International Immunopharmacology 71 (2019) 267–276

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

MiR-155 controls follicular Treg cell-mediated humoral autoimmune intestinal injury by inhibiting CTLA-4 expression

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ARTICLE INFO

Keywords:
Follicular regulatory T cell
MicroRNA-155
Foxp3
CTLA-4
IBD

ABSTRACT

High expression levels of miR-155 are involved in the pathogenesis of inflammatory bowel disease (IBD). We observed an increase in miR-155 in peripheral regulatory T (Treg) cells from IBD patients. Mice that specifically overexpress miR-155 in Foxp3+ Treg cells exhibit spontaneous autoimmunity and more severe dextran sulfate sodium (DSS)-induced intestinal injury. MiR-155 overexpression can lead to a lack of follicular Treg (Tfr) cells and central Treg (cTreg), whereas DSS treatment further depletes the Tfr cells. Furthermore, miR-155 can target the expression of CTLA-4 in cTreg and Tfr, directly inhibiting Tfr cell production and promoting enhanced germinal center (GC) B cell activation and autoantibody overproduction. This outcome may be the cause of severe intestinal injury in patients with autoimmune IBD.

1. Introduction

miR-155 is a key microRNA (miR) that regulates the immune response, and it plays an important role in infection, immunity and autoimmunity [1]. It is considered to be a tumor immunomodulatory molecule because it can regulate the antitumor activity of various immune cells [2]. The overexpression of miR-155 enhances the antigen-specific immune response and clonal proliferation of CD8+ T cells, and its defects lead to antitumor immune failure of T cells that can be restored by CTLA-4 antibody [2,3]. CTLA-4 is an important target for immune checkpoint blockade (ICB) therapy, which suggests that miR-155 has a potential regulatory effect on CTLA-4. Recently, miR-155 has been shown to bind to CTLA-4 mRNA 3′UTR in the form of competing endogenous RNA (ceRNA), enhancing the proliferative response of helper T (Th) cells [4]. miR-155 is significantly overexpressed in patients with atopic dermatitis and may increase Th cell proliferation by downregulating CTLA-4 [5]. Additional studies have reported a role for miR-155 in regulatory T (Treg) cells [6]; in children with acute Kawasaki disease, a reduction in Foxp3+ Treg may be directly related to the decreased expression of miR-155 [7].

Ulcerative colitis (UC) is an intestinal tract disease characterized by diffuse ulceration of the colon, which has been linked to immune regulation disorders [8]. Significant upregulation of miR-155 was observed in the peripheral blood of patients with UC [9], and significant upregulation of miR-155 was observed in the mucosa of patients with IBD [10]. Deficiency or inhibition of miR-155 protects mice from DSS-induced experimental colitis [11]. These studies indicate that excessive miR-155 exacerbates the pathological progression of IBD. CTLA-4 is also considered to play a key role in the progression of IBD. For example, CTLA-4 downregulation in SOCS1-deficient mice induces severe IBD [12], and there is a correlation between CTLA-4 deficiency and early-onset IBD [13]; moreover, CTLA-4-ICB therapy can cause adverse reactions of severe gastrointestinal ulceration [14]. Our previous studies showed that defects of CTLA-4 can lead to Tfr cell differentiation inhibition and severe autoimmune bowel injury [15]. However, it is unclear that miR-155 overexpression reduces CTLA-4 expression in Treg cells as well, and it is unknown whether miR-155 is associated with Tfr-mediated autoimmunity. In the current study, we hypothesize that miR-155 is involved in chronic inflammatory bowel disease, due to its up regulation in active clinical disease and corresponding reduction in Treg cells in IBD.

2. Materials and methods

2.1. Subjects

Blood samples were collected from 68 confirmed IBD patients and 24 healthy subjects with no bowel disease or autoimmune disease. In

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https://doi.org/10.1016/j.intimp.2019.03.009
Received 31 October 2018; Received in revised form 2 March 2019; Accepted 5 March 2019
Available online 28 March 2019

Table 1
The Montreal classification of 68 active IBD subjects.

