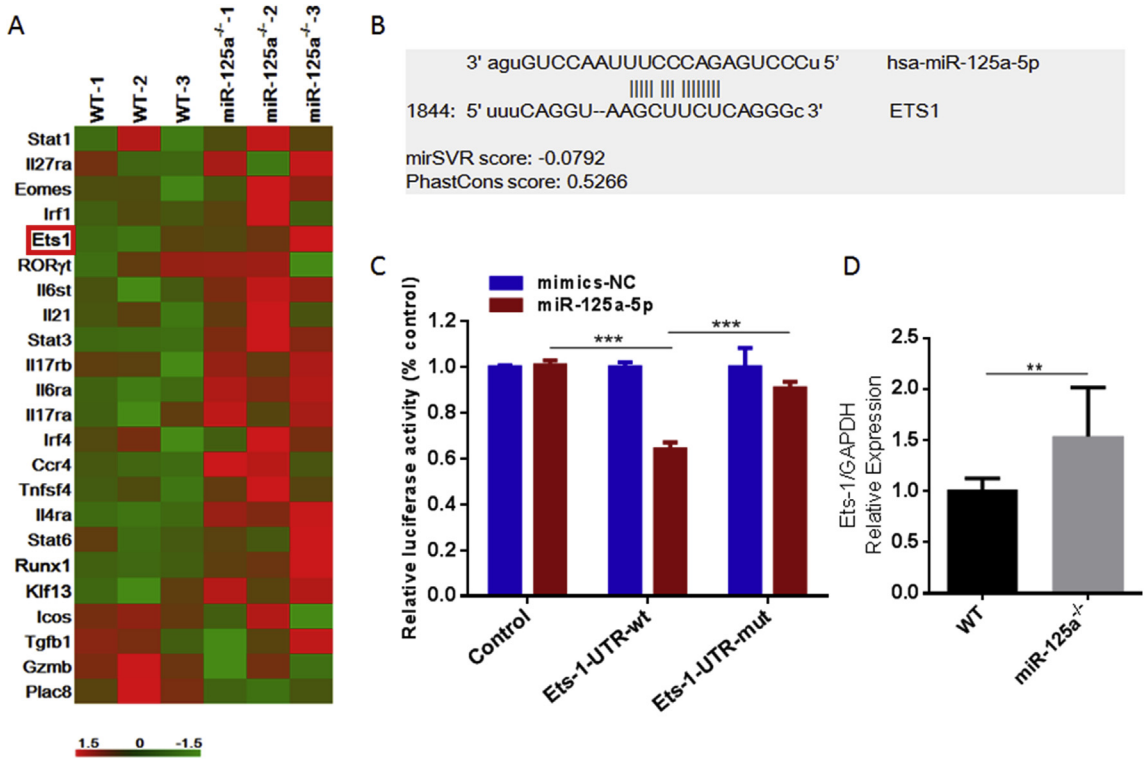

Fig. 4. miR-125a downregulates gene expression related to effector T cell differentiation, and targets ETS-1 in human $CD4^+$ T cells. (A) Heatmap shows differential genes between WT and miR-125a$^{-/-}$ $CD4^+$ T cells by RNA sequencing. Total RNAs of splenic $CD4^+$ T cells isolated from WT and miR-125a$^{-/-}$ mice (n = 3 each group) using anti-mouse CD4 magnetic particles were extracted for RNA sequencing. (B) Sequence alignment of miR-125a with reverse complementary ETS-1. (C) Activity of the luciferase gene linked to the 3′UTR of ETS-1. The pmirGLO luciferase reporter plasmids containing the WT or mutated (mut) 3′UTR sequences of ETS-1 were transiently transfected into HEK293 cells along with miR-125a mimics or negative control (mimics-NC) and a Renilla luciferase reporter for normalization. Luciferase activity was detected. The recombinant plasmid was normalized to an empty pmirGLO plasmid. Representative data are shown as mean ± SEM of four samples. ***p < .001. (D) An increase of Ets-1 expression in colonic tissue from miR-125a$^{-/-}$ mice. WT and miR-125a$^{-/-}$ mice were sacrificed at 6- to 8-week-old, and colonic tissues were then obtained to determine Ets-1 expression by qRT-PCR. GAPDH was served as a housekeeping gene. **p < .01 compared with WT mice.

studies indicate that miR-125a restrains Th1 cell differentiation by targeting ETS-1.

