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NF-κB-driven miR-34a impairs Treg/Th17 balance *via* targeting Foxp3

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A B S T R A C T

The subset of regulatory T (Treg) cells, with its specific transcription Foxp3, is a unique cell type for the maintenance of immune homeostasis by controlling effector T (Teff) cell responses. Although it is common that a defect in Treg cells with Treg/Teff disorder causes autoimmune diseases; however, the precise mechanisms are not thoroughly revealed. Here, we report that miR-34a could attenuate human and murine *Foxp3* gene expression *via* targeting their 3′ untranslated regions (3′ UTR). The human miR-34a, increased in peripheral blood mononuclear cells (PBMCs) and $CD4^+$ T cells from rheumatoid arthritis (RA) or systemic lupus erythematosus (SLE) patients, displayed a positive correlation with some serum markers of inflammation including rheumatoid factor (RF), anti-streptolysin antibody (ASO), erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) as well as Th17 signature gene RORγt, but inversely correlated with the mRNA expression levels of FOXP3. In addition, murine miR-34a levels were downregulated in TGF-β-induced Treg cells but upregulated in Th17 cells induced *in vitro* compared to activated $CD4^+$ T cells. It has also been demonstrated that elevated miR-34a disrupting Treg/Th17 balance *in vivo* contributed to the progress of pathogenesis of collagen induced arthritis (CIA) mice. Furthermore, IL-6 and TNF-α were responsible for the upregulation of miR-34a and downregulation of Foxp3, which was reverted by the addition of NF-κB/p65 inhibitor BAY11-7082, thus indicating that NF-κB/p65 inhibited Foxp3 expression in an miR-34a-dependent manner. Finally, IL-6 or TNF-α-activated p65 could bind to the *miR-34a* promotor and enhance its activity, resulting in upregulation of its transcription. Taken together, we show that NF-κB activated by inflammatory cytokines, such as IL-6 and TNF-α, ameliorates Foxp3 levels *via* regulating miR-34a expression, which provides a new mechanistic and therapeutic insight into the ongoing of autoimmune diseases.

1. Introduction

Treg cells with the $CD4^+$ $CD25^+$ $FOXP3^+$ phenotype have a vital role in immune tolerance by suppressing pathogenic T cell responses [1,2]. Although emerging evidence demonstrates that *Foxp3* gene mutation or downregulation is a remarkable immunological feature in many types of autoimmune diseases such as systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and multiple sclerosis (MS), etc. [3,4], the precise mechanisms leading to *Foxp3* gene downregulation in autoimmune diseases have not been well defined.

MicroRNAs (miRNAs) are proved to target 5′ or 3′ untranslated region (5′ or 3′ UTR) of genes and influence their expression patterns by promoting the degradation of target mRNA species or repressing mRNA translation [5,6]. Mounting evidence suggests that miRNAs are irreplaceable antagonists on part of Treg cell generation and/or function. For instance, miR-31 was downregulated in human umbilical cord blood-derived Treg cells and was verified as a bona repressor of human FOXP3 [7]. Simultaneously, peripherally derived-miR-31 was reported to target Gprc5a and negatively regulate Treg cell generation in an experimental autoimmune encephalomyelitis (EAE) model [8]. Reports from different laboratories confirmed miR-17-92 cluster as a repressor of Treg cell bioactivity. MiR-17, as an individual miRNA of the miR-17-92 cluster, could target Eos and other Foxp3 co-regulators, including Satb1 and IRF4, resulting in a decrease in Treg cell suppressive function [9]. MiR-19b, known as another member of the miR-17-92 cluster, was confirmed to repress TGF-β-induced Treg cell differentiation *via* controlling Pten expression [10]. However, whether other miRNAs participate in blocking Treg cell generation and how these miRNAs are

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regulated need further investigation.

In this study, we identified a new Foxp3 antagonist, miR-34a, which was downregulated in Treg cells and TGF-β induced Treg-like EL4 cells, but elevated in Th17 cells as well as in EL4 cells polarized under Th17-skewing condition. And we confirmed that miR-34a interacted with 3′ UTR of Foxp3, attenuating its transcription and translation levels. Meanwhile, miR-34a levels in peripheral blood mononuclear cells (PBMCs) from RA and SLE patients were positively correlated with several serum disease indexes, including rheumatoid factor (RF), anti-streptolysin antibody (ASO), erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP), suggesting a potential role of miR-34a in disease activity. Furthermore, miR-34a transgenic (miR-34a-TG) mice or mice injected with miR-34a agonist showed stronger disease phenotypes and more severe Treg/Th17 disorder upon collagen-induced arthritis (CIA) induction compared to wild-type mice or mice injected with the agonist control. More importantly, our experiments determined that the Th17-related cytokines IL-6 and TNF-α were involved in promoting miR-34a levels, thus lowering Foxp3 protein levels, which was reversed by NF-κB inhibitor BAY 11-7082. Moreover, NF-κB/p65 functioned as an upstream regulator of miR-34a-Foxp3 interaction through activating miR-34a promoter and thus enhancing its expression. Taken together, our findings show that IL-6 and TNF-α released from the microenvironment mediate the repressive effect of miR-34a on Foxp3 expression in an NF-κB-dependent manner, causing Treg/Th17 imbalance in rheumatoid arthritis. Thus, IL-6/TNF-α-NF-κB/p65-miR-34a-Foxp3 signaling pathway may be a novel therapeutic target for some autoimmune diseases.

2. Materials and methods

2.1. Mice

The specific-pathogen free (SPF) C57BL/6 mice were purchased from College of Veterinary Medicine of Yangzhou University. MiR-34a transgenic (miR-34a-TG) mice were kind gifts from Professor Jinping Zhang in Suchow University, P.R. China. The SPF DBA 1/J mice were purchased from Shanghai SLAC laboratory Animal Co., Ltd. (SLAC).

2.2. Reagents and antibodies

Restriction enzymes including BamH I (1010S), Xho I (1094S), Not I (1166S), Xba I (1093S), and Nhe I (1241S) from Takara, Japan. TIANgel Maxi Purification Kit (DP210, TIGEN Biotech, China), ClonExpress® Ultra One Step Cloning Kit (C115-02, Vazyme Biotech, China). PrimeSTAR® Max DNA Polymerase (R045Q), RNAiso Plus (9109), TB Green™ Fast qPCR Mix (RR430A) and TaKaRa Ex Taq® (RR001A) from Takara, Japan. Transfection reagents including Lipofectamine 2000 (11668-019, Invitrogen, USA) and Entranster™-R4000 (4000-4, Engreen Biosystem, China). Dual-Luciferase Reporter Assay System (E5311) and M-MLV Reverse Transcriptase, (M5301) from Promega, USA. The mmu-agomiR-NC (miR04201-1-10), mmu-antagomiR-NC (miR03201-1-10), mmu-agomiR-34a (miR40000542-1-10) and mmu-antagomiR-34a (miR20000542-1-5) from RIBOBIO, China. Histopaque®-1077 (10771), phorbol 12-myristate 13-acetate (PMA, P8139), ionomycin (407952), the bovine type II collagen (20021) and Freund's complete adjuvant (7024) from Sigma-Aldrich, USA. Brefeldin A (00-4506-51) and Fixation and Permeabilization kit (00-5521-00) from eBioscience, USA, and NF-κB/p65 inhibitor BAY 11-7082 (S2913, Selleck, USA) were used in this study. Reagents or antibodies used for western blotting were as follows: Protein extraction buffer including RIPA Lysis Buffer (P0013B), Protease and phosphatase inhibitor cocktail (P1045) and BCA protein assay kit (P0012S) from Beyotime, China. ECL Western Blotting Substrate (180-5001, Tanon, China), FOXP3 Rabbit Polyclonal antibody (22228-1-AP, Proteintech, USA), Phospho-NF-κB p65 antibody (Ser536) (3033, Cell Signaling Technology, USA), NF-κB p65 antibody (8242, Cell Signaling Technology, USA), mouse m-IgGκ BP-HRP antibody (sc-516102, Santa Cruz Biotechnology, USA) and Horseradish peroxidase (HRP)-conjugated anti-Rabbit IgG (A0208, Beyotime, China). For human $CD4^+$ T cell and mouse naïve T cell purification, EasySep™ Direct Human $CD4^+$ T Cell Isolation Kit (19662) and EasySep™ Mouse $CD4^+$ $CD62L^+$ T Cell Isolation Kit (18765) from STEMCELL, Canada were used. For Th cell differentiation, purified anti-mouse CD3 (100201), anti-mouse CD28 (102108), IFN-γ (517901) and IL-4 antibody (504101) from BioLegend, USA. Recombinant mouse IL-2 (402-ML-020), recombinant human TGF-β1 (240-B-002/CF), recombinant human IL-1β (201-LB-005) and recombinant mouse IL-6 (406-ML-025) from R&D, USA as well as recombinant human IL-23 (200-12p40, PeproTech, USA) were used in this study. The antibodies for Flow cytometry detection were as follows: FITC anti-human CD3 (300305), FITC anti-mouse CD4 (100405), APC anti-human CD4 (A161A1), PerCP/Cyanine5.5 anti-human CD8 (344709), PE anti-mouse/rat/human FOXP3 (320007), APC anti-mouse IL-17 A (506915) and PE anti-human IL-17 A (512306) from BioLegend, USA.

2.3. Patient characteristics

A total of 35 RA patients (including 10 new cases), 22 SLE patients and 37 health controls (including 10 new volunteers) were enrolled in the present study. RA patients fulfilled the 2009 revised criteria of the American College of Rheumatology/European League Against Rheumatism (ACR/EULAR) collaborative initiative [11]. SLE patients fulfilled the 1997 revised criteria of the ACR [12]. Age-matched healthy volunteers were recruited as negative controls. The blood samples of RA, SLE patients and healthy donors, without clinical therapy from 18 January 2011 to 3 March 2019 in the Outpatient Department of Affiliated Hospital of Jiangsu University, were collected in this study. The parameters regarding serum markers of inflammation including rheumatoid factor (RF), anti-streptolysin antibody (ASO), erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) were obtained and collected from the Department of Laboratory Medicine in Affiliated Hospital of Jiangsu University. The clinical data describing the studied subjects are summarized in Table I. This study was approved by the Medical Ethics Committee of Jiangsu University.

2.4. Blood samples and human peripheral blood mononuclear cell (PBMC) and $CD4^+$ T cell isolation

Approximately, 15 ml of venous blood was drawn by venipuncture from RA or SLE patients and healthy controls. 10 ml samples of them prepared were heparinized for the isolation of peripheral blood mononuclear cells (PBMCs) by Ficoll-Hypaque density gradient centrifugation. Trypan blue staining revealed the viability of freshly isolated cells to be greater than 95%. Besides, 4–5 ml whole blood samples from RA patients or matched healthy donors were used for $CD4^+$ T cell enrichment according to the manufacturer's instructions.

2.5. Cell culture

HEK-293 T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS, 10099-141, Gibco®, Thermo Fisher Scientific, USA), penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37 °C under 5% CO_2. Primary and induced T cells (naïve T, Treg or Th17 cells) and EL4 mouse lymphoma cells were maintained in RPMI-1640 with 10% FBS at 37 °C under 5% CO_2.

2.6. Induction of Treg or Treg-like EL4 cells and Th17 or Th17-like EL4 cells

In vitro generation of Treg cells was conducted according to a manufacturer's instructions [13]. In brief, $CD4^+$ T cells obtained from mouse spleens were isolated by EasySep™ Mouse $CD4^+$ $CD62L^+$ T Cell

Table 1
The clinical data depicting the RA, SLE patients, as well as the corresponding healthy donors.

Patient	Age (Years)	Sex	miR-34a/U6	RF (U/ml)	ASO (U/ml)	ESR (mm/h)	CRP (mg/l)
RA 01	30	Female	0.0000707 (PBMCs)	22.4	104.0	31.0	79.60
RA 02	66	Female	0.000602 (PBMCs)	37.8	110.0	11.0	4.83
RA 03	61	Female	0.000178 (PBMCs)	20.0	25.0	7.0	3.12
RA 04	37	Female	0.0000221 (PBMCs)	20.0	5.0	26.0	1.55
RA 05	57	Male	0.000152 (PBMCs)	23	20.0	5.0	2.30
RA 06	78	Male	0.000873 (PBMCs)	517.0	25.0	51.0	42.60
RA 07	40	Male	0.000160 (PBMCs)	1800.0	48.5	6.0	2.48
RA 08	64	Male	0.0000465 (PBMCs)	480.0	44.4	104.0	8.31
RA 09	51	Male	0.000300 (PBMCs)	20.0	49.4	18.0	1.36
RA 10	46	Male	0.003210 (PBMCs)	590.0	25.0	86.0	21.00
RA 11	39	Female	0.000649 (PBMCs)	20.0	31.2	5.0	1.48
RA 12	69	Female	0.0000929 (PBMCs)	1040.0	25.0	56.0	22.10
RA 13	54	Female	0.000178 (PBMCs)	160.0	48.0	10.0	22.60
RA 14	45	Female	0.000443 (PBMCs)	682.0	64.2	15.0	4.40
RA 15	41	Female	0.005740 (PBMCs)	3460.0	147.0	135.0	90.50
RA 16	55	Male	0.000924 (PBMCs)	24.5	25.0	20.0	7.47
RA 17	51	Female	0.000836 (PBMCs)	57.4	73.6	12.0	14.80
RA 18	31	Female	0.0000157 (PBMCs)	20.0	64.7	6.0	4.50
RA 19	66	Male	0.000213 (PBMCs)	52.4	25.0	4.0	3.94
RA 20	51	Male	0.004930 (PBMCs)	1340.0	46.4	7.0	9.06
RA 21	53	Female	0.0000338 (PBMCs)	234.0	38.5	12.0	5.89
RA 22	56	Male	0.002090 (PBMCs)	365.0	144.0	12.0	57.00
RA 23	57	Female	0.000763 (PBMCs)	22.2	33.5	42.0	30.80
RA 24	44	Female	0.0000934 (PBMCs)	452.0	22.0	6.0	1.00
RA 25	35	Female	0.001850 (PBMCs)	20.8	106.0	20.0	65.50
RA 26	29	Female	0.041235 ($CD4^+$ T cells)	126	/	131	0.5
RA 27	64	Female	0.046071 ($CD4^+$ T cells)	981	/	53	49.7
RA 28	55	Male	0.006615 ($CD4^+$ T cells)	227	/	20.1	2.7
RA 29	50	Female	0.002228 ($CD4^+$ T cells)	66.3	/	14	1
RA 30	58	Female	0.004581 ($CD4^+$ T cells)	115	/	–	10.9
RA 31	37	Female	0.011924 ($CD4^+$ T cells)	291	/	22	0.5
RA 32	53	Female	0.027017 ($CD4^+$ T cells)	148	/	82	21.4
RA 33	67	Female	0.006346 ($CD4^+$ T cells)	70.3	/	52	12.5
RA 34	84	Male	0.040386 ($CD4^+$ T cells)	155	/	92	151
RA 35	49	Female	0.032352 ($CD4^+$ T cells)	110	/	110	59.7
SLE 01	48	Female	0.000147977 (PBMCs)	20.6	10.0	32.0	29.00
SLE 02	47	Male	0.001202268 (PBMCs)	98.0	25.0	63.0	6.89
SLE 03	31	Female	0.000206145 (PBMCs)	20.0	37.7	3.0	1.00
SLE 04	29	Female	0.000246667 (PBMCs)	317.0	473.0	162.0	45.70
SLE 05	34	Female	0.000109394 (PBMCs)	20.0	25.0	1.0	1.30
SLE 06	65	Female	0.000880519 (PBMCs)	150.0	25.0	36.0	27.80
SLE 07	28	Female	0.000136364 (PBMCs)	27.8	25.0	47.0	5.91
SLE 08	23	Female	0.000243921 (PBMCs)	4.0	52.9	3.2	1.18
SLE 09	40	Female	0.000390921 (PBMCs)	20.0	31.0	60.0	2.85
SLE 10	32	Female	0.001250 (PBMCs)	204.0	207.0	122.0	135.00
SLE 11	30	Female	0.000245238 (PBMCs)	20.0	25.0	16.8	2.70
SLE 12	39	Female	0.000998 (PBMCs)	24.9	156.0	15.0	1.55
SLE 13	64	Male	0.000318471 (PBMCs)	26.0	80.0	16.0	87.00
SLE 14	44	Female	0.000433962 (PBMCs)	96.0	15.0	1.0	1.00
SLE 15	46	Female	0.000404894 (PBMCs)	37.6	93.9	102.0	1.19
SLE 16	48	Female	0.000499069 (PBMCs)	1.0	30.7	20.0	7.00
SLE 17	41	Male	0.000545455 (PBMCs)	20.9	65.6	25.0	45.00
SLE 18	49	Female	0.001149502 (PBMCs)	234.0	116.0	48.0	63.30
SLE 19	49	Female	0.001000 (PBMCs)	33.4	25.0	128.0	3.94
SLE 20	31	Female	0.000517123 (PBMCs)	21.2	67.0	64.0	57.00
SLE 21	20	Female	0.000210744 (PBMCs)	89.0	100.0	70.0	56.00
SLE 22	29	Female	0.002085954 (PBMCs)	302.0	554.0	180.0	232.00
HD 01	49	Female	0.00010046 (PBMCs)	/	/	/	/
HD 02	36	Female	0.000150342 (PBMCs)	/	/	/	/
HD 03	58	Female	0.0000231034 (PBMCs)	/	/	/	/
HD 04	29	Male	0.0000269444 (PBMCs)	/	/	/	/
HD 05	63	Male	0.00000184713 (PBMCs)	/	/	/	/
HD 06	61	Female	0.0000734317 (PBMCs)	/	/	/	/
HD 07	42	Female	0.00006875 (PBMCs)	/	/	/	/
HD 08	31	Male	0.00012994 (PBMCs)	/	/	/	/
HD 09	35	Female	0.0000850633 (PBMCs)	/	/	/	/
HD 10	53	Male	0.0000259442 (PBMCs)	/	/	/	/
HD 11	26	Female	0.0000329819 (PBMCs)	/	/	/	/
HD 12	24	Female	0.000410684 (PBMCs)	/	/	/	/
HD 13	33	Female	0.0000329082 (PBMCs)	/	/	/	/
HD 14	33	Female	0.0000819383 (PBMCs)	/	/	/	/
HD 15	37	Female	0.0000124844 (PBMCs)	/	/	/	/
HD 16	60	Female	0.000233592 (PBMCs)	/	/	/	/
HD 17	61	Male	0.00000948936 (PBMCs)	/	/	/	/