Clinical classification of active UC subjects		
Subjects	Montreal classification of extent	Montreal classification of severity
1	E2	S2
2	E2	S1
3	E2	S1
4	E1	S2
5	E2	S1
6	E1	S2
7	E2	S1
8	E1	S1
9	E1	S2
10	E1	S2
11	E2	S2
12	E2	S2
13	E3	S3
14	E2	S2
15	E2	S2
16	E2	S2
17	E2	S2
18	E2	S3
19	E3	S2
20	E2	S3
21	E2	S3
22	E3	S3
23	E2	S3
24	E3	S3
25	E2	S3
26	E3	S3
27	E2	S3
28	E2	S3
29	E3	S3
30	E3	S3
31	E3	S3

Clinical characteristics of active CD subjects			
	Age at diagnosis	Location	Behaviour
1	A2	L3	B2
2	A3	L1	B1
3	A3	L1	B2
4	A2	L3	B2
5	A3	L1	B2
6	A2	L3	B1
7	A2	L3	B1
8	A2	L2	B2
9	A2	L3	B2
10	A2	L3	B1
11	A3	L2	B2
12	A3	L1	B2
13	A3	L1	B1
14	A2	L2	B1
15	A3	L1	B2
16	A3	L1	B2
17	A3	L2	B1
18	A1	L2	B2
19	A3	L1	B3
20	A3	L1	B3
21	A2	L3	B2
22	A2	L2	B1
23	A2	L3	B1
24	A1	L2	B2
25	A3	L1	B1
26	A3	L1	B1
27	A2	L2	B2
28	A3	L1	B3
29	A3	L1	B1
30	A2	L2	B2
31	A2	L3	B2
32	A2	L3	B2
33	A2	L2	B1
34	A3	L1	B3
35	A3	L1	B2

Table 1 (*continued*)

Clinical characteristics of active CD subjects			
	Age at diagnosis	Location	Behaviour
36	A2	L3	B2
37	A3	L1	B2

this study, written consents were obtained for all subjects, and the research protocol was approved by the Xi'an Jiaotong University Ethics Review Committee. All authors and experimenters of this study have read the Code of Ethics of the World Medical Association (Declaration of Helsinki) and strictly abided by it. The Mayo Score Index (MSI) and the Crohn's disease activity index (CDAI) were used to assess the severity of UC and CD, respectively.

2.2. Mice

Rosa26$^{miR\text{-}155}$Foxp3^{Cre} (miR-155^{Foxp3}) C57BL/6J background mice and the corresponding homologous WT mice were created by Shanghai Model Organisms Center (Shanghai, CHN). Briefly, the exogenous targeting vector CAG-LoxP-neo-stop-loxP-Pre-mir155 based on CRISPR-Pro technology was introduced into the Rosa26 site of the Rosa26 tool WT mice to obtain Rosa26-flox-Stop-flox-miR-155 fl/− mice (Stop-155$^{fl/-}$). Stop-155$^{fl/-}$ mice do not express miR-155 due to the presence of the STOP element between the Rosa26 CAG-promoter and mir-155 genomic. When hybridized with Fox3p + -driven Cre-expressed tool mice, Cre recombinase removes “STOP” and the Rosa26 promoter initiates mir-155 expression. C57BL/6 CD45.1, CD45.2 WT mice and Rag2 − / − mice were purchased from Weitong Lihua (Beijing, CHN). Mice were treated with 5% (w/v) DSS dissolved in the drinking water for 5 days and are referred to as the DSS-treated experimental mice. According to the approval of the Animal Ethics and Use Committee of the Medical College of Xi'an Jiaotong University, the mice were kept free from specific pathogens compliant with the guidelines of the Animal Care and Welfare Institute. Total CD4 + T cells were isolated from miR-155^{Foxp3} and WT mice using the Mouse CD4 + T Cell Enrichment kit (STEMCELL Technologies).