3.6. Deficiency of miR-125a aggravates TNBS-induced colitis in mice

After generating miR-125a$^{-/-}$ mice, we first did phenotypic study and isolated $CD4^+$ T cells, $CD8^+$ T cells, and B cells from spleen, MLN and LP of WT and miR-125a$^{-/-}$ mice. Flow cytometric analysis revealed no significant differences in the frequencies of these cell population between miR-125a$^{-/-}$ and WT mice (Fig. S6). To further investigate the protective role of miR-125a in the pathogenesis of intestinal mucosal inflammation, an experimental colitis model was established in miR-125a$^{-/-}$ and WT mice by means of administration of TNBS intrarectally. Colon tissues were obtained for further analysis on day 6 after TNBS administration. As shown in Fig. 6A–D, miR-125a$^{-/-}$ mice showed more severe colitis compared with WT mice on TNBS exposure, characterized by more significant weight loss, higher levels of DAI and pathological scores, and shorter colon length. H&E staining of colon sections further revealed more severe structural damages and inflammatory infiltrations in the LP of colon in miR-125a$^{-/-}$ mice compared with those in WT mice (Fig. 6E and F).

In addition, we observed that the levels of IL-17A, RORγt, IFN-γ, T-bet, TNF-α, IL-6 and Ets-1 mRNA were markedly increased in inflamed colon of miR-125a$^{-/-}$ mice than those in WT mice (Fig. 7A–F and S7A), while no significant changes in GATA3, IL-4 and IL-10 mRNA expression were seen (Figs. S7B–D). Moreover, flow cytometric analysis revealed the increased frequencies of IFN-γ^+CD4^+ and IL-$17A^+CD4^+$ T cells in the LP of miR-125a$^{-/-}$ mice compared with those in WT mice after TNBS administration (Fig. 7G and H). Taken together, these results indicate that miR-125a negatively regulates Th1/Th17 cell-mediated immune responses during inflammation in gut mucosa.

4. Discussion

In the current study, using *in vitro* experiments and TNBS-induced colitis model in mice, we demonstrated that the lack of miR-125a expression markedly promoted the Th1/Th17 cell differentiation and proinflammatory production including TNF-α, IFN-γ and IL-17A. TNF-α and IL-6 could downregulate miR-125a expression, and effective *anti*-TNF treatment could reverse its expression. ETS-1, as one of its target genes, was proven to play an important role in facilitating $CD4^+$ T cell differentiation. RNA-seq revealed an increase of Stat3 and Ets-1 expression in miR-125a$^{-/-}CD4^+$ T cells, which were verified as the targets of miR-125a. These results delineate an important role of miR-125a in regulating Th1/Th17 cell immune response in gut mucosa during inflammation.

Substantial advances have regarded the dysregulated $CD4^+$ T cell-mediated immune response as one of key pathophysiological changes in the pathogenesis of IBD. Th1 cells and their related cytokines (e.g., IFN-γ, TNF-α) are considered to be involved in IBD development, and *anti*-TNF-α mAb treatment has been applied in the clinical trials demonstrating a powerful approach in the management of disease [30,31]. In recent years, increasing lines of evidence have identified IL-17A as crucial proinflammatory cytokines in IBD development, which is produced by IL-23-driven Th17 cells [32]. However, the precise mechanism whereby $CD4^+$ T cells regulate inflammatory progress in the gut mucosa remains to be ascertained.