(*continued on next page*)

Table 1 (*continued*)

Patient	Age (Years)	Sex	miR-34a/U6	RF (U/ml)	ASO (U/ml)	ESR (mm/h)	CRP (mg/l)
HD 18	48	Female	0.000754717 (PBMCs)	/	/	/	/
HD 19	52	Female	0.000352349 (PBMCs)	/	/	/	/
HD 20	58	Female	0.000685271 (PBMCs)	/	/	/	/
HD 21	55	Female	0.00064497 (PBMCs)	/	/	/	/
HD 22	38	Female	0.00051131 (PBMCs)	/	/	/	/
HD 23	30	Female	0.000910448 (PBMCs)	/	/	/	/
HD 24	29	Male	0.000149331 (PBMCs)	/	/	/	/
HD 25	49	Female	0.000246154 (PBMCs)	/	/	/	/
HD 26	57	Female	0.000449398 (PBMCs)	/	/	/	/
HD 27	60	Female	0.000723404 (PBMCs)	/		/	/
HD 28	27	Female	0.001106 ($CD4^+$ T cells)	/	/	/	/
HD 29	31	Male	0.005299 ($CD4^+$ T cells)	/	/	/	/
HD 30	43	Female	0.006003 $CD4^+$ T cells)	/	/	/	/
HD 31	58	Male	0.002801 $CD4^+$ T cells)	/	/	/	/
HD 32	64	Female	0.003826 $CD4^+$ T cells)	/	/	/	/
HD 33	33	Female	0.00734 $CD4^+$ T cells)	/	/	/	/
HD 34	48	Female	0.00282 $CD4^+$ T cells)	/	/	/	/
HD 35	67	Male	0.001106 $CD4^+$ T cells)	/	/	/	/
HD 36	59	Female	0.00162 $CD4^+$ T cells)	/	/	/	/
HD 37	65	Female	0.002388 $CD4^+$ T cells)	/	/	/	/

Isolation Kit according the protocols. 2×10^6/ml or 4×10^6/ml $CD4^+$ T cells were seeded in anti-mouse CD3 (5 mg/ml) coated 24- or 12-well plates (Nunc™/Thermo Fisher, USA) cultured in RPMI 1640 medium in the presence of soluble anti-mouse CD28 (2 mg/ml), recombinant mouse IL-2 (50 U/ml) and recombinant human TGF-β1 (5 ng/ml) for another 2 days. For Foxp3 induction in EL4 cells, cells were cultured as Treg cell induction described above and treated with recombinant mouse IL-2 (50 U/ml) and recombinant human TGF-β1 (5 ng/ml) for 6 h, 12 h, 24 h and 48 h, respectively. For *in vitro* differentiation of Th17 cells, naïve $CD4^+$ T cells isolated and activated as Treg cell induction described above were maintained in RPMI 1640 medium in the presence of recombinant human IL-1β (20 ng/ml), recombinant mouse IL-6 (20 ng/ml); recombinant human TGF-β1 (2 ng/ml); recombinant human IL-23 (5 ng/ml), anti-IFN-γ (10 μg/ml) and anti-IL-4 (10 μg/ml) for 3 days.

2.7. Plasmid construction

The pLV-miR-rpuro lentivirus vector is a product of Biosettia Inc. in San Diego, CA, USA. The MGP (pMSCV-puro) and MGP-pre-miR-34a retrovirus vectors were kind gifts from Professor David Baltimore at California Institute of Technology, USA. Sequences of mouse pre-miR-34a and pre-miR-31 were PCR-amplified from EL4 genomic DNA by primers with BamH I and Xho I and inserted into pCDNA-3.1 vector. For MGP-31 retrovirus vector construction, the pre-miR-31 sequence was amplified using primers with NotI and XhoI restriction sites and inserted into the MGP lentivirus vector as previously described [14]. For construction of pLV-miR-34a-rpuro and pLV-miR-31-rpuro lentivirus vector, the sequences of pre-miR-34a and pre-miR-31 were amplified using primers with BamHI and XbaI and cloned into the pLV-miR-rpuro lentivirus vector. For generation of mouse *Foxp3* gene containing its 3′ UTR in pCDNA3.1 (pCDNA-mFoxp3-3′ UTR) and pCDNA-mLumin vector (pCDNA-mLumin-mFoxp3-3' UTR), complementary DNA (cDNA) from primary T cells was amplified and digested with Not I and XbaI or XhoI and XbaI, and inserted into pCDNA3.1 or pCDNA-mLumin vector, respectively. The mouse Foxp3-3′ UTR cloned from cDNA of primary T cells was also then inserted into psiCHECK-2 luciferase reporter vector (a kind gift of Professor Yongchang Chen at School of Medicine, Jiangsu University, P.R. China.) at Xho I and Not I sites. The psiCHECK-2-mFoxp3-3′ UTR-mutants (M34a, M31) were generated using primers according to an overlap PCR. For generation of different forms of mouse miR-34a promoter vectors, genomic DNA extracted from EL4 cells was cloned in pGL3-basic vector *via* PCR using primers with NheI and XhoI restriction sites. The amplified production of mouse miR-34a promoter was then purified and inserted into pGL3-basic vector by homologous recombination using ClonExpress® Ultra One Step Cloning Kit according to manufacturer's instructions. Primer sequences for DNA constructs are listed in Table II.

2.8. RNA reverse transcription and reverse transcription-polymerase chain reaction (RT-PCR) or real-time quantitative PCR (qRT-PCR)

For evaluation of the levels of miR-34a or several other genes in PBMCs from RA or SLE patients, total RNA was extracted using RNAiso Plus. The cDNA was synthesized using a reverse transcriptase by M-MLV Reverse Transcriptase. The RT-PCR and qRT-PCR were carried out with the TaKaRa Ex Taq® in Thermal Cyclers for PCR (Bio-Rad Laboratories, China) and the TB Green™ Fast qPCR Mix in CFX96™ Real-Time PCR Detection Systems (Bio-Rad Laboratories, USA), respectively. U6 small RNA (U6 snRNA) or Gapdh was specially amplified as an internal standard for mature miRNA-34a and indicated mRNA. The transcription levels of miRNAs (miR-34a and miR-31) and the indicated mRNA in PBMCs of RA and SLE patients were calculated using the standard curve according to the manufacturer's protocol [15]. For analysis of miR-34a and miR-31 expression as well as the transcription factor (FOXP3, RORγt) and inflammatory cytokine transcription levels in purified human $CD4^+$ T cells, induced murine Treg, Th17 cells, Treg-like EL4 or Th17-like EL4 cells and isolated spleen or lymph node from mice *in vitro* or *in vivo*, quantification of the transcription levels of miRNAs (miR-34a and miR-31) and mRNAs was measured by the comparative CT method, normalized to endogenous U6, Gapdh or β-actin expression and determined by formula $2^{-\Delta\Delta ct}$. Each experiment was independently repeated three times. All primers used for qRT-PCR are listed in Table II.

Table 2
Specific primers used in expression plasmids construction, promoter luciferase reporter construction and PCR reaction (RT-PCR and qRT-PCR).

Plasmids/Genes	Primers	Sequences
pCDNA-pre-miR-34a (mouse)	Forward	GGATCCGGGCTGGTCTTGAACTCCTG
	Reverse	CTCGAGAAGTCCTGGCGTCTCCCACT
pCDNA-pre-miR-31 (mouse)	Forward	GGATCCGGCGAGACAGACCAAGTCA
	Reverse	CTCGAGCAACGAAGAGGGATGGTATTGC
MGP-31 (mouse)	Forward	GCGGCCGGCGAGACAGACCAAGTCA
	Reverse	CTCGAGCAACGAAGAGGGATGGTATTGC
pLV-pre-miR-34a (mouse)	Forward	GGATCCGGGCTGGTCTTGAACTCCTG
	Reverse	TCTAGAAAGTCCTGGCGTCTCCCACT
pLV-pre-miR-31 (mouse)	Forward	GGATCCGGCGAGACAGACCAAGTCA
	Reverse	TCTAGACAACGAAGAGGGATGGTATTGC
pCDNA- mFoxp3-3' UTR	Forward	GCGGCCGCATTGCGGCCGCCCTCAAAACCAAGAAAAGGTG
	Reverse	CTCGAGCCGCTCGAGGCCCTGATGGATGTCTCCTAAC
pCDNA- mLumin-mFoxp3-3' UTR	Forward	CATCTCGAGCCTCAAAACCAAGAAAAGGTG
	Reverse	CCGTCTAGAGCCCTGATGGATGTCTCCTAAC
psiCHECK-2-mFoxp3-3' UTR (WT)	Forward	CCGCTCGAGCCTCAA AACCAAGAAAAGGTG
	Reverse	ATTGCGGCCGCGCCCTGATGGATGTCTCCTAAC
psiCHECK-2-mFoxp3-3' UTR (M34a)	Forward	TTCTGCTTACTCAATGCCAAGGCAGATATG
	Reverse	CATATCTGCCTTGGCATTGAGTAAGCAG
psiCHECK-2-mFoxp3-3' UTR (M31)	Forward	GGGTCAGTACGGTTTGAGACGTGGGGTAC
	Reverse	GTACCCCACGTCTCAAACCGTACTGACCC
pGL3-basic-miR-34a promoter (FL/-3254～+200)	Forward	cgagctcttacgcgtgctagcGGTGATAGGCGTTGACTATCTTGC
	Reverse	cttagatcgcagatctcgagCCAGCCCAGGCTGAGGCG
pGL3-basic-miR-34a promoter (M1/-2509～+200)	Forward	cgagctcttacgcgtgctagcAAGATGATATGGGGAGTGTGGTCA
	Reverse	cttagatcgcagatctcgagCCAGCCCAGGCTGAGGCG
pGL3-basic-miR-34a promoter (M2/-2275～+200)	Forward	cgagctcttacgcgtgctagcCACACACAGGCAGAGGTGAGG
	Reverse	cttagatcgcagatctcgagCCAGCCCAGGCTGAGGCG
pGL3-basic-miR-34a promoter (M3/-1289～+200)	Forward	cgagctcttacgcgtgctagcGAGCCCCTCCCCAGTTCA
	Reverse	acttagatcgcagatctcgagGAGGGACAGCGCCACCAG
pGL3-basic-miR-34a promoter (M4/+74～+200)	Forward	cgagctcttacgcgtgctagcTCCTGTGCTAGCGTGCTGG
	Reverse	acttagatcgcagatctcgagGAGGGACAGCGCCACCAG
miR-34a	Reverse transcriptase	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACAACC
	Forward (qRT-PCR)	GGGTGGCAGTGTCTTAGCT
	Reverse (qRT-PCR)	CAGTGCGTGTCGTGGAGT
miR-31	Reverse transcriptase	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACAGCTAT
	Forward (qRT-PCR)	AGGCAAGATGCTGGCATAGCTG
	Reverse (qRT-PCR)	CAGTGCGTGTCGTGGAGT
human FOXP3	Forward (qRT-PCR)	AGATGGTACAGTCTCTGGAGCAG
	Reverse (qRT-PCR)	AAGTAGTCCATGTTGTGGAGGAA
mouse Foxp3	Forward (RT-PCR)	AGGAGAAAGCGGATACCA
	Reverse (RT-PCR)	TGTGAGGACTACCGAGCC
	Forward (qRT-PCR)	AAGTGCTTTGTGCGAGT
	Reverse (qRT-PCR)	TCAAGGGCAGGGATTGG
human RORγt	Forward (qRT-PCR)	GTGGGGACAAGTCGTCTGG
	Reverse (qRT-PCR)	AGTGCTGGCATCGGTTTCG
mouse RORγt	Forward (qRT-PCR)	CGCCTCACCTGACCTACCC
	Reverse (qRT-PCR)	TGGCTGTCTGGACCCTGTTC
mouse Tbet	Forward (qRT-PCR)	CCTGGACCCAACTGTCAACT
	Reverse (qRT-PCR)	AACTGTGTTCCCGAGGTGTC
mouse IL-1β	Forward (qRT-PCR)	TTCAGGCAGGCAGTATCACTC
	Reverse (qRT-PCR)	GAAGGTCCACGGGAAAGACAC
mouse IL-6	Forward (qRT-PCR)	TTCCATCCAGTTGCCTTCT
	Reverse (qRT-PCR)	ATTTCCACGATTTCCCAGAG
human IL-17A	Forward (qRT-PCR)	AGATTACTACAACCGATCCACCT
	Reverse (qRT-PCR)	GGGGACAGAGTTCATGTGGTA
mouse IL-17a	Forward (qRT-PCR)	ATCCACCTCACACGAGGCA
	Reverse (qRT-PCR)	AGATGAAGCTCTCCCTGGACTC
mouse IFNγ	Forward (qRT-PCR)	TGAAAGACAATCAGGCCATC
	Reverse (qRT-PCR)	TTGCTGTTGCTGAAGAAGGT
U6	Forward (qRT-PCR)	CTCGCTTCGGCAGCACATAT
	Forward (qRT-PCR)	ACGCTTCACGAATTTGCGTG
human Gapdh	Forward (qRT-PCR)	CATGAGAAGTATGACAACAGCCT
	Reverse (qRT-PCR)	AGTCCTCCACGATACCAAAGT
mouse β-actin	Forward (RT-PCR)	CTGTCCCTGTATGCCTCTG
	Reverse (RT-PCR)	ATGTCACGCACGATTTCC
	Forward (qRT-PCR)	TGCTGTCCCTGTATGCCTCT
	Reverse (qRT-PCR)	AGGTCTTTACGGATGTCAACG

2.9. Protein extraction and western blotting

Cells were lysed using RIPA lysis buffer containing phosphatase inhibitor, and the protein concentrations were determined using the BCA protein assay kit. To analyze NF-κB p65 phosphorylation and Foxp3 protein levels, western blotting was performed on total lysates as described previously [16]. In brief, an equal amount of protein samples (80 μg/lane) were loaded and separated in 12% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) depending on the molecular weight of the proteins and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked in 5% BSA for 2 h at room temperature with gentle shaking and then incubated with primary antibodies

(Foxp3, 1:1000; p-p65, 1:1000; t-p65, 1:1000; β-actin, 1:10000) overnight at 4 °C, followed by incubation with secondary antibodies (HRP)-conjugated anti-Rabbit IgG (1:10000) at room temperature for 1 h. The proteins were subsequently visualized using enhanced chemiluminescence. Finally, the densities of radiographic bands (versus β-actin) onto PVDF membranes were analyzed by using the software of Quantity One, and all the results were normalized to the corresponding groups. Each experiment was independently repeated for three times.