2.3. MiRNA detection

Total RNA from Treg of the peripheral blood of the IBD patients was isolated by flow cytometry using an RNAeasy small RNA isolation kit (Thermo, MA, USA). Reverse transcription of miRNA was performed using a miRCURY LNA universal RT microRNA cDNA synthesis kit; miRCURY LNA with the miR-155 mature sequence UniRT PCR primers (Qiagen, Dusseldorf, GER) was used to detect the miR-155 expression. Hsa-miR-155 and mmu-miR-155 Stem-loop RT-PCR primer: 5′-ctcaactggtgtcgtggagtcggcaattcagttgagagcattaa-3′; Amplification primer (forward): 5′-gccgaggggatagtgctaa-3′; Amplification primer (reverse): 5′-tggtgtcgtggagtcg-3′. U6 Stem-loop RT-PCR primer: 5′-aacgcttcacgaatttgcgt-3′; Forward primer: 5′-gggctcgcttcggcagcaca-3′; Reverse primer: 5′-tggtgtcgtggagtcg-3′.

2.4. Flow cytometry

CD38 (clone 90, APC), CD4 (clone GK1.5, eFluor450, PE, APC, FITC), CD44 (clone IM7, FITC, PE), GL.7 (clone GL7, FITC), Foxp3 (clone FJK-16s, PE, APC, FITC), CD19 (clone 1D3, eFluor450, APC), CXCR5 (clone SPRCL5, Per-CP eFluor710, FITC), PD-1 (clone RMP1-30, APC, FITC), and CD45.1 (A20, PE, FITC) were purchased from Abcam (Cambridge, UK). Anti-CTLA4 (clone D4E9I, PE) was purchased from Cell Signaling Technology (MA, USA). Anti-IgA, IgG and IgM were purchased from Abcam. Cells from the peripheral blood of the IBD patients, miR-155^{Foxp3} or WT mouse spleen or lymph nodes were