miR-125a has been reported to regulate diverse biological functions,

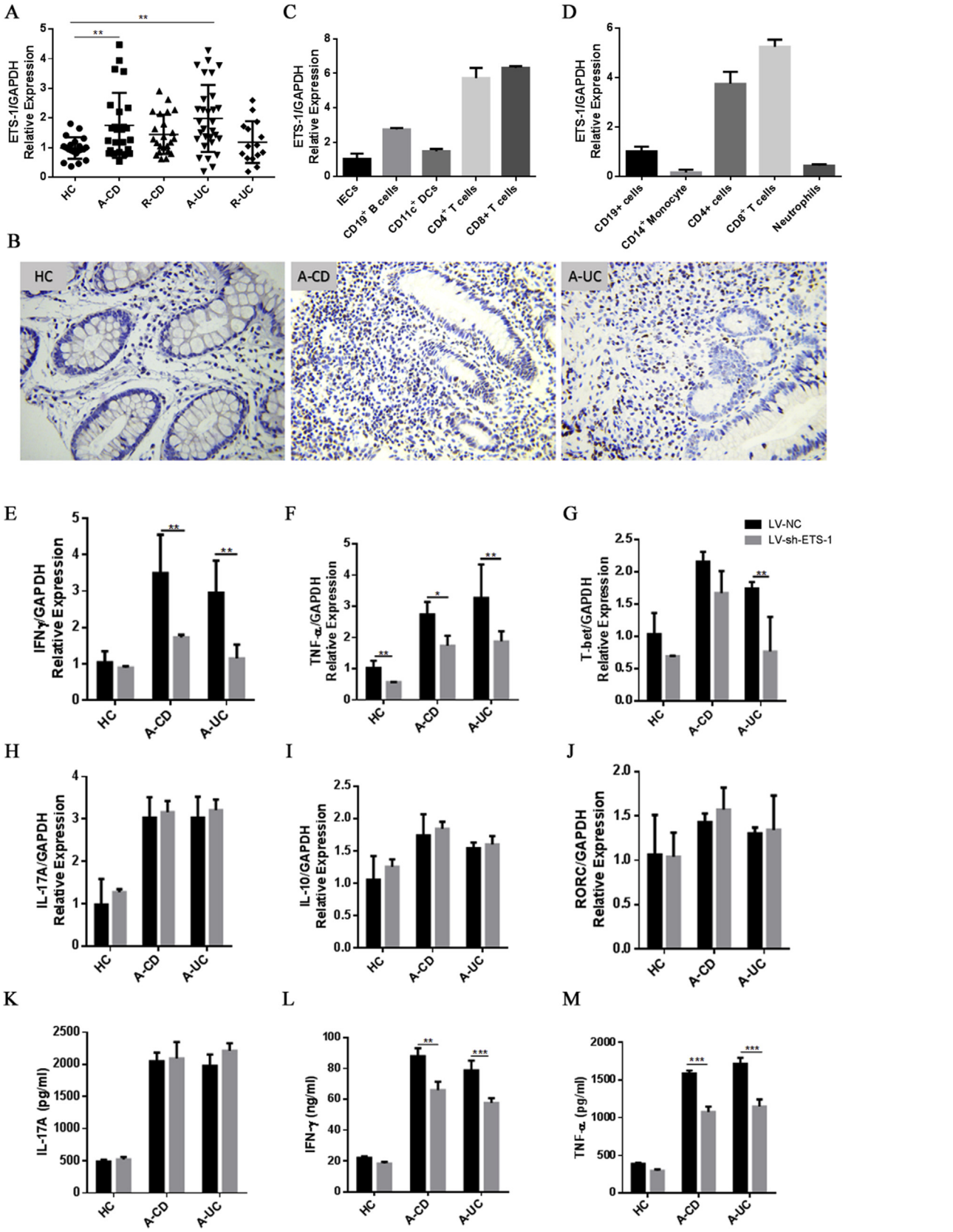

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Fig. 5. ETS-1 is upregulated in patients with IBD, and knockdown of ETS-1 inhibits Th1 cell differentiation and TNF-α production. (A) ETS-1 expression in colon biopsies was analyzed by qRT-PCR. Colon biopsies were collected from patients with A-CD (n = 25), patients with R-CD (n = 23), patients with A-UC (n = 30), patients with R-UC (n = 15), and HCs (n = 20). **p < .01, ***p < .001 versus HCs. (B) Immunohistochemical staining for ETS-1 of representative sections from colonic mucosa of patients with A-CD, patients with A-UC, or HC. Original magnification × 400. (C) LP-CD19$^+$ B cells, CD11c$^+$ dendritic cells (DCs), CD4$^+$ and CD8$^+$ T cells and IECs (1×10^6 for each subset) were isolated from normal intestinal mucosa from 6 patients who underwent colectomy for colon cancer, and ETS-1 expression was determined using qRT-PCR. (D) PB-CD19$^+$ B cells, CD14$^+$ monocytes, neutrophils, CD4$^+$ and CD8$^+$ T cells were isolated from 6 healthy donors, and ETS-1 expression was determined by qRT-PCR. GAPDH was served as the endogenous reference gene in each sample. Data are representative of 3 independent experiments. (E–J) Peripheral blood CD4$^+$ T cells (1×10^5/well) isolated from patients with A-CD (n = 18), A-UC (n = 16), or HC (n = 12) were cultured with immobilized anti-CD3 (5 μg/mL) and anti-CD28 (2 μg/mL) mAbs *in vitro* for 48 h, and transduced with LV-ETS1-sh or LV-NC, respectively, for 5 h. After transduction, cells were then cultured with immobilized anti-CD3 (5 μg/mL) and anti-CD28 (2 μg/mL) mAbs *in vitro* for a further 5 days. Cultured cells were then collected to perform mRNA expression of IFN-γ (E), TNF-α (F), T-bet (G), IL-17A (H), IL-10 (I), and RORC (J) by qRT-PCR. GAPDH was used as a housekeeping gene. *p < .05, **p < .01 versus LV-NC. (K–M) Supernatants were collected to determine levels of IL-17A (K), IFN-γ (L) and TNF-α (M) by ELISA. **p < .01, ***p < .001 versus LV-NC. Representative results from 3 independent experiments are shown.