2.10. Cell transfection

For assess of Foxp3 mRNA and protein levels in HEK-293 T cells, cells (1×10^6/ml) were co-transfected with 2 μg pre-miRNA-34a or pre-miRNA-31 plasmid (pSMCV-puro- or pLV-miR-rpuro-) and 0.5 μg pCDNA-mFoxp3-3′ UTR for 2 days using Lipofectamine 2000 in a 6-well plate. For detection of miR-34a and Foxp3 levels in $CD4^+$ T or EL4 cells, cells (2×10^6/ml) were pre-transfected with 100 nM agomiR-34a or 200 nM antagomiR-34a according to the manufacturer's protocol and then induced towards different T cell subsets (Treg, Th17) as previously described [13]. For some experiments, $CD4^+$ T or EL4 cells were pre-treated with BAY 11-7082 (5 μM) for 30 min, transfected with agomiR-34a or antagomiR-34a for 24 h and then induced towards different T cell subsets (Treg, Th17) or treated with cytokines (IL-2, TGF-β, IL-6, TNF-α) as previously described. Each experiment was independently repeated for three times.

2.11. Assays of Foxp3-3' UTR activity

For analysis of Foxp3-3′ UTR activity by flow cytometry, HEK-293 T cells (3×10^5/ml) were co-transfected with 200 ng pCDNA3.1-pre-miR-34a or pCDNA3.1-pre-miR-31 plasmid, 50 ng pCDNA-mLumin-Foxp3-3′ UTR plasmid and 200 ng pYFP (internal reference) for 48 h by lipofectamine 2000 in a 24-well plate. The cells were detached from the plate surface with 0.25% trypsin-EDTA, washed three times with PBS and resuspended in PBS. The Foxp3-3′ UTR activity was analyzed by the ratio of $mLumin^+$ cells/YFP^+ cells according to the fluorescence intensity using a FACS Calibur (BD Biosciences). For analysis of Foxp3-3′ UTR activity by luciferase reporter assay, 400 ng pCDNA3.1-pre-miR-34a or pCDNA3.1-pre-miR-31 plasmids and 80 ng psiCHECK-2-mFoxp3-3′ UTR plasmid or psiCHECK-2-mFoxp3-3′ UTR-mutants were co-transfected into HEK-293 T cells in a 24-well plate described as above by Lipofectamine 2000 reagent for 48 h. Cell extracts were prepared after transfection, and the luciferase activity was measured using a dual luciferase reporter assay system according to manufacturer's instructions. Each experiment was independently repeated for three times.

2.12. Assays of mouse miR-34a promoter activity

For measurement of the activity of mouse miR-34a promoter, 100 ng pGL3-miR-34a promoter and 50 ng pRL-SV40 plasmid were co-transfected into HEK-293 T cells (2×10^5/ml) in a 24-well plate by Lipofectamine 2000 reagent for 48 h in the presence of 500 ng pCDNA-p65 vector (a kind gift obtained from Prof. Zhiqiang Xiao, Xiangya Hospital, Central South University, P.R. China). After 48 h, cell extracts were prepared and the luciferase activity was measured using a dual luciferase reporter assay system according to manufacturer's instructions. For NF-κB p65 inhibitor BAY 11-7082 treatment, HEK-293 T cells pre-treated with BAY 11-7082 (5 μM) for 30min were transfected with plasmids mentioned above for 28 h, starved for 12 h and then treated with IL-6 or TNF-α for 8 h. Cell extracts were prepared and the luciferase activity was measured using a dual luciferase reporter assay system according to manufacturer's instructions. Each experiment was independently repeated for three times.

2.13. Induction of collagen induced arthritis (CIA) mice

To induce collagen-induced arthritis (CIA), miR-34a-TG (C57B7 mice) or DBA 1/J mice were intradermally immunized with 0.1 ml of bovine type II collagen (200 mg) emulsified in Freund's complete adjuvant (0.2 ml) on day 0. On day 21, type II bovine collagen/PBS (200 mg in 0.2 ml) was delivered by intravenous injection. AgomiR-34a or antagomiR-34a, along with the corresponding negative control, was injected i.v. into DBA 1/J mice on day 28, 31, 34, and 40. Mice were monitored for the progression of arthritis by micro-caliper measurements of paw swelling. In addition, a clinical severity score was used as described [17] in which 0 = no reaction; 1 = mild but definite redness and swelling of the ankle/wrist/digits; 2 = moderate-to-severe redness and swelling of the ankle/wrist; 3 = redness and swelling of the entire paw including digits; and 4 = maximally inflamed limb with involvement of multiple joints. Mice were sacrificed with CO_2 anesthesia on day 42 and cells from spleens and lymph nodes and other tissues were isolated. Two researchers measured paw swelling (one of them blind); and both evaluated histology sections blind to experimental condition. Spleen index = spleen weight divided by body weight × 10. Each experiment was independently repeated for three times.

2.14. Hematoxylin-eosin staining

Hematoxylin-eosin staining was performed as described [18]. In brief, mice were sacrificed with CO_2 anesthesia. Thereafter, whole knees and/or ankle joints were re-moved and fixed for 4 days in 10% formalin. After decalcification in 5% formic acid, the specimens were processed for paraffin embedding. Tissue sections (4 μm) were stained with hematoxylin and eosin. Each experiment was independently repeated for three times.

2.15. Flow cytometry analysis

Cells were stimulated with phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (500 ng/ml) for 5 h in the presence of brefeldin A. Anti-CD3-FITC, anti-CD4-FITC, anti-CD4-APC or anti-CD8-PerCy5.5 staining was performed at 4 °C for 45 min. Intracellular staining was performed with the Fixation and Permeabilization kit according to the manufacturer's instructions. Anti-Foxp3-PE and anti-IL-17 A-PE or anti-IL-17 A-APC staining were performed at 4 °C for 30 min. Each experiment was independently repeated for three times.

2.16. Molybdenum target X-rays

Joint damage in the left posterior ankle was observed by molybdenum target X-ray machine (Giotto, Italy) at day 42 following primary immunization. The stage of arthritis was subsequently determined according to the American Rheumatism Association criteria for the classification of rheumatoid arthritis [17], as following: Normal (0), osteoporosis period (+), bone destruction period (++), bone serious destruction period (+++) and ankylosis period (++++). Each experiment was independently repeated for three times.

2.17. Statistical analysis

Statistical analysis was performed with GraphPad Prism5 software. Unless indicated otherwise, data were expressed as mean ± standard deviation (SD). Statistical comparisons of the results between groups were performed using the Student's *t*-test, one-way analysis of variance and two-way analysis of variance. $p < 0.05$ was considered statistically significant.

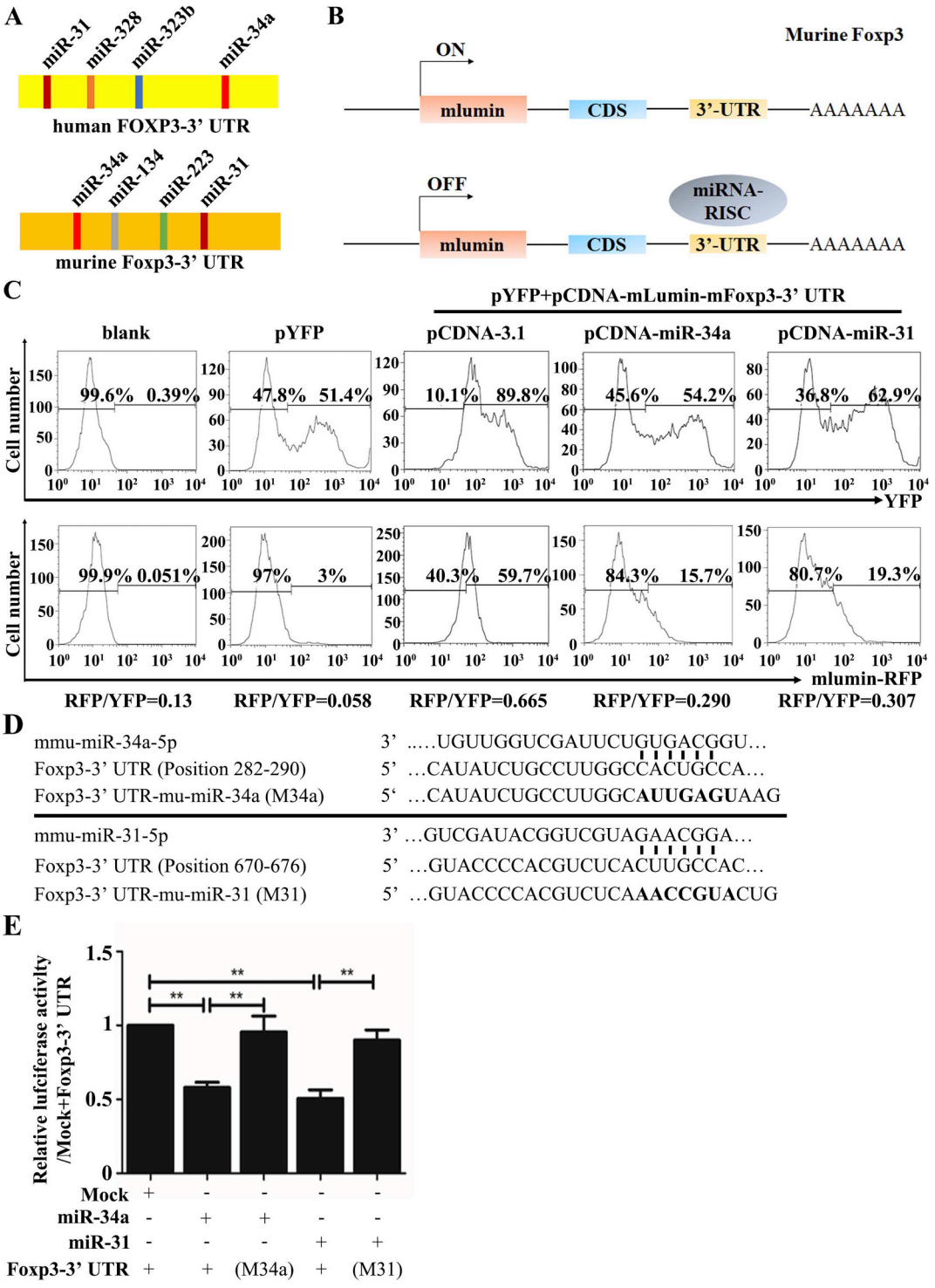


Fig. 1. MiR-34a is a negative regulator of Foxp3 by targeting its 3′ UTR. A, Prediction of different miRNAs that target human and murine Foxp3 3′ UTR by Targetscan, miRanda and Microinspector. **B,** A schematic diagram shows the modulation of miRNAs on mlumin-Foxp3-3′ UTR activity. **C,** HEK-293 T cells were co-transfected with control (Mock) or pCDNA-pre-miRNAs, pCDNA-mLumin-Foxp3-3′ UTR and pYFP (internal control) for 24 h, the Foxp3 3′ UTR activity was re-presented as the percentage of RFP^+ cells/YFP^+ cells according to the fluorescence intensity by flow cytometry analysis. **D,** A schematic diagram displays the Foxp3-3′ UTR regions targeting the seed sequences miR-34a or miR-31 and corresponding mutant sequences (M34a/M31). **E,** HEK-293 T cells were co-transfected with the psiCHECK-2-Foxp3-3′ UTR luciferase reporter vector (Foxp3-3′ UTR) or its corresponding mutant forms (M34a/M31) and control (Mock) or pCDNA-pre-miRNAs, and the luciferase activity was measured after 24 h according to the protocol described in materials and methods. **p < 0.01 vs Mock + Foxp3-3′ UTR, miR-34a + Foxp3-3′ UTR or miR-31 + Foxp3-3′ UTR, respectively. The luciferase activity of Foxp3-3′ UTR in Mock + Foxp3-3′ UTR was set as 1. Data are presented as means ± SD from three independent experiments.

3. Results

3.1. MiR-34a is a negative regulator of Foxp3 by targeting its 3' UTR

To identify miRNAs that can directly repress both human and murine Foxp3 expression, we analyzed miRNA candidates which potentially might bind to 3′ UTR of human and murine Foxp3 by Targetscan (http://www.targetscan.org/vert_72/), miRanda (http://www.microrna.org/) and Microinspector (https://omictools.com/microinspector-tool). The predicted results showed that miR-31, a miRNA that was verified to target FOXP3-3′ UTR [7], along with miR-328, miR-323b and miR-34a contained a seed region associated with human FOXP3-3′ UTR (Fig. 1A), miR-31, miR-34a, miR-134 and miR-223 were identified to target murine Foxp3-3′ UTR (Fig. 1A), suggesting that miR-34a and miR-31 are conserved miRNAs that may regulate Foxp3 expression. To confirm the predicted results, murine Foxp3-3′ UTR was inserted into a pCDNA- or psiCHECK-2-based plasmid fused with mLumin fluorescent protein [19] as an indicator, and the activity of Foxp3-3′ UTR was positively correlated with fluorescence intensity of mLumin transfected-cells (Fig. 1B). MiR-34a, as well as the positive control miR-31, significantly attenuated mLumin fluorescence intensity (Fig. 1C). We further generated wild-type and mutant Foxp3-3′ UTR vectors according to the sequences complementary to seed regions of miR-34a and miR-31 (Fig. 1D) and measured their luciferase activities. We found that miR-34a, as well as miR-31, significantly decreased the activity of wild-type Foxp3-3′ UTR, whereas neither of them had repressive effect on mutant Foxp3-3′ UTR, as shown in Fig. 1E. These findings indicated that miR-34a is a novel and conserved inhibitor of Foxp3 by paring with its 3' UTR.