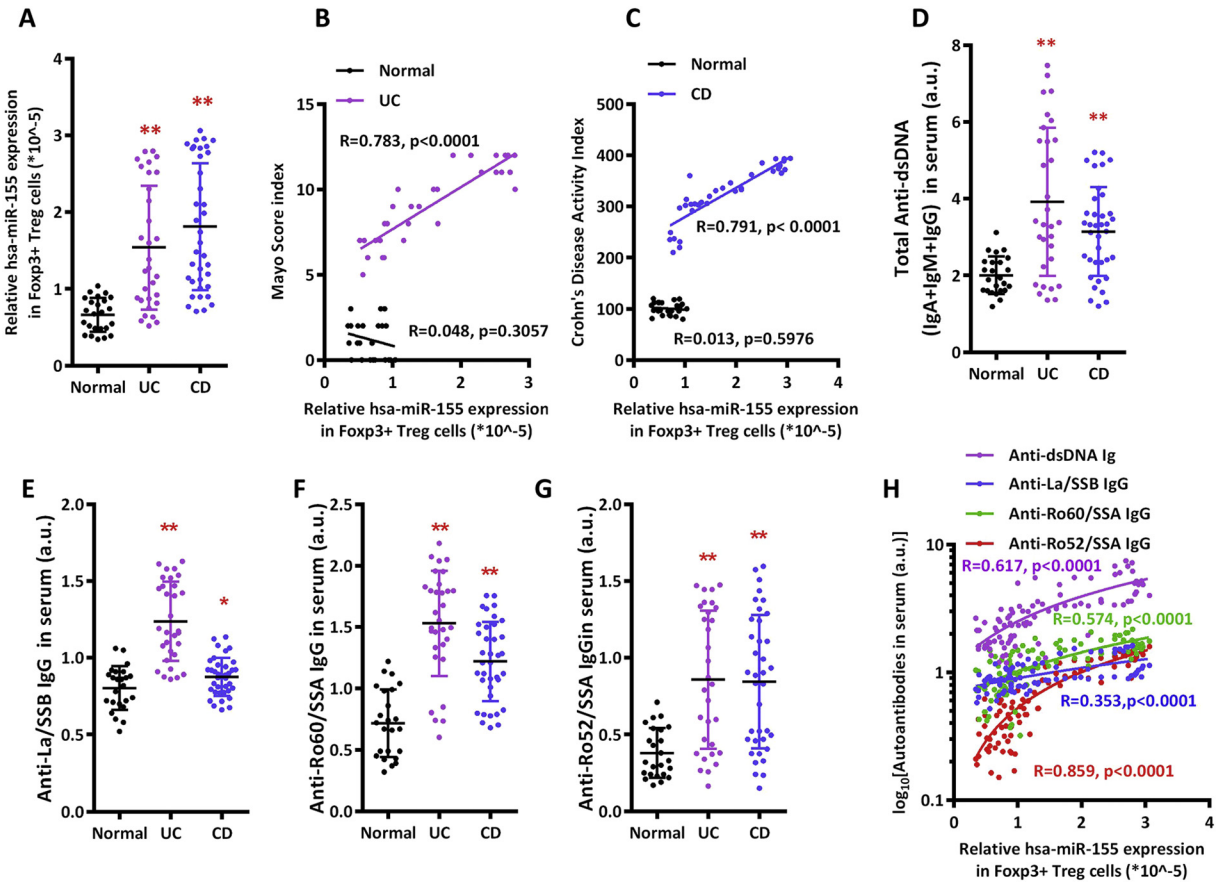

Fig. 1. Increased miR-155 in the Treg cells in the patients with autoimmune IBD.
qPCR was used to detect the expression levels of hsa-miR-155 in the peripheral blood Treg cells of the patients with IBD and healthy subjects normalized to RNAU6 (A). Correlations between hsa-miR-155 levels and Mayo scores (B) and CDAI scores (C) in patients with UC and CD. Total anti-dsDNA levels in the peripheral blood of patients with IBD (D); anti-La (SSB) IgG (E), anti-Ro60 (SSA) (F) and anti-Ro52 (SSA) (G). Correlations between the expression levels of hsa-miR-155 and autoantibodies in patients with IBD (H). One-way ANOVA was used to compare differences between the normal and UC or CD; *$p < 0.05$, **$p < 0.01$, mean ± SD, n = 24–31. Spearman's correlation coefficient was used to assess the relationship between Normal and UC or CD.

isolated by washing with 0.1% BSA in cold PBS. Anti-FcgRII/FcgRIII (clone 2.4G2, eBioscience, CA, USA) was used for blocking. For fluorescently labeled leukocyte differentiation antigen antibodies, incubation was 1 h at 4 °C in the dark. For the detection of the intracellular factors Foxp3 and CTLA4, cells were treated and permeabilize after blocking using an intracellular fixation buffer (Thermofisher, Waltham, MA, USA). Samples were analyzed or sorted using a BD FACSCanto II flow cytometer and further analyzed using Flowjo v.8.7 and v.10.0.8r1 (FlowJo, LLC) software.

2.5. BM chimera mice

2.5×10^6 BM cells from the WT (CD45.1) mice and 2.5×10^6 BM cells from the miR-155^{Foxp3} (CD45.2) mice were stripped and transplanted to the Rag2$^{-/-}$ mice irradiated with a lethal X-ray dose (5G y × 2); cell frequencies were analyzed by flow cytometry after 20 weeks.