including cell cycle regulation, cell differentiation and the host immune response [14,18,33]. Foxp3-expressing Treg cells provide new insights into the mucosal inflammatory response [34]. It has been shown that miR-125a is essential for the homeostasis of Treg-mediated immune response [25,35]. Currently, the roles of miR-125a in regulating the effector T cell-mediated immune response are still not clear. Luciferase assay has identified TNF-α, IL-6, and STAT3 as direct targets of miR-125a, implying its protective role in regulating immune response, particularly in T cell activation and differentiation [17,25]. In our study, we observed that miR-125a expression was decreased in inflamed intestinal mucosa of IBD patients and that TNF-α and IL-6 could significantly downregulate miR-125a expression in PB-CD4$^+$ T cells, indicating that decreased miR-125a expression might be the consequence of intestinal mucosal inflammation. Consistently, miR-125a$^{-/-}$ mice had more severe intestinal inflammation after TNBS administration and more aggravated Th1 and Th17 cell infiltrations in inflamed mucosa.

Our findings also demonstrated that ETS-1 is a functional target of miR-125a. ETS-1, which belongs to the large family of the ETS domain family of transcription factors, has been reported to be involved in carcinoma progression and immunity [36]. It is highly expressed in a variety of immune cells, including B, T, NK, and NK T cells [37]. Th1 cells and its IFN-γ production are dominant proinflammatory factors related to the disease activity of IBD patients [38,39]. It has been