3.2. MiR-34a attenuates Foxp3 expression at the transcriptional level

In order to explore miR-34a roles in the regulation of Foxp3 expression, miR-34a and Foxp3-3' UTR plasmids were co-transfected into HEK-293 T cells, qRT-PCR and immunoblot analysis showed that miR-34a and miR-31 had a negative effect on both the mRNA and protein levels of Foxp3 (Fig. 2A and B and supplementary Fig. 2A and 2B). Next, to know whether miR-34a influenced Treg cell differentiation, we transfected agomiR-34a or antagomiR-34a into $CD4^+$ T cells and the treated $CD4^+$ T cells were induced to express Foxp3 under the Treg-skewing condition. As shown in Fig. 2C and E, overexpression of miR-34a in $CD4^+$ T cells strongly inhibited Foxp3 expression, while down-regulation of miR-34a promoted Foxp3 expression (Fig. 2D and F). The T lymphoma cell line EL4 has been reported to express Foxp3 upon anti-CD3/CD28 activation and TGF-β stimulation [20]. We found that TGF-β induced Foxp3 expression in EL4 cells in a time-dependent manner in the presence of IL-2, and Foxp3 expression reached a peak at 12 h (supplementary Fig. 1). Then, we used this cell line to examine the process of Foxp3 induction regulated by miR-34a. Consistent with the data in Fig. 2C–F, enhanced miR-34a expression in EL4 cells lowered Foxp3 expression in both naïve and inducing condition (supplementary Fig. 2C and 2E), while reduced miR-34a significantly elevated Foxp3 protein levels (supplementary Fig. 2D and 2F). Taken together, these results indicated that miR-34a could suppress Treg cell generation *in vitro*.

3.3. MiR-34a plays a potential role in disease activity in RA and SLE patients

Given that miR-34a could limit Treg cell differentiation *via* repressing Foxp3 expression, we wanted to know whether miR-34a played important roles in the pathogenesis of Treg cell-involved diseases, such as RA, SLE. We first determined the levels of miR-34a, as well as other signatures of Treg (FOXP3) or Th17 (RORγt and IRF4) cells [21], in isolated peripheral blood mononuclear cells (PBMCs) of RA and SLE patients. As displayed in Fig. 3, the transcription levels of miR-34a, together with those of Th17 lineage-specific transcription factor RORγt and IRF4 that help Th17 cell differentiation, substantially increased (Fig. 3A, C and D), and FOXP3 expression levels were remarkedly decreased in RA or SLE patients compared to the healthy donors (Fig. 3B). Besides, the percentages of Treg cells were significantly lower, but the ratios of Th17 cells were significantly higher in RA and SLE patients than those of in healthy donors (supplementary Fig. 3). To test whether miR-34a could be a high-risk factor of RA or SLE, we analyzed the correlation of miR-34a with some common serum markers of autoimmune diseases. Interestingly, miR-34a expression levels in PBMCs were positively associated with serum rheumatoid factor (RF), anti-streptolysin antibody (ASO), erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP) levels from RA and SLE patients (Fig. 3E–L), indicating a potential role of miR-34a as a biomarker of disease progress. Meanwhile, we also concerned whether the expression levels of miR-34a related to the mRNA expression levels of FOXP3 and RORγt in PBMCs of RA and SLE patients. The data displayed that the transcription levels of miR-34a were intensively negatively correlated with FOXP3 mRNA but positively correlated with RORγt mRNA levels in PBMCs of RA patients (supplementary Fig. 4A). Surprising, it was also demonstrated that there was a positive correlation between miR-34a and RORγt expression in PBMCs of SLE patients. Further, a merely slight correlation, with no statistical significance ($r = -0.2612$, $p = 0.1201$), was observed between miR-34a and FOXP3 levels (supplementary Fig. 4B). Moreover, we analyzed the transcription levels of the above genes in purified human $CD4^+$ T cells. As shown in supplementary Fig. 5, the results not only exhibited that miR-34a as well as the mRNA abundances of RORγt and IL-17 strongly increased, but also showed that the FOXP3 mRNA levels dramatically decreased in $CD4^+$ T cells of RA patients. Those also suggested a negative correlation between miR-34a and FOXP3 and a positive correlation between miR-34a and RORγt in the human $CD4^+$ T cell subset. It further implied a pathogenic role of miR-34a in RA.

3.4. MiR-34a overexpressed mice show increased susceptibility to arthritis induced by collagen

To further study the function of miR-34a in the regulation of Foxp3 expression, we used miR-34a transgenic (miR-34a-TG) mice as experimental animals to induce the CIA animal model. We first confirmed high transcription levels of miR-34a in miR-34a-TG mice (supplementary Fig. 6A). Meanwhile, we found that miR-34a-TG mice expressed high levels of inflammatory cytokines and Teff-related transcription factors (supplementary Fig. 6B). To investigate the impact of miR-34a on immunologic disarrangement *in vivo*, we took advantage of these mice to evaluate the pathogenic role of miR-34a in the development of RA by inducing collagen-induced arthritis (CIA) model. First, we focused on the bodyweight change of wild-type or miR-34a-TG mice upon collagen immunization. The bodyweight of both wild-type and miR-34a-TG mice steadily increased under the naïve condition (Fig. 4A). However, when these mice were intradermally immunized with bovine type II collagen (CII) to induce arthritis, an obvious weight reduction was observed in the later phase of both wild-type and miR-34a-TG mice (Fig. 4A), indicating a link between RA and metabolic disorder. At the meantime, there was no significant difference of bodyweight between wild-type and miR-34a-TG mice in CIA-inducing group, though the latter group displayed more weight loss than the former one did (Fig. 4A). Next, we compared several physical parameters of the mice from different groups. As displayed in Fig. 4B, mice under CIA-inducing condition developed more severe symptoms, evidenced by swollen hind ankles. More importantly, molybdenum target X-rays showed that both strains exhibited signs of osteoporosis, bone and articular cartilage destruction and narrowed joint spaces on the 42nd day following primary immunization, but with more deteriorative phenotypes in miR-34a-TG mice (Fig. 4C). In accord with the result of molybdenum target X-rays, histopathological data also confirmed that the WT mice with

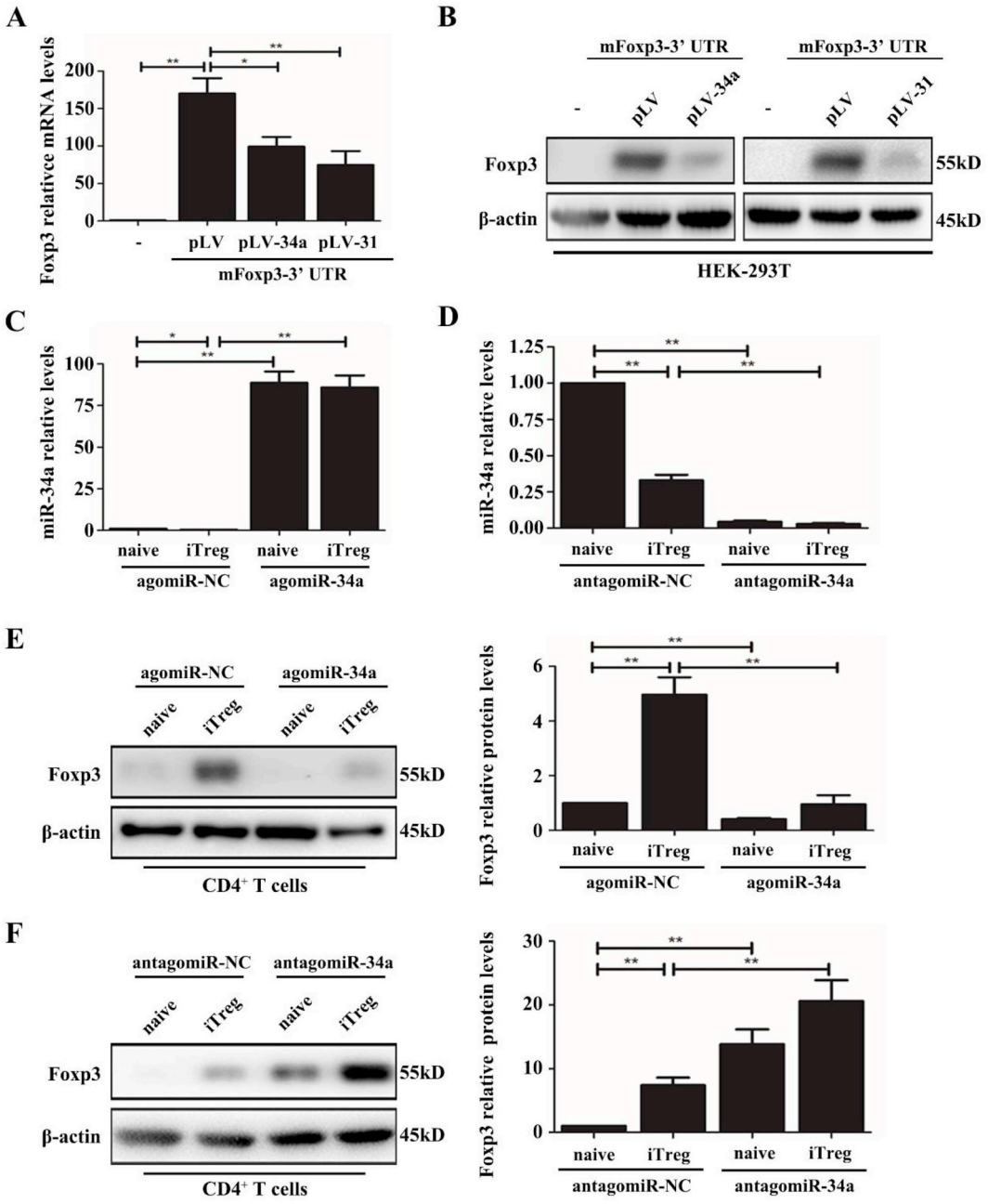


Fig. 2. MiR-34a attenuates Foxp3 expression at the transcriptional level in HEK-293T and CD4$^+$ T cells. A-B, HEK-293 T cells were co-transfected with pLV, pLV-34a or pLV-31 and pCDNA3.1-mFoxp3-3′ UTR for 48 h, the Foxp3 mRNA **(A)** and protein **(B)** levels were analyzed by qRT-PCR and immunoblot respectively. *p $<$ 0.05 vs pLV + Foxp3-3′ UTR, **p $<$ 0.01 vs blank, pLV + Foxp3-3′ UTR. **C-F:** CD4$^+$ T cells pre-transfected with 100 nM agomiR-NC or agomiR-34a, 200 nM antagomiR-NC or antagomiR-34a respectively for 24 h were polarized towards iTreg-skewing condition for an additional 12 h according to the protocol described in materials and methods. MiR-34a transcription levels **(C, D)** were analyzed by qRT-PCR. Foxp3 protein levels **(E, F)** were analyzed by immunoblot. *p $<$ 0.05 vs agomiR-NC, **p $<$ 0.01 vs naïve condition (agomiR-NC), iTreg-skewing condition (agomiR-NC), naïve condition (antagomiR-NC) or iTreg-skewing condition (antagomiR-NC). The expression of miR-34a or mRNA expression ratio of Foxp3 versus U6 snRNA or β-actin and the protein expression ratio of Foxp3 versus β-actin in indicated control groups were set as 1, respectively. Data are presented as means ± SD from three independent experiments.

CIA symptoms were accompanied with massive pannus, destruction of cartilages, narrowed joint spaces, and infiltration of inflammatory cells, which was further augmented in miR-34a-TG mice (Fig. 4D). Interestingly, miR-34a overexpression promoted splenomegaly as well as lymph node enlargement in both naïve- and CIA-inducing context compared to wild-type mice (Fig. 4E-G). Moreover, we assessed the severity of CIA by measuring arthritis pathology score, the result showed that miR-34a-TG mice had more severe phenotypes of arthritis (Fig. 4H). Finally, we detected the effects of miR-34a overexpression on the relative abundance of Treg cells. Flow cytometric assay exhibited that miR-34a overexpression in naïve C57BL/6 mice apparently repressed Foxp3 expression in CD4$^+$ T cells, with the similar degree as wild-type CIA mice did, which was more apparent in miR-34a-TG mice inoculated with collagen (Fig. 4I). Given that the suppressive function of Treg cells was impaired by miR-34a, we reasoned that miR-34a might also alter Th17 cell generation. As we anticipated, miR-34a facilitated CD4$^+$ T cells to produce more IL-17 in naïve condition and to a less extent than CIA condition (Fig. 4J). We also induced CIA using DBA 1/J mice and treated them with the miR-34a agonist (agomiR-34a) or antagonist (antagomiR-34a) to overexpress or silence miR-34a gene *in*

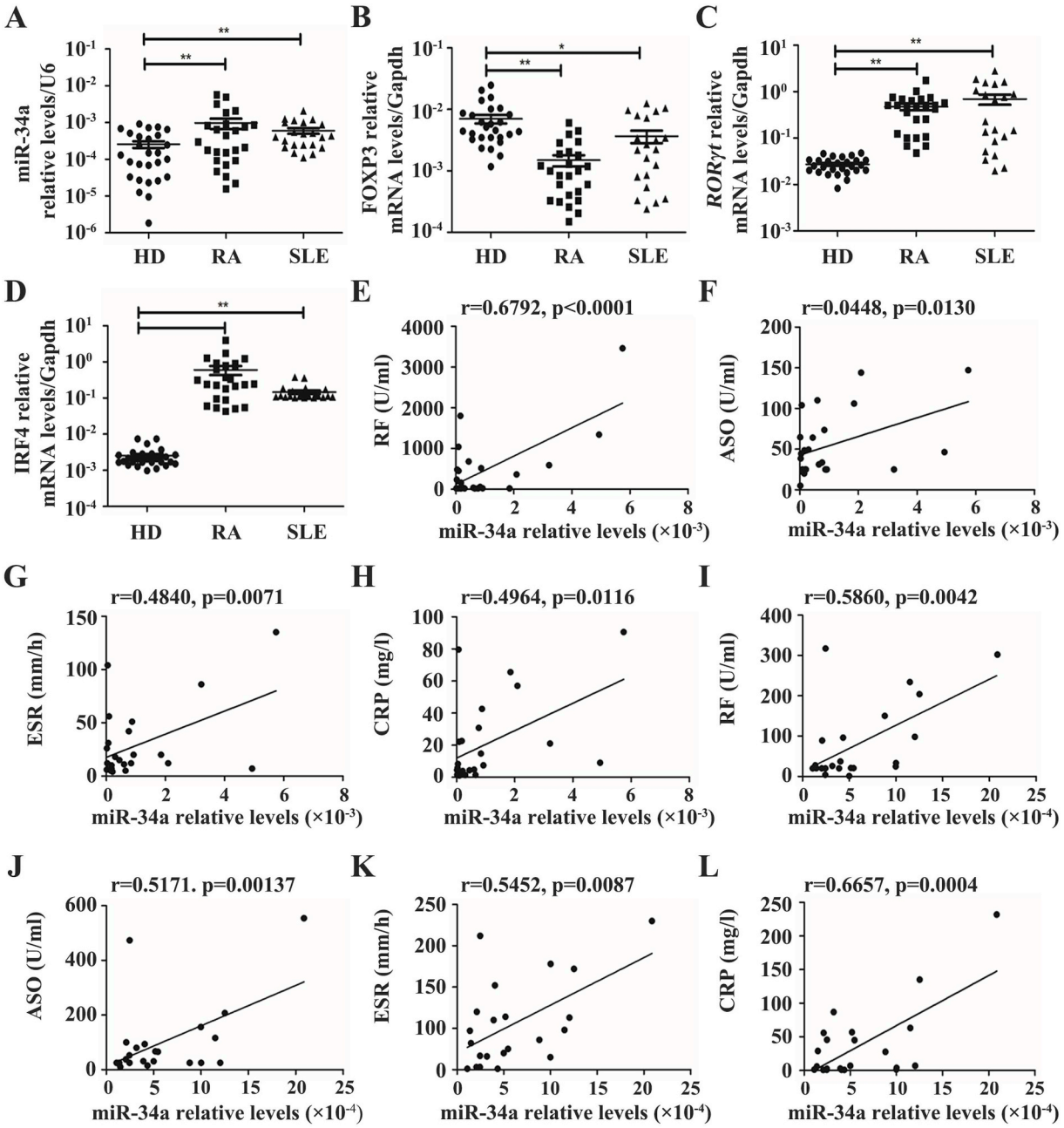


Fig. 3. MiR-34a plays a potential role in disease activity in RA and SLE patients. A-D, PBMCs were isolated from RA patients (RA, n = 25), SLE patients (n = 22) or healthy donors (HD, n = 27). Expression levels of miR-34a **(A)**, *FOXP3* **(B)**, *RORγt* **(C)** and *IRF4* **(D)** were analyzed by qRT-PCR. *p < 0.05 or **p < 0.01 vs HD. The expression levels of miR-34a and the mRNA expression ratio of the transcription factors or the inflammatory cytokines versus U6 snRNA or Gapdh were calculated using the standard curve. **E-L,** Correlation of miR-34a levels in PBMCs from RA and SLE patients with serum RF **(E, I)**, ASO **(F, J)**, ESR **(G, K)** and CRP **(H, L)** was analyzed.