2.6. ELISA

The total serum levels of IgM, IgG, and IgA in the IBD patients and mice were measured according to the product specifications (Abcam, MA, USA) and absorbance at 450 nm was measured using a SpectraMax M5 plate reader (Molecular Devices, MA, USA). For the detection of autoantibodies in the mouse serum, ELISA plates were precoated with polylysine (Aladdin, MO, USA) and treated with the mouse serum and calf thymus dsDNA (2.5 μg/ml, Sigma); recombinant La, Ro52, Ro60 (10 μg/ml, Sigma) or natural Ro60 protein (5 μg/ml, Arotec Diagnostics) were incubated overnight, and autoantibody titers in the serum samples were assayed with goat anti-mouse IgM, IgG, IgA and IgE as detection antibodies (Abcam) Self-antibody titer in serum. Colons were homogenized in PBS buffer solution (1 mL/0.1 g). Then, collect supernatants to detect the expression of IL-6, TNF-α, MCP-1 and PGE2. Sandwich enzyme-linked immunosorbent assay were performed with cytokine-specific kits (Abcam).

2.7. Immunohistochemistry

Mouse colonic tissue samples were fixed in 4% PFA for 24 h and embedded in paraffin. A microtome (Leica, Eisfeld, GER) was used to cut the samples into 3 μm thick sections that were fixed on the polylysine precoated slides. Endogenous peroxidase activity was inactivated by 3% H_2O_2, and the slides were blocked in a TBST solution containing 5% goat serum at room temperature. Slides were incubated overnight with rabbit anti-Ig-HRP (Abcam) at 4 °C. After washing with TBST, a DAB substrate kit was used for staining and the tissue was counterstained with hematoxylin. After dehydration, the sections were observed using an inverted microscope (Olympus IX53).

2.8. Tissue fxation, paraffn embedding, and histological scoring

Colons were removed, longitudinal cut, and washed with PBS followed by fxation with 4% PFA. Tissue was embedded in parafn, cut into 3-μm sections, and stained with hematoxylin and eosin (H&E).