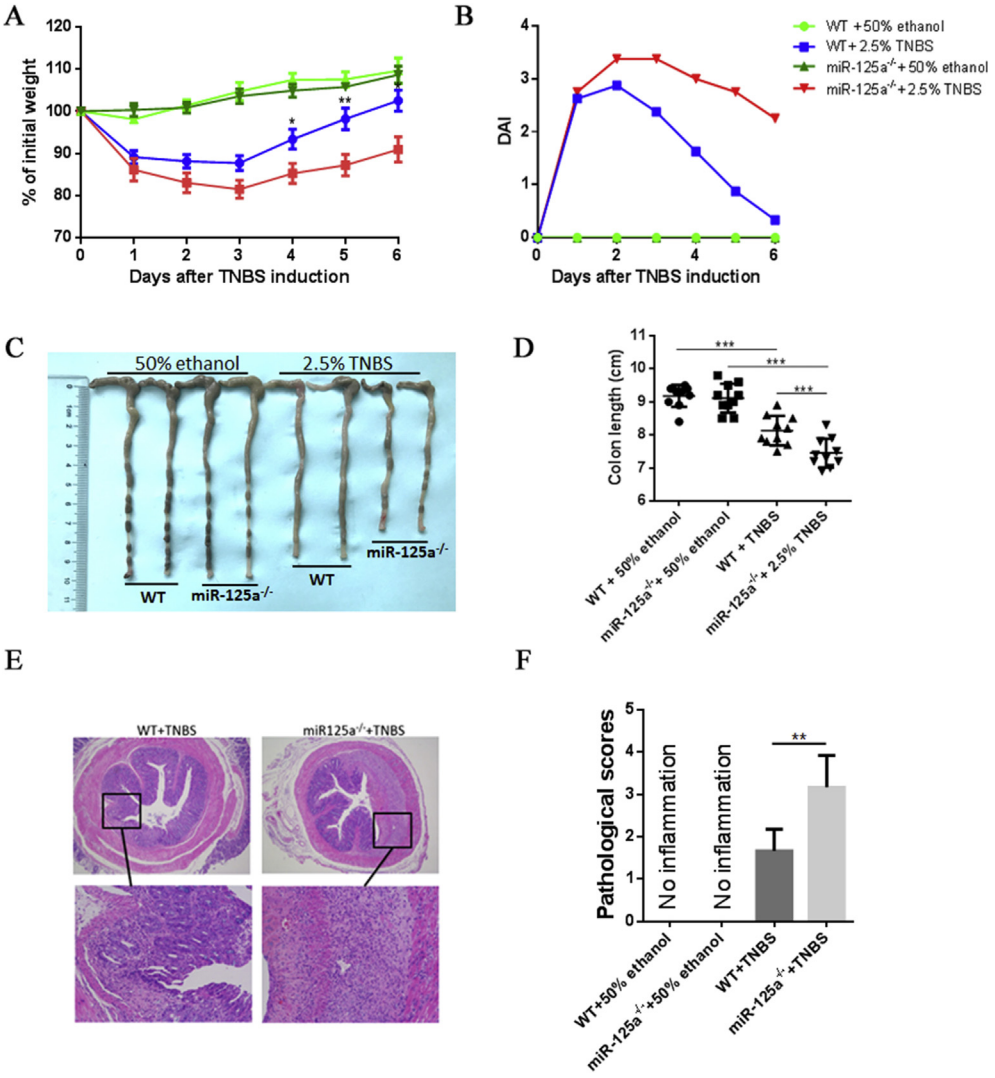

Fig. 6. miR-125a deficiency aggravates TNBS-induced colitis in mice. TNBS-induced acute colitis was performed in WT (n = 12) and miR-125a$^{-/-}$ (n = 12) mice as indicated. All mice were sacrificed on day 6. (A) Changes of body weigh over a period of observation were indicated as a percentage of original weight at the start of the experiments. *p < .05, **p < .01 versus the TNBS-treated WT group on the same day. (B) Changes of disease activity index (DAI) score in WT and miR-125a$^{-/-}$ mice during TNBS-induced colitis. (C) Gross morphology of the large bowels on day 6 after TNBS administration. (D) Colon length was recorded and shown in the chart. ***p < .001. (E) Histological appearance of colonic sections after hematoxylin and eosin (H&E). Original magnification × 200. (F) Pathological scores of colonic sections were observed as indicated. **p < .01 versus TNBS-treated WT group. Data are representative of 3 independent experiments.