vivo (supplementary Fig. 7A and 7B). Similar to the miR-34a TG-mice, CIA mice had more severe bodyweight loss compared with naïve mice, which was slightly aggravated by agomiR-34a injection (supplementary Fig. 7C). Conversely, the bodyweight loss of mice under CIA-inducing condition was notably ameliorated when antagomiR-34a was injected (supplementary Fig. 7C). We further detected some physical signs of DBA1/J mice with arthritic phenotypes. The results shown in supplementary Fig. 7D-7F demonstrated that collagen-induced damage in CIA mice was further enhanced in agomiR-34a-treated group but attenuated in antagomiR-34a-treated group. As we expected, splenomegaly as well as lymph node enlargement was also observed in CIA and agomiR-34a-injected CIA mice, which was rescued in antagomiR-34a-injected mice (supplementary Fig. 7G and 7H). The result of arthritis pathology score in supplementary Fig. 7I showed that CIA mice treated with agomiR-34a had more severe phenotypes of arthritis, which was dramatically reversed by antagomiR-34a treatment compared to the negative control group. In summary, these above data together implied that miR-34a in CIA mice represses Foxp3 expression as well as Treg cell number, causing the limitation of the suppressive function and the expanded subpopulation of Th17 cell generation, as well as the exacerbation of RA.

3.5. MiR-34a is increased in inflammatory T cells by IL-6 and TNF-α, however decreased in Foxp3^{high} T cells by TGF-β treatment

It is well-established that Treg/Th17 disorder plays an important

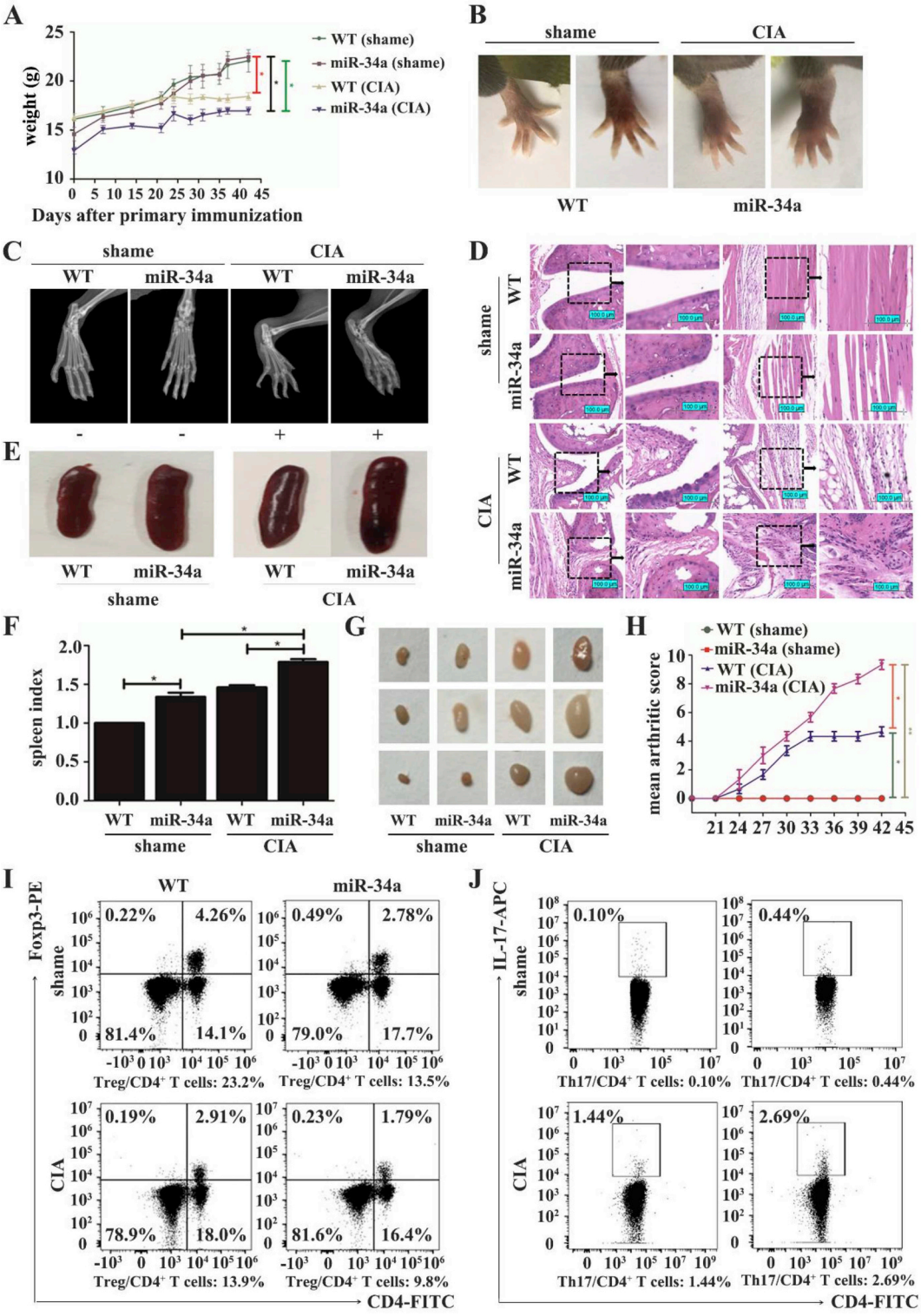


Fig. 4. MiR-34a overexpressed mice show increased susceptibility to arthritis induced by collagen. A-D, WT (n = 5) and miR-34a transgenic (miR-34a-TG; n = 5) were challenged with bovine type II collagen (CII) to induce arthritis (CIA). **A,** WT and miR-34a-TG mice showed comparable weights under both naïve and CIA condition. *p < 0.05 vs WT (shame) or miR-34a-TG (shame). **B,** Swellings of paws and joints were more apparent in miR-34a TG mice compared to WT mice under CIA condition. **C,** Local pathological alterations of ankle joints from WT and miR-34a-TG mice under naïve- or CIA-induction condition were detected by molybdenum target X-rays. **D,** Pathological sections of joints from WT and miR-34a-TG mice were detected by H&E staining. **E-G,** MiR-34a-TG mice showed increased susceptibility to splenomegaly **(E, F)** and lymphadenectasis **(G)** under both naïve and CIA condition. Spleen index was calculated in WT or miR-34a-TG mice under both naive- or CIA-inducing condition. The spleen index of WT mice at the shame state was set as 1. *p < 0.05 vs WT (shame), miR-34a-TG (shame) or WT (CIA). **H,** Average arthritic scores of WT and miR-34a-TG mice under both naïve and CIA condition were evaluated. *p < 0.05 vs WT (shame), miR-34a (shame) or WT (CIA), **p < 0.01 vs WT (shame) or miR-34a-TG (shame). **I-J,** Splenic cells treated with PMA, ionomycin and Brefeldin A were stained with anti-CD4-FITC and anti-Foxp3-PE or anti-IL-17-APC, the percentages of Treg **(I)** and Th17 cells **(J)** in $CD4^+$ T cells were analyzed by flow cytometry. Data are presented as means ± SD from three independent experiments.

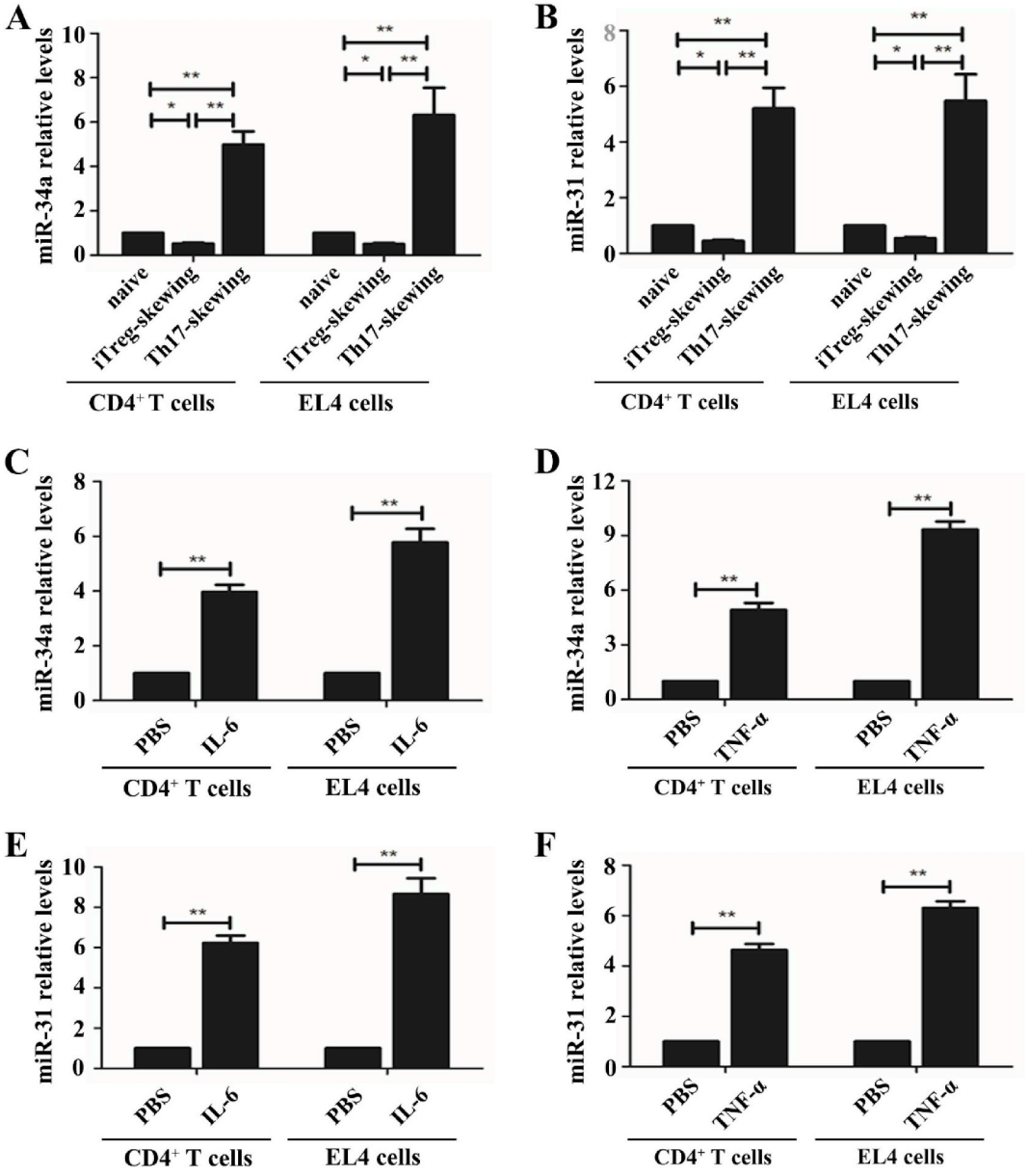


Fig. 5. MiR-34a is increased in inflammatory T cells by IL-6 and TNF-α, however decreased in Foxp3^{high} cells by TGF-β. A-B, $CD4^+$ T or EL4 cells activated by anti-CD3/CD28 for 12 h were polarized towards iTreg- or Th17-skewing condition for an additional 12 h according to the protocol described in materials and methods. The miR-34a **(A)** and miR-31 **(B)** levels were analyzed by qRT-PCR. *p < 0.05 vs naïve condition, **p < 0.01 vs naïve condition or iTreg-skewing condition. **C-F,** $CD4^+$ T or EL4 cells activated by anti-CD3/CD28 for 12 h were stimulated with IL-6 (20 ng/mL) or TNF-α (5 ng/mL) for an additional 12 h. Expression levels of miR-34a **(C, D)** and miR-31 **(E, F)** were analyzed by qRT-PCR. **p < 0.01 vs PBS. The expression ratio of miR-34a or miR-31 versus U6 snRNA in indicated control group was set as 1. Data are presented as means ± SD from three independent experiments.

role in the occurrence of autoimmune diseases [22,23]. With the goal to evaluate whether miR-34a expression patterns were variant in Treg and Th17 cells, we tested the abundance of miR-34a in $CD4^+$ T cells or EL4 cells cultured with the addition of Treg- or Th17-related cytokines. MiR-31 was used as a positive control that had been reported to regulate Treg/Th17 ratio [7,8]. As shown in Fig. 5, miR-34a and miR-31 were significantly declined under the Treg-skewing condition, but dramatically risen in the presence of Th17-skewing condition (Fig. 5A and B). Thus, these observations indicated that the expression of miR-34a was oppositely regulated under differentiated skewing conditions of Treg and Th17 cells. Although the pathogenic role of miR-34a in Treg/Th17 homeostasis and the onset of RA was primarily identified, the mechanisms underlying miR-34a enhancement in Th17 cells remain unknown. To determine whether cytokines that are critical for Th17 cell differentiation could initiate miR-34a transcription, we selected IL-6 and TNF-α as candidates due to their indispensable roles in occurrence of autoimmune diseases [24,25], Th17 cell induction [26–28], as well as in repressing Treg cell polarization [26,29]. Strikingly, both IL-6 and TNF-α robustly upregulated miR-34a expression (Fig. 5C and D), to the similar degree with miR-31 (Fig. 5E and F), in both primary $CD4^+$ T cells and EL4 cells.

3.6. IL-6 and TNF-α are responsible for inhibition of Foxp3 expression through enhancing miR-34a levels

To further determine the impact of IL-6 or TNF-α on Foxp3 abundance, $CD4^+$ T cells or EL4 cells activated by plate-coated anti-CD3/CD28 were cultured with IL-2 and TGF-β to induce Foxp3 expression. As shown in Fig. 6A and B as well as in supplementary Fig. 8A and 8B, TGF-β-augmented Foxp3 levels in $CD4^+$ T or EL4 cells were robustly

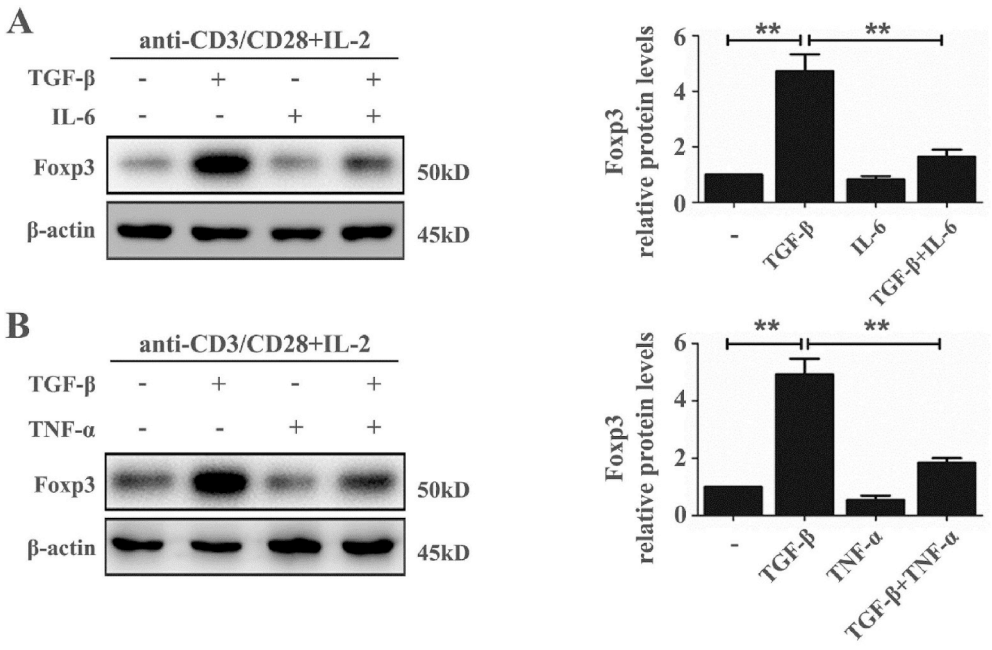
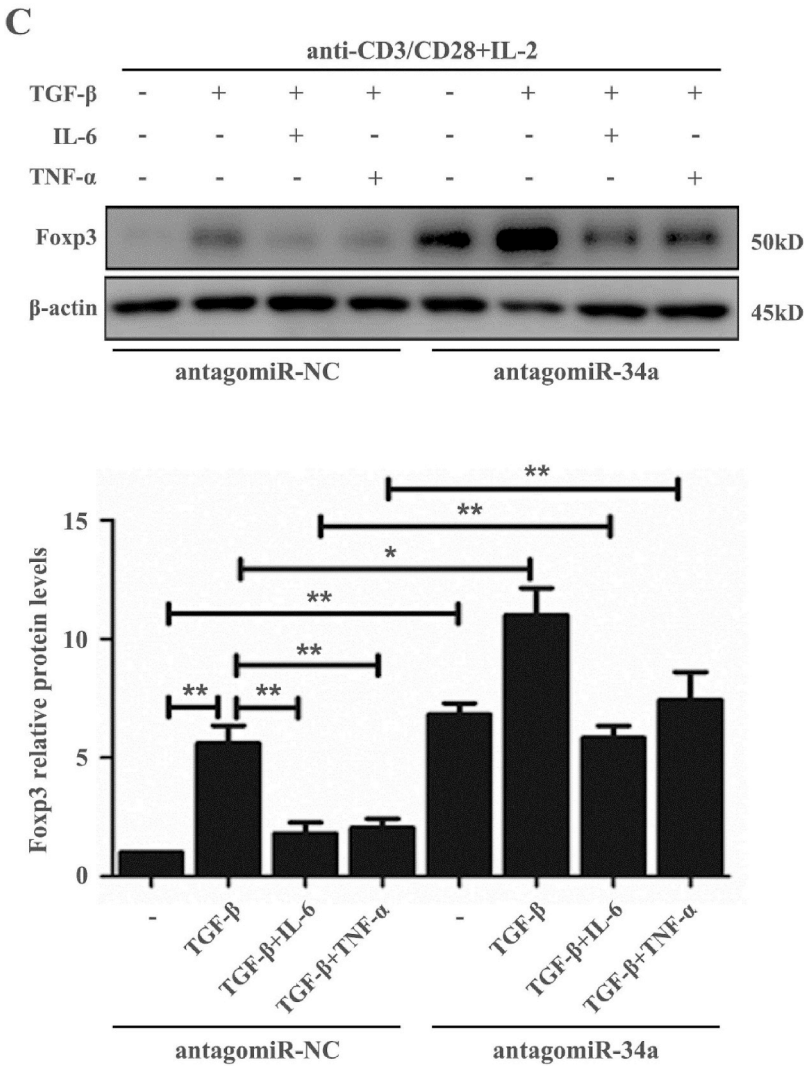


Fig. 6. IL-6 and TNF-α are responsible for inhibition of Foxp3 expression through enhancing miR-34a levels in CD4$^+$ T cells. A-B, CD4$^+$ T cells activated by anti-CD3/CD28 for 12 h were polarized towards iTreg-skewing condition for an additional 12 h in the presence or absence of IL-6 **(A)** or TNF-α **(B)**, the Foxp3 expression levels were determined by immunoblot. **p < 0.01 vs the negative control (-) or TGF-β treated group (TGF-β). **C,** CD4$^+$ T pre-transfected with 200 nM antagomiR-NC or antagomiR-34a for 24 h were treated with indicated cytokines for another 12 h, the Foxp3 expression levels were determined by immunoblot. *p < 0.05 vs TGF-β (antagomiR-NC), **p < 0.01 vs - (antagomiR-NC), TGF-β (antagomiR-NC), TGF-β + IL-6 (antagomiR-NC) or TGF-β + TNF-α (antagomiR-NC). The protein expression ratio of Foxp3 versus β-actin in indicated control groups was set as 1. Data are presented as means ± SD from three independent experiments.



reverted in the presence of IL-6 or TNF-α, confirming that IL-6 or TNF-α may be an inhibitory factor of Foxp3 as reported by other laboratories [28,30]. We next determined whether IL-6 or TNF-α inhibited Foxp3 protein levels in an miR-34a-dependent manner. Purified $CD4^+$ T cells or EL4 cells pre-transfected with a negative control (antagomiR-NC) or miR-34a antagonist (antagomiR-34a) were then co-cultured with IL-2 and TGF-β to generate Foxp3 in the presence or absence of IL-6 or TNF-α. The results showed that IL-6 and TNF-α significantly limited Foxp3 translation, while inhibition of miR-34a expression partially rescued the inhibitory effect observed above (Fig. 6C and supplementary Fig. 8C). Taken together, these data suggested that IL-6 or TNF-α is the inducer of miR-34a enhancement and mediates Foxp3 downregulation.

3.7. NF-κB p65 is involved in Foxp3 downregulation via regulating miR-34a expression

Plenty of previous studies reported that IL-6 and TNF-α triggers NF-κB signaling to initiate inflammatory responses [31,32]. Meanwhile, NF-κB subunit p65 could upregulate human miR-34a levels as a direct transcription factor [33,34]. Therefore, we first detected NF-κB/p65 activity in $CD4^+$ T cells or EL4 cells treated with IL-6 or TNF-α in the presence of TGF-β. As shown in Fig. 7A and supplementary Figure 9A, the addition of TGF-β had no influence on p65 phosphorylation at serine 536, whereas TGF-β, together with IL-6 or TNF-α, clearly enhanced p65 phosphorylation levels. These data strongly suggested that NF-κB p65 subunit was highly activated in $CD4^+$ T or EL4 cells upon IL-6 or TNF-α treatment. More importantly, pretreatment of $CD4^+$ T cells or EL4 cells with BAY 11-7082, an IκBα inhibitor, notably ameliorated the repressive effect on miR-34a together with miR-31 transcription levels mediated by IL-6 or TNF-α (Fig. 7B–E and supplementary Fig. 9B-9E), indicating that the transcription factor p65 was indeed involved in regulating the expression of miR-34a and miR-31. We then determined the consequence of NF-κB inhibition on Foxp3 protein levels, the results of immunoblot analysis in Fig. 7F and supplementary Figure 9F showed that IL-6 or TNF-α promoted p65 phosphorylation and suppressed TGF-β-induced Foxp3 elevation as previously displayed, and this suppression was partially inverted by BAY 11-7082. All these data strongly indicated that the p65 subunit is the core effector of IL-6 and TNF-α to attenuate the expression of Foxp3 and inhibition of p65 activity can significantly induce TGF-β-mediated Foxp3 expression. To ensure that miR-34a overexpression could partly rescue the suppressive effect on Foxp3 expression in the presence of Bay 11-7082, we pre-transfected miR-34a and demonstrated that cells transfected with miR-34a limited Foxp3 elevation induced by BAY 11-7082 (Fig. 7G and supplementary Figure 9G). Consequently, our results demonstrated that p65 represses TGF-β-induced Foxp3 in an miR-34a-dependent manner.

3.8. NF-κB/p65 interacts with miR-34a promoter to induce its transcription

In order to identify the response elements of p65 on miR-34a promoter, we generated a FLAG-tagged vector encoding murine p65 gene (pCDNA-p65) and introduced it into HEK-293 T cells with another vector of murine miR-34a promoter that had been predicted to associate with p65. Luciferase reporter assay displayed that p65 robustly enhanced the activity of miR-34a promoter by almost 5 folds (Fig. 8A). Furthermore, we confirmed miR-34a promoter activity using IL-6 or TNF-α in the presence or absence of BAY 11-7082. As we expected, IL-6 as well as TNF-α strongly increased the promoter activity, while inhibition of p65 activity notably reversed it (Fig. 8B). Finally, we designed various promoter deletion mutants of miR-34a to uncover the binding site(s) for p65 on miR-34a promoter (Fig. 8C). The M3 truncated promoter fragment (−1289 to +200) co-transfected with p65 obviously displayed reduced promoter activity compared to the FL, M1 or M2 group (Fig. 8D), implying the binding element sites might be located within −2275 to −1289 nt. Surprisingly, we observed lowered activity of miR-34a promoter when co-transfecting the M4 fragment (+74 to +200 nt) compared to the M3 fragment (−1289 to +200 nt) with pCDNA-p65 plasmid into HEK-293 T cells (Fig. 8D), hinting that the main binding site was indeed located in −1289 to +74 nt. Taken together, we concluded that NF-κB signaling triggered by IL-6 or TNF-α mediates miR-34a elevation through interacting with the region in −2275 to +74 nt of the miR-34a promoter, causing a sharp decrease in Foxp3 protein levels.

4. Discussion

The lineage of Treg cells is a unique subpopulation of $CD4^+$ T cells that is critical for the maintenance of internal homeostasis *via* directly contacting with effector immune cells by regulating the functions of molecules including CTLA-4 (CD152) and lymphocyte-activation gene 3 (LAG3) [35] or releasing soluble cytokines i.e., IL-10, IL-35 and TGF-β, etc. [36–38]. In contrast to Treg cells, Th17 cells mount responses to extracellular bacterial and fungal infections in the intestine and the airways [39]. Meanwhile, they were also implicated in autoimmune and chronic inflammatory diseases [40]. Although imbalance of Treg/Th17 ratio has been reported to be a common feature of autoimmunity [22,41] and several cytokines responsible for Treg/Th17 imbalance have been identified [42,43], the underlying mechanisms mediated by miRNAs remain not fully understood. Here, we identified that miR-34a was a conserved repressor of Foxp3 and involved in CIA pathogenesis through impairing the Treg/Th17 balance. More importantly, we found that IL-6 or TNF-α released from inflammatory environment could activate NF-κB pathway, enhancing miR-34a promoter activity and increasing the expression levels of miR-34a, leading to Foxp3 downregulation.

In this study, we first predicted miRNAs responsible for targeting 3′ UTR of Foxp3. The results appeared that only miR-34a and miR-31 might be capable of binding to both human and murine Foxp3-3′ UTR. Next, we confirmed the results by flow cytometry and luciferase reporter assay. Our data demonstrated that miR-34a along with miR-31, robustly decreased the activity of Foxp3-3′ UTR. Similarly, recent published work has also reported that miR-34a functions as a repressor of human FOXP3 in cervical cancer and gastric cancer [44,45]. We then transfected miR-34a overexpression plasmids, agonist or antagonist into HEK-293 T cells, primary $CD4^+$ T cells or EL4 cells to determine whether there was indeed an inhibitory effect of miR-34a on Foxp3 expression. The results reflected that miR-34a altered Foxp3 expression at the transcription level, indicating its role in regulating the mRNA stability of Foxp3. Accumulating evidence suggested that there exists a potential link between miR-34a and Treg cell differentiation and function. TGF-β and its downstream signal have been widely recognized as key inducers of Treg cells [20,46–49]. Expanded reports indicated that miR-34a could target Smad4 for its mRNA degradation, leading to the termination of TGF-β signal [50,51], our investigation first reported that miR-34a could directly interact with Foxp3-3' UTR in primary $CD4^+$ T cells together with Treg-like EL4 cells.

Aberrant expression of miR-34a was verified to play an important role in the development of RA, SLE and multiple sclerosis (MS) [52–54]. To reveal the potential role of miR-34a in RA progress, we determined the levels of miR-34a as well as the signatures of Treg and Th17 cells in PBMCs or $CD4^+$ T cells isolated from RA and SLE patients. An elevated expression of miR-34a and Th17 cell signatures and a decrease in FOXP3 levels were observed in both PBMCs and $CD4^+$ T cells of RA or SLE patients. Meanwhile, we analyzed the correlation between the expression levels of miR-34a and some serum markers of autoimmune diseases as well as the signature genes of Treg and Th17 cells. Surprisingly, we found a positive link between the levels of miR-34a and these serum markers as well as the Th17 signature genes (RORγt, IL-17) and a negative link between miR-34a transcription levels and FOXP3 mRNA levels. More importantly, miR-34a was also increased in murine $CD4^+$ T cells or EL4 cells under the Th17-skewing condition whereas it was notably decreased under the Treg-skewing condition, which was in