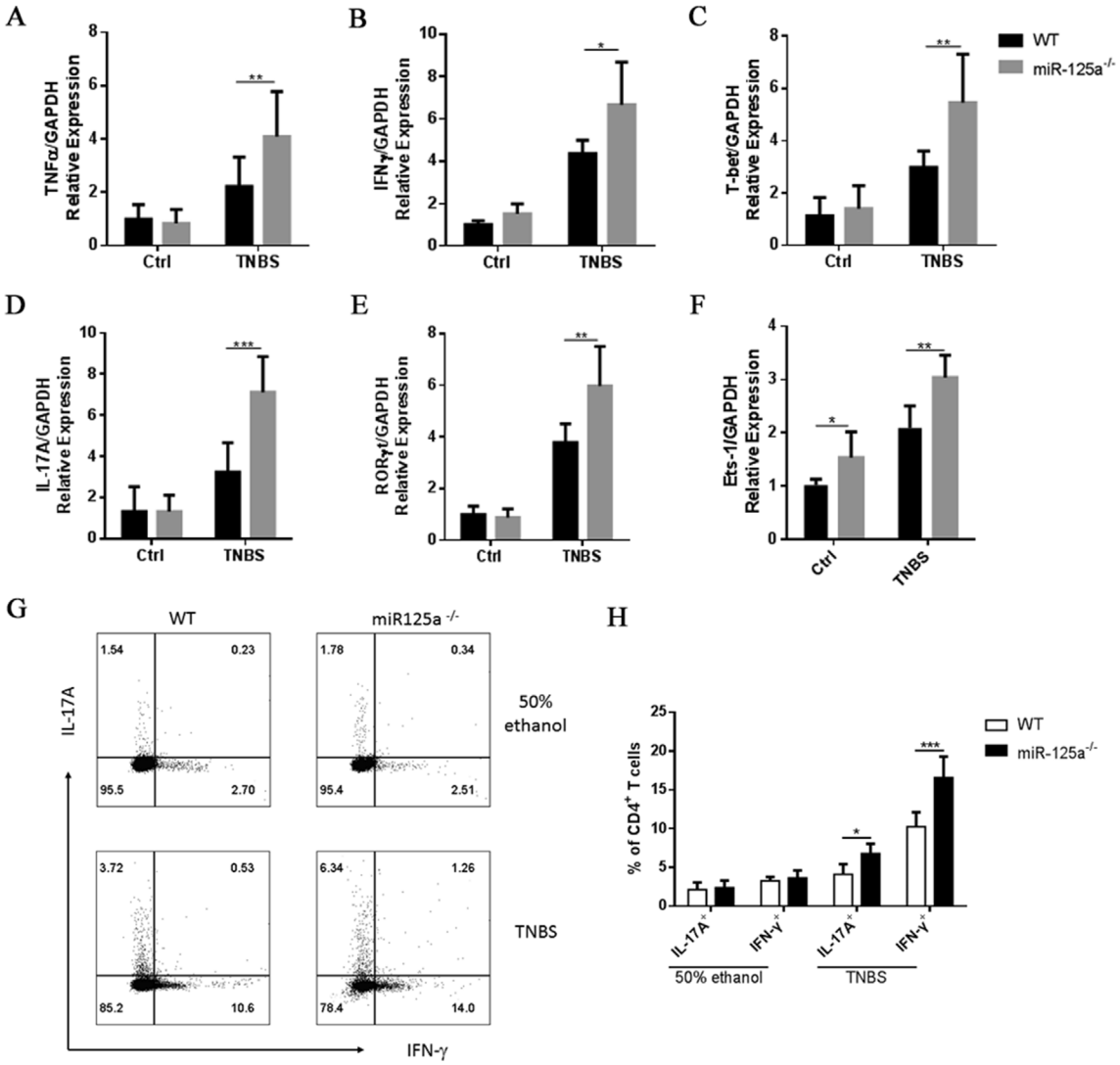

Fig. 7. miR-125a inhibits Th1 and Th17 cell immune responses *in vivo*. (A–F) Colon tissues were obtained from colitis model induced by TNBS. Total RNA was extracted to determine the expression of TNF-α (A), IFN-γ (B), T-bet (C), IL-17A (D), ROR-γt (E) and Ets-1 (F) mRNA using qRT-PCR. GAPDH was used as a housekeeping gene. *p < .05, **p < .01, ***p < .001. (G) LPMC were obtained from colonic mucosa of mice as described in Fig. 6, and flow cytometric analysis was performed to examine intracellular expression of IL-17A and IFN-γ. (H) Percentages of IL-17A$^+$ and IFN-γ$^+$ CD4$^+$ T cells are shown in the bar chart. *p < .05, **p < .01, ***p < .001.