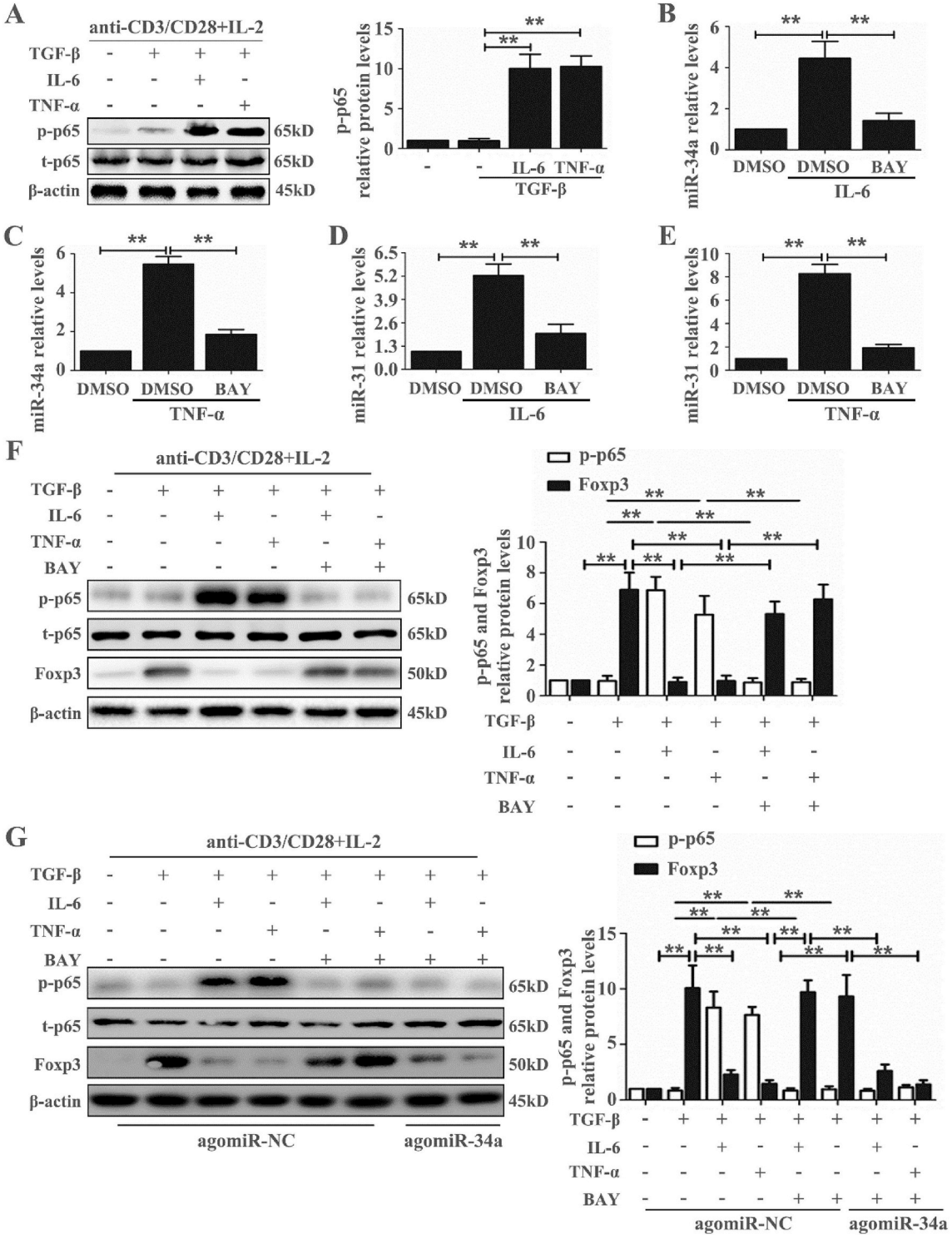


Fig. 7. NF-κB p65 is involved in Foxp3 downregulation *via* regulating miR-34a expression in CD4$^+$ T cells. A, CD4$^+$ T cells activated by anti-CD3/CD28 for 12 h were co-cultured with indicated cytokines for an additional 12 h, p65 phosphorylation (Ser 536) and total p65 (t-p65) were measured by immunoblot. **p < 0.01 vs the negative control (-) or TGF-β treated group (TGF-β). **B-E,** CD4$^+$ T cells pre-treated with BAY 11-7082 (5 μM) for 30min were activated by anti-CD3/CD28 in the presence or absence of IL-6 or TNF-α. The miR-34a **(B, C)** and miR-31 **(D, E)** expression levels were analyzed by qRT-PCR. **p < 0.01 vs DMSO, IL-6 + DMSO or TNF-α + DMSO. **F-G,** CD4$^+$ T cells pre-treated with BAY 11-7082 (5 μM) for 30min were activated by anti-CD3/CD28 in the presence or absence of indicated cytokines and agomiR-34a. Phosphorylated p65 and Foxp3 protein levels were analyzed by immunoblot. **p < 0.01 vs -, TGF-β, TGF-β + IL-6, or TGF-β + TNF-α in **F**. **p < 0.01 vs - (agomiR-NC), TGF-β (agomiR-NC), IL-6 + TGF-β (agomiR-NC), TGF-β + TNF-α (agomiR-NC), TGF-β + IL-6 + BAY 11-7082 (agomiR-NC) or TGF-β + TNF-α + BAY 11-7082 (agomiR-NC) in **G**. The protein expression or phosphorylation ratio of target proteins versus β-actin or t-p65 and the expression ratio of the miR-34a or miR-31 versus U6 snRNA were set as 1. Data are presented as means ± SD from three independent experiments.

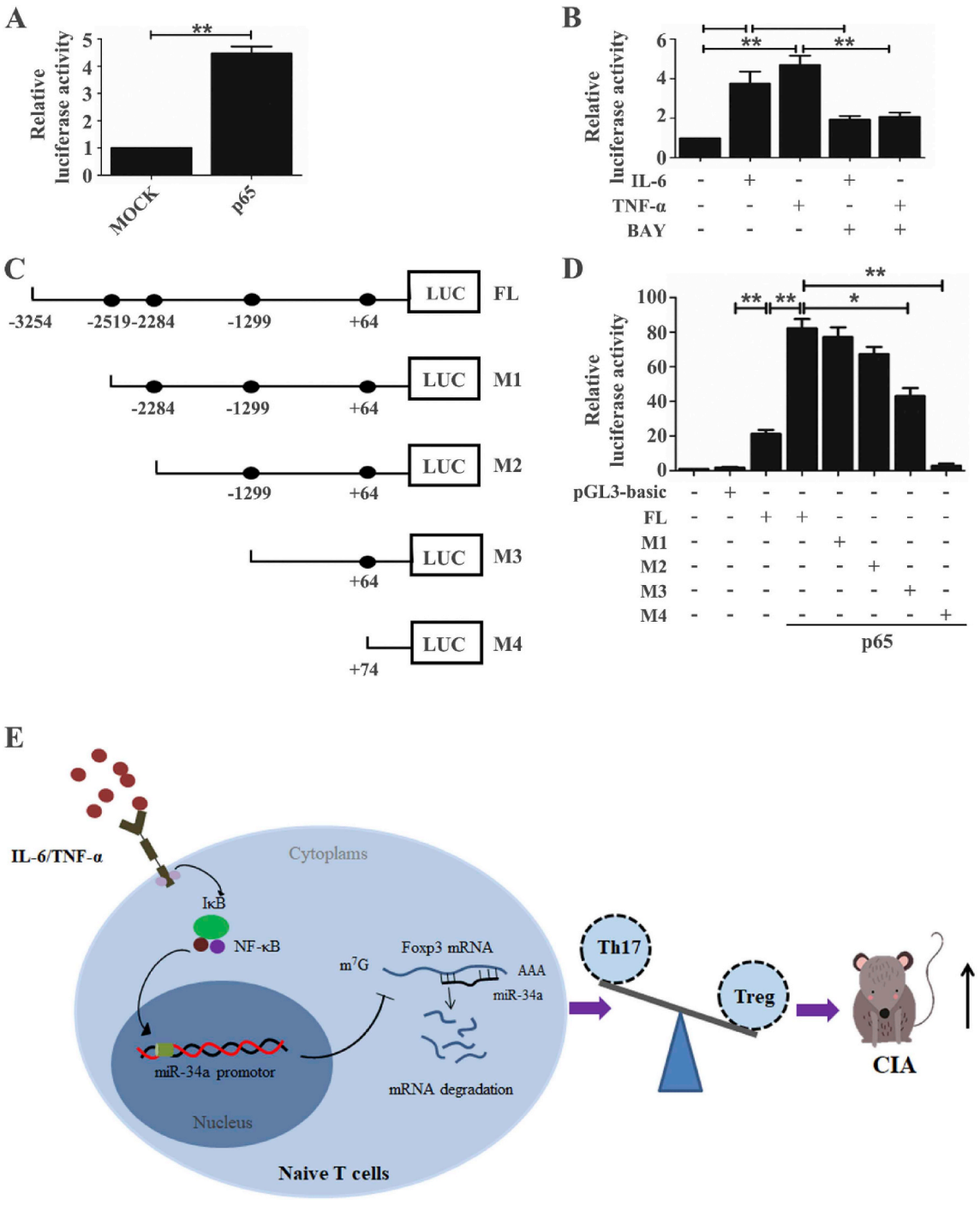


Fig. 8. NF-κB/p65 interacts with miR-34a promoter to induce its transcription. A and D, HEK-293 T cells were co-transfected with the pGL3-basic, WT and mutant miR-34a promoter vectors (FL/M1/M2/M3/M4) in the presence of pCDNA (Mock) or pCDNA-p65, and the luciferase activity was measured after 24 h. *p < 0.05 vs Mock + pGL3-basic, **p < 0.01 vs Mock, Mock + pGL3-basic or p65 + pGL3-miR-34a promoter FL. **B,** HEK-293 T cells pre-transfected with miR-34a promoter vector (pGL3-basic) were treated with IL-6 or TNF-α in the presence or absence of BAY 11-7082. The luciferase activity was measured. **p < 0.01 vs -, p < 0.01 vs IL-6 or TNF-α. The luciferase activities of the miR-34a promoter in indicated control groups were set as 1. Data are presented as means ± SD from three independent experiments. **C,** A schema graph exhibits the wildtype (WT) and mutant (M1/M2/M3/M4) miR-34a promoter. **E,** A schema graph displays NF-κB-driven miR-34a-Foxp3 axis in the process of CIA. IL-6 and TNF-α released from the inflammatory microenvironment mediate the repressive effect of miR-34a on Foxp3 levels in an NF-κB-dependent manner, resulting in Treg/Th17 imbalance in rheumatoid arthritis.

accord with the result of a miRNA array published by Dong's laboratory [55]. Recent studies about the involvement of miR-34a in RA process mainly included: a, MiR-34a targets the 3′UTR of the transcription factor Foxp1 [14], a negative regulator of follicular helper T (Tfh) cell differentiation [56], leading to Tfh cell differentiation and RA development [57,58]. **b,** MiR-34a targets tyrosine kinase receptor AXL in dendritic cells (DCs) to attenuate the inhibitory effect of AXL on DCs' function and facilitate the interaction of DCs and T cells, resulting in T cell activation, inflammatory cytokine synthesis and RA development [17]. **c,** MiR-34a is capable of targeting the class III histone deacetylase Sirtuin-1 (Sirt-1) [59], a well-defined anti-inflammatory agent against RA [60], which relieves the preventive and therapeutic function of Sirt-

1 on RA progress. **d,** MiR-34a could reverse T cell tolerance mediated by Sirt-1 and trigger T cell activation to aggravate RA [61]. In this study, we described that miR-34a overexpression or downregulation in CIA mice could strengthen or ameliorate joint lesions and leukocyte infiltration by altering Treg/Th17 balance. Further study should focus on the potential role of miR-34a in other autoimmune diseases.

Finally, we attempted to identify the upstream factors responsible for miR-34a elevation in T cells. Although the tumor suppressor p53 has been broadly proved as the transcription factor promoting miR-34a expression [62,63], it was mainly reported to exert an anti-inflammatory function in autoimmune diseases [64]. Thus, other transcription factor(s) that might aggravate inflammation through positively regulating miR-34a expression need (s) to be identified. IL-6 and TNF-α are two key agents to initiate inflammatory responses in different autoimmune diseases. As previously reported, neutralization against IL-6 or TNF-α on patients with autoimmune symptoms has achieved certain curative effects [65–69]. More importantly, IL-6 and TNF-α have been confirmed to increase miR-31 levels [31]. So, we chose both IL-6 and TNF-α as candidates to evaluate their influences on miR-34a as well as Foxp3 expression levels. As we expected, both IL-6 and TNF-α could significantly inhibit Foxp3 expression in an miR-34a-dependent manner. Therefore, we concluded that miR-34a might be a downstream effector of IL-6 or TNF-α to suppress Foxp3 translation. Our further investigation identified that NF-κB p65 subunit, a transcription factor, can initiate miR-34a transcription and mediate Foxp3 decrease induced by IL-6 or TNF-α. NF-κB p65 subunit was identified as a putative contributor for its indispensable position in autoimmune inflammation [70–72] and the impact on miR-34a expression [33,34]. Despite NF-κB activation was proved to be involved in Foxp3 defect *via* decreasing IL-2 expression [73,74], the mechanisms remain poorly investigated, especially when the concentration of IL-2 is abundant in T cells. Our data described that p65 phosphorylation at the Ser 536 site in $CD4^+$ T cells or EL4 cells was significantly increased upon IL-6 or TNF-α treatment. Furthermore, lowering IκBα activity robustly downregulated miR-34a, but significantly promoted Foxp3 expression, suggesting that p65 is involved in Foxp3 downregulation *via* modulating miR-34a levels. Our recue experiments demonstrated that miR-34a is orchestrated by p65 to regulate Foxp3 translation. Thus, we considered that besides miR-31, NF-κB could also be capable of penetrating miR-34a upregulation to negatively regulate Foxp3 expression. Finally, we took advantage of luciferase reporter assay and identified that the region of −2275 to +74 nt might contain the binding sites on the miR-34a promoter responsible for p65 binding.

In summary, we describe herein that under inflammatory environment, NF-κB p65 activated by IL-6 or TNF-α binds the promoter of miR-34a and enhances its expression. In RA patients or arthritis mouse model, sustained expression of miR-34a in $CD4^+$ T cells controls cellular Foxp3 expression that governs Treg cell polarization and generation, which exacerbates RA progress as a result of Treg/Th17 imbalance (Fig. 8E). Treatment based on IL-6/TNF-α-NF-κB-miR-34a-Foxp3 axis might be a novel combined strategy for therapeutic purpose of autoimmune diseases.

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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.2019.04.018.

References

[1] A.M. Bilate, J.J. Lafaille, Induced CD4+Foxp3+ regulatory T cells in immune tolerance, Annu. Rev. Immunol. 30 (2012) 733–758.
[2] C.A. Akdis, M. Akdis, Mechanisms of immune tolerance to allergens: role of IL-10 and Tregs, J. Clin. Investig. 124 (2014) 4678–4680.
[3] M. Miyara, Y. Ito, S. Sakaguchi, TREG-cell therapies for autoimmune rheumatic diseases, Nat. Rev. Rheumatol. 10 (2014) 543–551.
[4] M. Piedavent-Salomon, A. Willing, J.B. Engler, K. Steinbach, S. Bauer, B. Eggert, et al., Multiple sclerosis associated genetic variants of CD226 impair regulatory T cell function, Brain 138 (2015) 3263–3274.
[5] M. Ha, V.N. Kim, Regulation of microRNA biogenesis, Nat. Rev. Mol. Cell Biol. 15 (2014) 509–524.
[6] W. Gu, Y. Xu, X. Xie, T. Wang, J.H. Ko, T. Zhou, The role of RNA structure at 5' untranslated region in microRNA-mediated gene regulation, RNA 20 (2014) 1369–1375.
[7] R. Rouas, H. Fayyad-Kazan, N. El Zein, P. Lewalle, F. Rothe, A. Simion, et al., Human natural Treg microRNA signature: role of microRNA-31 and microRNA-21 in FOXP3 expression, Eur. J. Immunol. 39 (2009) 1608–1618.
[8] L. Zhang, F. Ke, Z. Liu, J. Bai, J. Liu, S. Yan, et al., MicroRNA-31 negatively regulates peripherally derived regulatory T-cell generation by repressing retinoic acid-inducible protein 3, Nat. Commun. 6 (2015) 7639.
[9] H.Y. Yang, J. Barbi, C.Y. Wu, Y. Zheng, P.D. Vignali, X. Wu, et al., MicroRNA-17 modulates regulatory T cell function by targeting Co-regulators of the Foxp3 transcription factor, Immunity 45 (2016) 83–93.
[10] S. Jiang, C. Li, V. Olive, E. Lykken, F. Feng, J. Sevilla, et al., Molecular dissection of the miR-17-92 cluster's critical dual roles in promoting Th1 responses and preventing inducible Treg differentiation, Blood 118 (2011) 5487–5497.
[11] D. Aletaha, T. Neogi, A.J. Silman, J. Funovits, D.T. Felson, C.O. Bingham 3rdet al., Rheumatoid arthritis classification criteria: an American College of Rheumatology/European League against Rheumatism collaborative initiative, Arthritis Rheum. 62 (2010) 2569–2581.
[12] M.C. Hochberg, Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus, Arthritis Rheum. 40 (1997) 1725.
[13] M.C. Fantini, S. Dominitzki, A. Rizzo, M.F. Neurath, C. Becker, In vitro generation of CD4+ CD25+ regulatory cells from murine naive T cells, Nat. Protoc. 2 (2007) 1789–1794.
[14] D.S. Rao, R.M. O'Connell, A.A. Chaudhuri, Y. Garcia-Flores, T.L. Geiger, D. Baltimore, MicroRNA-34a perturbs B lymphocyte development by repressing the forkhead box transcription factor Foxp1, Immunity 33 (2010) 48–59.
[15] X. Liu, Q. Zhao, X. Peng, S. Xia, W. Shen, Y. Zong, et al., PTD-mediated intracellular delivery of mutant NFAT minimum DNA binding domain inhibited the proliferation of T cells, Int. Immunopharmacol. 19 (2014) 110–118.
[16] X. Liu, B. Ji, M. Sun, W. Wu, L. Huang, A. Sun, et al., Cell-penetrable mouse forkhead box protein 3 alleviates experimental arthritis in mice by up-regulating regulatory T cells, Clin. Exp. Immunol. 181 (2015) 87–99.
[17] M. Kurowska-Stolarska, S. Alivernini, E.G. Melchor, A. Elmesmari, B. Tolusso, C. Tange, et al., MicroRNA-34a dependent regulation of AXL controls the activation of dendritic cells in inflammatory arthritis, Nat. Commun. 8 (2017) 15877.
[18] E. Lubberts, M.I. Koenders, B. Oppers-Walgreen, L. van den Bersselaar, C.J. Coenen-de Roo, L.A. Joosten, et al., Treatment with a neutralizing anti-murine interleukin-17 antibody after the onset of collagen-induced arthritis reduces joint inflammation, cartilage destruction, and bone erosion, Arthritis Rheum. 50 (2004) 650–659.
[19] J. Chu, Z. Zhang, Y. Zheng, J. Yang, L. Qin, J. Lu, et al., A novel far-red bimolecular fluorescence complementation system that allows for efficient visualization of protein interactions under physiological conditions, Biosens. Bioelectron. 25 (2009) 234–239.