confirmed that Ets-1 binds to the region of the IFN-γ promoter and enhances the activity of T-bet [26,40], and Ets-1$^{-/-}$CD45RB^{high} CD4$^+$ T cells fail to induce intestinal mucosal inflammation in SCID mice because of the defect of Th1 cell differentiation and IFN-γ reduction [26]. This may explain the reason why knockdown of ETS-1 in CD4$^+$ T cells restrains the Th1 cell-mediate immune response and the production of IFN-γ and TNF-α. In addition, evidence has also indicated that Ets-1 enhances the expression of IL-2 by recruiting NFAT proteins to the IL-2 promoter [41], and that IL-2 constrains Th17 cell generation [42]. Consistently, Ets-1 deficiency is also shown to promote Th17 cell differentiation in an IL-2-dependent manner [43]. However, low levels of IL-17A are expressed by either Ets-1$^{-/-}$ or WT Th cells when the Th cells are differentiated in the absence of skewing cytokines (Th0), which is probably caused by the absence of TGF-β1 [43]. This may explain why there is no significant difference in IL-17A production when ETS-1 expression was silenced in CD4$^+$ T cells in our study (Fig. 5H and K). Previous study has shown that ETS-1 is also highly expressed in cholangiocytes of patients with primary sclerosing cholangitis (PSC), a disease often associated with IBD [44]. Increased expression of ETS-1 could promote LPS-induced cholangiocyte senescence, a potentially pathologic state of secreting proinflammatory cytokines such as IL-6 and IL-8, thus contributing to fibroinflammatory processes of PSC. Moreover, another work demonstrated increased Th1 response in the intestinal mucosa of patients with PSC-associated IBD [45]. There may exist some connection between ETS-1 and PSC-associated IBD. Since IBD patients in our study were not accompanied by PSC, this study provided us with new perspective of understanding the role of ETS-1 in IBD. In this study, we found that ETS-1 expression was upregulated in inflamed colon mucosa from both IBD patients and miR-125a$^{-/-}$ mice, and that knockdown of Ets-1 expression decreased Th1 cell differentiation and proinflammatory cytokines, including IFN-γ and TNF-α. Thus, Ets-1 may be considered as an important disease-causing gene in the pathogenesis of IBD.

Since our RNA-seq analysis showed an increase of STAT3 expression in miR-125a$^{-/-}$CD4$^+$ T cells, the underlying mechanism whereby miR-125a regulates Th17 cell-mediated immune response in pathogenesis of IBD may be also associated with STAT3. Previous work has demonstrated that STAT3 is recognized as a target of miR-125a [46], and that STAT3 is an essential transcription factor in Th17 cell differentiation. Therefore, miR-125a might inhibit Th17 cell-mediated

immune response by targeting STAT3 directly. Moreover, IL-6 is found to trigger the activation of STAT3 by binding to IL-6 receptor (IL-6R) in Th17 cell-mediated immune response, and induce Treg cells to differentiate into Th17 cells [47,48]. In our study, we observed that IL-6 could markedly decrease miR-125a expression in $CD4^+$ T cells *in vitro*, but dramatically increased in intestinal mucosa of miR-$125a^{-/-}$ mice after TNBS treatment. Take altogether, it can be envisaged that there is a positive feedback regulation between IL-6 and miR-125a, and that miR-125a may downregulate Th17 cell differentiation by suppressing the IL-6/STAT3 pathway.

There truly exists some limitations to our study. Firstly, our findings were from a single-center, and we will seek multi-center cooperation to further strengthen the study of miR-125a, especially on the key issues in its translational clinical research. Secondly, although we clarified the protective role of miR-125a by suppressing Th1/Th17 cell differentiation in IBD, we believed it might be only the tip of the iceberg. For further understanding the roles of miR-125a in the pathogenesis of IBD, more experiments are required to determine its effect on intestinal microbes, permeability of epithelial cell barrier and other immune cells such as interepithelial lymphocytes (IELs) and neutrophils. Thirdly, although TNBS-induced colitis model in mice is widely used to mimic the T-cell-mediated immune response in IBD, no animal models can exactly capture the complexity of human IBD because of their different gene expression profile and diversity of intestinal microbiota. Finally, all studies about ETS-1 were only performed *in vitro*, yet the question remains as to whether these observations represent *in vivo* performance.

In conclusion, our data indicate that miR-125a suppresses Th1 cell differentiation by binding to ETS-1, and Th17 cell differentiation by targeting STAT3 (Fig. S8). miR-125a may act as a biomarker to evaluate the therapeutic effect, disease prognosis, and recurrence in IBD. Therefore, our study provides new insight into the role of miR-125a in the pathogenesis of IBD, and both miR-125a and ETS-1 may serve as potential therapeutic targets for IBD treatment in future.

Author contributions

ZL designed and conducted the experiments, acquired and analyzed the data; YG, MS, WW, and CM performed all experiments; CZ, CH, JL, YC, and DZ analyzed the data; MS and ZL contributed to the clinical data and specimens; YG and ZL wrote the manuscript. All authors discussed and revised the manuscript.

Conflicts of interest

The authors declare no competing financial interests.

Patient consent

Before initialing the study, patient consents were obtained.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2019.04.014.

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