[20] Y. Tone, K. Furuuchi, Y. Kojima, M.L. Tykocinski, M.I. Greene, M. Tone, Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer, Nat. Immunol. 9 (2008) 194–202.
[21] Y. Kishi, T. Kondo, S. Xiao, N. Yosef, J. Gaublomme, C. Wu, et al., Protein C receptor (PROCR) is a negative regulator of Th17 pathogenicity, J. Exp. Med. 213 (2016) 2489–2501.
[22] M. Noack, P. Miossec, Th17 and regulatory T cell balance in autoimmune and inflammatory diseases, Autoimmun. Rev. 13 (2014) 668–677.
[23] J. Barbi, D. Pardoll, F. Pan, Metabolic control of the Treg/Th17 axis, Immunol. Rev. 252 (2013) 52–77.
[24] C.A. Hunter, S.A. Jones, IL-6 as a keystone cytokine in health and disease, Nat. Immunol. 16 (2015) 448–457.
[25] F. Atzeni, M. Turiel, F. Capsoni, A. Doria, P. Meroni, P. Sarzi-Puttini, Autoimmunity and anti-TNF-alpha agents, Ann. N. Y. Acad. Sci. 1051 (2005) 559–569.
[26] T. Korn, M. Mitsdoerffer, A.L. Croxford, A. Awasthi, V.A. Dardalhon, G. Galileos, et al., IL-6 controls Th17 immunity in vivo by inhibiting the conversion of conventional T cells into Foxp3+ regulatory T cells, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 18460–18465.
[27] D.Y. Chen, Y.M. Chen, H.H. Chen, C.W. Hsieh, C.C. Lin, J.L. Lan, Increasing levels of circulating Th17 cells and interleukin-17 in rheumatoid arthritis patients with an inadequate response to anti-TNF-alpha therapy, Arthritis Res. Ther. 13 (2011) R126.
[28] S. Heink, N. Yogev, C. Garbers, M. Herwerth, L. Aly, C. Gasperi, et al., Trans-presentation of IL-6 by dendritic cells is required for the priming of pathogenic TH17 cells, Nat. Immunol. 18 (2017) 74–85.
[29] C. Kuhn, R.M. Rezende, H. M'Hamdi, A.P. da Cunha, H.L. Weiner, IL-6 inhibits upregulation of membrane-bound TGF-beta 1 on CD4+ T cells and blocking IL-6 enhances oral tolerance, J. Immunol. 198 (2017) 1202–1209.
[30] H. Nie, Y. Zheng, R. Li, T.B. Guo, D. He, L. Fang, et al., Phosphorylation of FOXP3 controls regulatory T cell function and is inhibited by TNF-alpha in rheumatoid arthritis, Nat. Med. 19 (2013) 322–328.
[31] S. Yan, Z. Xu, F. Lou, L. Zhang, F. Ke, J. Bai, et al., NF-kappaB-induced microRNA-31 promotes epidermal hyperplasia by repressing protein phosphatase 6 in psoriasis, Nat. Commun. 6 (2015) 7652.
[32] D.A. Altomare, C.W. Menges, J. Pei, L. Zhang, K.L. Skele-Stump, M. Carbone, et al., Activated TNF-alpha/NF-kappaB signaling via down-regulation of Fas-associated factor 1 in asbestos-induced mesotheliomas from Arf knockout mice, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 3420–3425.
[33] J. Li, K. Wang, X. Chen, H. Meng, M. Song, Y. Wang, et al., Transcriptional activation of microRNA-34a by NF-kappa B in human esophageal cancer cells, BMC Mol. Biol. 13 (2012) 4.
[34] V.K. Sharma, V. Raimondi, K. Ruggero, C.A. Pise-Masison, I. Cavallari, M. Silic-Benussi, et al., Expression of mir-34a in T-cells infected by human T-lymphotropic virus 1, Front. Microbiol. 9 (2018) 832.
[35] M. Miyara, S. Sakaguchi, Natural regulatory T cells: mechanisms of suppression, Trends Mol. Med. 13 (2007) 108–116.
[36] A. Spence, J.E. Klementowicz, J.A. Bluestone, Q. Tang, Targeting Treg signaling for the treatment of autoimmune diseases, Curr. Opin. Immunol. 37 (2015) 11–20.
[37] S. Huber, N. Gagliani, E. Esplugues, W. O'Connor Jr., F.J. Huber, A. Chaudhry, et al., Th17 cells express interleukin-10 receptor and are controlled by Foxp3(-) and Foxp3+ regulatory CD4+ T cells in an interleukin-10-dependent manner, Immunity 34 (2011) 554–565.
[38] S. Sakaguchi, T. Yamaguchi, T. Nomura, M. Ono, Regulatory T cells and immune tolerance, Cell 133 (2008) 775–787.
[39] N. Hernandez-Santos, S.L. Gaffen, Th17 cells in immunity to Candida albicans, Cell Host Microbe 11 (2012) 425–435.
[40] J. Zepp, L. Wu, X. Li, IL-17 receptor signaling and T helper 17-mediated autoimmune demyelinating disease, Trends Immunol. 32 (2011) 232–239.
[41] F. Pan, H. Fan, L. Lu, Z. Liu, S. Jiang, The yin and yang of signaling in Tregs and TH17 cells, Sci. Signal. 4 (2011) mr4.
[42] A. Kimura, T. Kishimoto, IL-6: regulator of Treg/Th17 balance, Eur. J. Immunol. 40 (2010) 1830–1835.
[43] R. Basu, S.K. Whitley, S. Bhaumik, C.L. Zindl, T.R. Schoeb, E.N. Benveniste, et al., IL-1 signaling modulates activation of STAT transcription factors to antagonize retinoic acid signaling and control the TH17 cell-iTreg cell balance, Nat. Immunol. 16 (2015) 286–295.
[44] J. Xi, J. Feng, S. Zeng, P. Huang, Long noncoding RNA UFC1 is activated by E2F1 and exerts oncogenic properties by functioning as a ceRNA of FOXP3, Cancer Med (2018) 3301–3310.
[45] J.P. Li, X.H. Liao, Y. Xiang, A. Yao, L.J. Fan, H. Li, et al., MKL1/miR34a/FOXP3 axis regulates cell proliferation in gastric cancer, J. Cell. Biochem. (2018) 7814–7824.
[46] L. Lu, J. Wang, F. Zhang, Y. Chai, D. Brand, X. Wang, et al., Role of SMAD and non-SMAD signals in the development of Th17 and regulatory T cells, J. Immunol. 184 (2010) 4295–4306.
[47] J.R. Grainger, K.A. Smith, J.P. Hewitson, H.J. McSorley, Y. Harcus, K.J. Filbey, et al., Helminth secretions induce de novo T cell Foxp3 expression and regulatory function through the TGF-beta pathway, J. Exp. Med. 207 (2010) 2331–2341.
[48] S.M. Schlenner, B. Weigmann, Q. Ruan, Y. Chen, H. von Boehmer, Smad3 binding to the foxp3 enhancer is dispensable for the development of regulatory T cells with the exception of the gut, J. Exp. Med. 209 (2012) 1529–1535.
[49] S.S. Hwang, S.W. Jang, M.K. Kim, L.K. Kim, B.S. Kim, H.S. Kim, et al., YY1 inhibits differentiation and function of regulatory T cells by blocking Foxp3 expression and activity, Nat. Commun. 7 (2016) 10789.
[50] P. Qiao, G. Li, W. Bi, L. Yang, L. Yao, D. Wu, microRNA-34a inhibits epithelial mesenchymal transition in human cholangiocarcinoma by targeting Smad4 through transforming growth factor-beta/Smad pathway, BMC Canc. 15 (2015) 469.
[51] X.J. Li, Z.J. Ren, J.H. Tang, MicroRNA-34a: a potential therapeutic target in human cancer, Cell Death Dis. 5 (2014) e1327.
[52] L. Classen, L.O. Tykocinski, F. Wiedmann, C. Birr, P. Schiller, C. Tucher, et al., Extracellular vesicles mediate intercellular communication: transfer of functionally active microRNAs by microvesicles into phagocytes, Eur. J. Immunol. 47 (2017) 1535–1549.
[53] B.G. Garchow, O. Bartulos Encinas, Y.T. Leung, P.Y. Tsao, R.A. Eisenberg, R. Caricchio, et al., Silencing of microRNA-21 in vivo ameliorates autoimmune splenomegaly in lupus mice, EMBO Mol. Med. 3 (2011) 605–615.
[54] N. Ghadiri, N. Emamnia, M. Ganjalikhani-Hakemi, K. Ghaedi, M. Etemadifar, M. Salehi, et al., Analysis of the expression of mir-34a, mir-199a, mir-30c and mir-19a in peripheral blood CD4+T lymphocytes of relapsing-remitting multiple sclerosis patients, Gene 659 (2018) 109–117.
[55] K. Ichiyama, A. Gonzalez-Martin, B.S. Kim, H.Y. Jin, W. Jin, W. Xu, et al., The MicroRNA-183-96-182 cluster promotes T helper 17 cell pathogenicity by negatively regulating transcription factor Foxo1 expression, Immunity 44 (2016) 1284–1298.
[56] H. Wang, J. Geng, X. Wen, E. Bi, A.V. Kossenkov, A.I. Wolf, et al., The transcription factor Foxp1 is a critical negative regulator of the differentiation of follicular helper T cells, Nat. Immunol. 15 (2014) 667–675.
[57] S. Onuora, Autoimmunity: TFH cells link gut microbiota and arthritis, Nat. Rev. Rheumatol. 12 (2016) 133.
[58] J. Collison, Rheumatoid arthritis: new player in RA pathogenesis brought to light, Nat. Rev. Rheumatol. 13 (2017) 195.
[59] M. Yamakuchi, M. Ferlito, C.J. Lowenstein, miR-34a repression of SIRT1 regulates apoptosis, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 13421–13426.
[60] S. Kong, P. Yeung, D. Fang, The class III histone deacetylase sirtuin 1 in immune suppression and its therapeutic potential in rheumatoid arthritis, J Genet Genomics 40 (2013) 347–354.
[61] J. Zhang, S.M. Lee, S. Shannon, B. Gao, W. Chen, A. Chen, et al., The type III histone deacetylase Sirt1 is essential for maintenance of T cell tolerance in mice, J. Clin. Investig. 119 (2009) 3048–3058.
[62] M. Rokavec, H. Li, L. Jiang, H. Hermeking, The p53/miR-34 axis in development and disease, J. Mol. Cell Biol. 6 (2014) 214–230.
[63] C. Liu, K. Kelnar, B. Liu, X. Chen, T. Calhoun-Davis, H. Li, et al., The microRNA miR-34a inhibits prostate cancer stem cells and metastasis by directly repressing CD44, Nat. Med. 17 (2011) 211–215.
[64] C. Munoz-Fontela, A. Mandinova, S.A. Aaronson, S.W. Lee, Emerging roles of p53 and other tumour-suppressor genes in immune regulation, Nat. Rev. Immunol. 16 (2016) 741–750.
[65] T. Tanaka, M. Narazaki, T. Kishimoto, IL-6 in inflammation, immunity, and disease, Cold Spring Harb Perspect Biol 6 (2014) a016295.
[66] U.K. Samavedam, K. Kalies, J. Scheller, H. Sadeghi, Y. Gupta, M.F. Jonkman, et al., Recombinant IL-6 treatment protects mice from organ specific autoimmune disease by IL-6 classical signalling-dependent IL-1ra induction, J. Autoimmun. 40 (2013) 74–85.
[67] X. Yao, J. Huang, H. Zhong, N. Shen, R. Faggioni, M. Fung, et al., Targeting interleukin-6 in inflammatory autoimmune diseases and cancers, Pharmacol. Ther. 141 (2014) 125–139.
[68] S. Garces, J. Demengeot, E. Benito-Garcia, The immunogenicity of anti-TNF therapy in immune-mediated inflammatory diseases: a systematic review of the literature with a meta-analysis, Ann. Rheum. Dis. 72 (2013) 1947–1955.
[69] I. Cleynen, S. Vermeire, Paradoxical inflammation induced by anti-TNF agents in patients with IBD, Nat. Rev. Gastroenterol. Hepatol. 9 (2012) 496–503.
[70] P.K. Gregersen, C.I. Amos, A.T. Lee, Y. Lu, E.F. Remmers, D.L. Kastner, et al., REL, encoding a member of the NF-kappaB family of transcription factors, is a newly defined risk locus for rheumatoid arthritis, Nat. Genet. 41 (2009) 820–823.
[71] Y. Ben-Neriah, M. Karin, Inflammation meets cancer, with NF-kappaB as the matchmaker, Nat. Immunol. 12 (2011) 715–723.
[72] M. Trenkmann, M. Brock, R.E. Gay, B.A. Michel, S. Gay, L.C. Huber, Tumor necrosis factor alpha-induced microRNA-18a activates rheumatoid arthritis synovial fibroblasts through a feedback loop in NF-kappaB signaling, Arthritis Rheum. 65 (2013) 916–927.
[73] L.L. Molinero, M.L. Miller, C. Evaristo, M.L. Alegre, High TCR stimuli prevent induced regulatory T cell differentiation in a NF-kappaB-dependent manner, J. Immunol. 186 (2011) 4609–4617.
[74] S. Jana, P. Jailwala, D. Haribhai, J. Waukau, S. Glisic, W. Grossman, et al., The role of NF-kappaB and Smad3 in TGF-beta-mediated Foxp3 expression, Eur. J. Immunol. 39 (2009) 2571–2583.