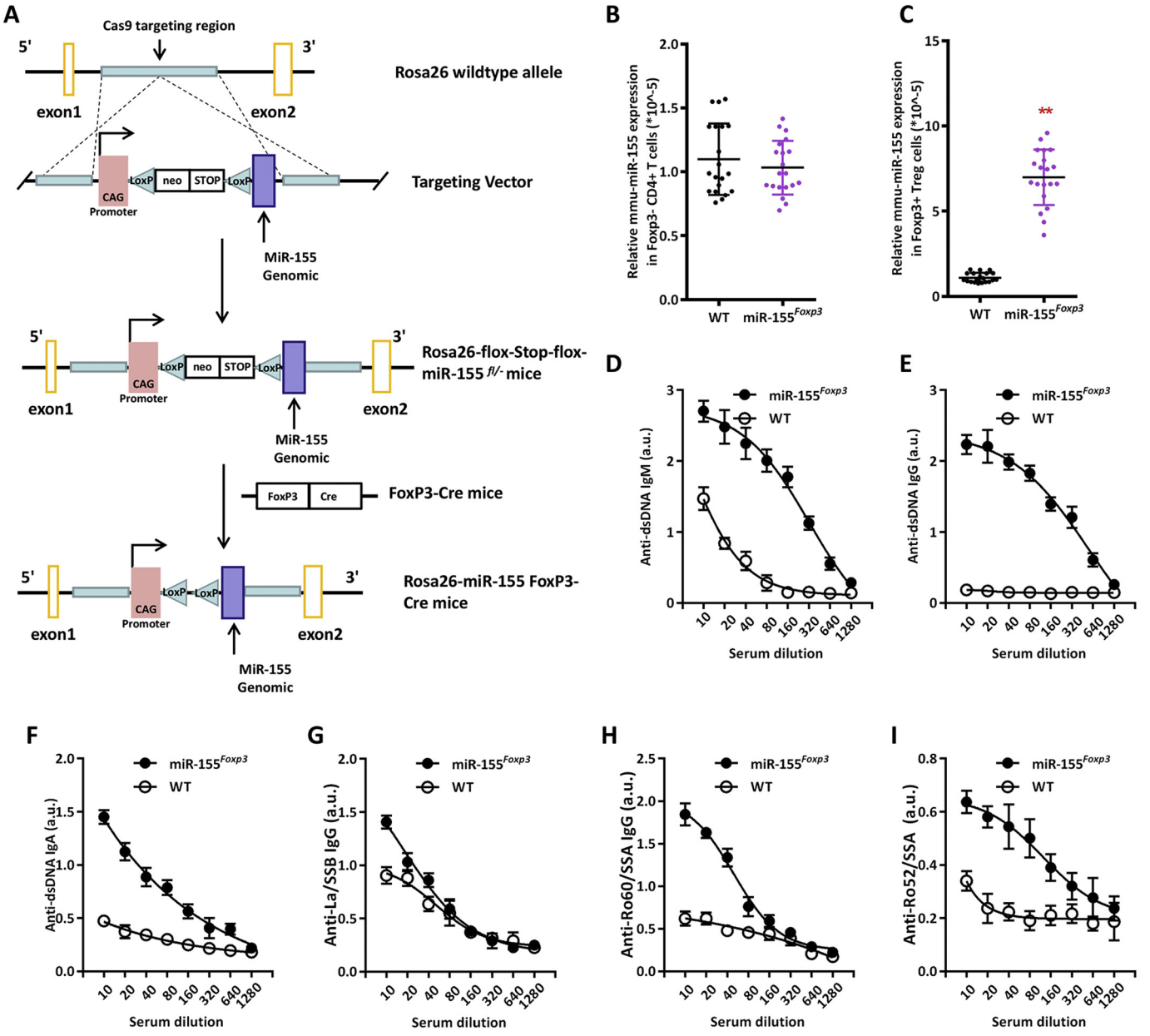

Fig. 2. miR-155 overexpression in Foxp3 + Treg causes humoral autoimmunity.
Structures of the miR-155^{Foxp3} transgenes (A). Foxp3 + Treg (B) and Foxp3-CD4 + T cells (C) from miR-155^{Foxp3} mice showed a significant decrease in miR-155 expression compared with Foxp3 + Treg Foxp3-CD4 + T cells from WT mice. Anti-dsDNA IgM (D), IgG (E), and IgA (F) antinuclear antibodies, anti-La (SSB) IgG (G) and anti-Ro60 (SSA) (H) and anti-Ro52 (SSA) (I) titers absorbance unit (a.u.) curve in the serum of 20-week-old WT and miR-155^{Foxp3} mice, n = 3. A Student's paired *t*-test was used to compare differences between the WT and miR-155^{Foxp3} mice, *p < 0.05, **p < 0.01, mean ± SD, n = 20.

Histological assessment of colitis was performed by a board-certified pathologist, who was blinded to experimental details, as described [16] (McNamee et al., 2010).

2.9. Cell transfection

pGL3-luc-CTLA4 3′UTR, miR-155 mimics and inhibitor were constructed by Genepharm (Shanghai, CHA). For cell transfection, 1×10^5 sorted cTreg and Tfr cells were seeded in 12-well plates; the plasmids and miRNAs were transfected using lentiviral packet transfection system (System Biosciences, CA, USA) according to the instruction manual; luciferase activity was measured using a multifunctional fluorescent plate reader (Molecular Devices).

2.10. Photoactivatable ribonucleoside-enhanced cross-linking immunoprecipitation (PAR-CLIP) analysis

Enrichment of miR-155 in the RNA-induced silencing complex (RISC) was performed according to the method of Feng [17]. Briefly, the cTreg and Tfr cells were pretreated with M4-thiouridine (Sigma). After UV irradiation, the cells were lysed on ice for 20 min. The cell pellet was resuspended and immunoprecipitated overnight using agarose beads (Roche) and anti-AGO2 antibody (Cell Signaling Technology). qPCR was performed to detect the expression levels of the CTLA4 mRNA in the final precipitate; GAPDH, B2M and RPL13A were used as internal reference genes.

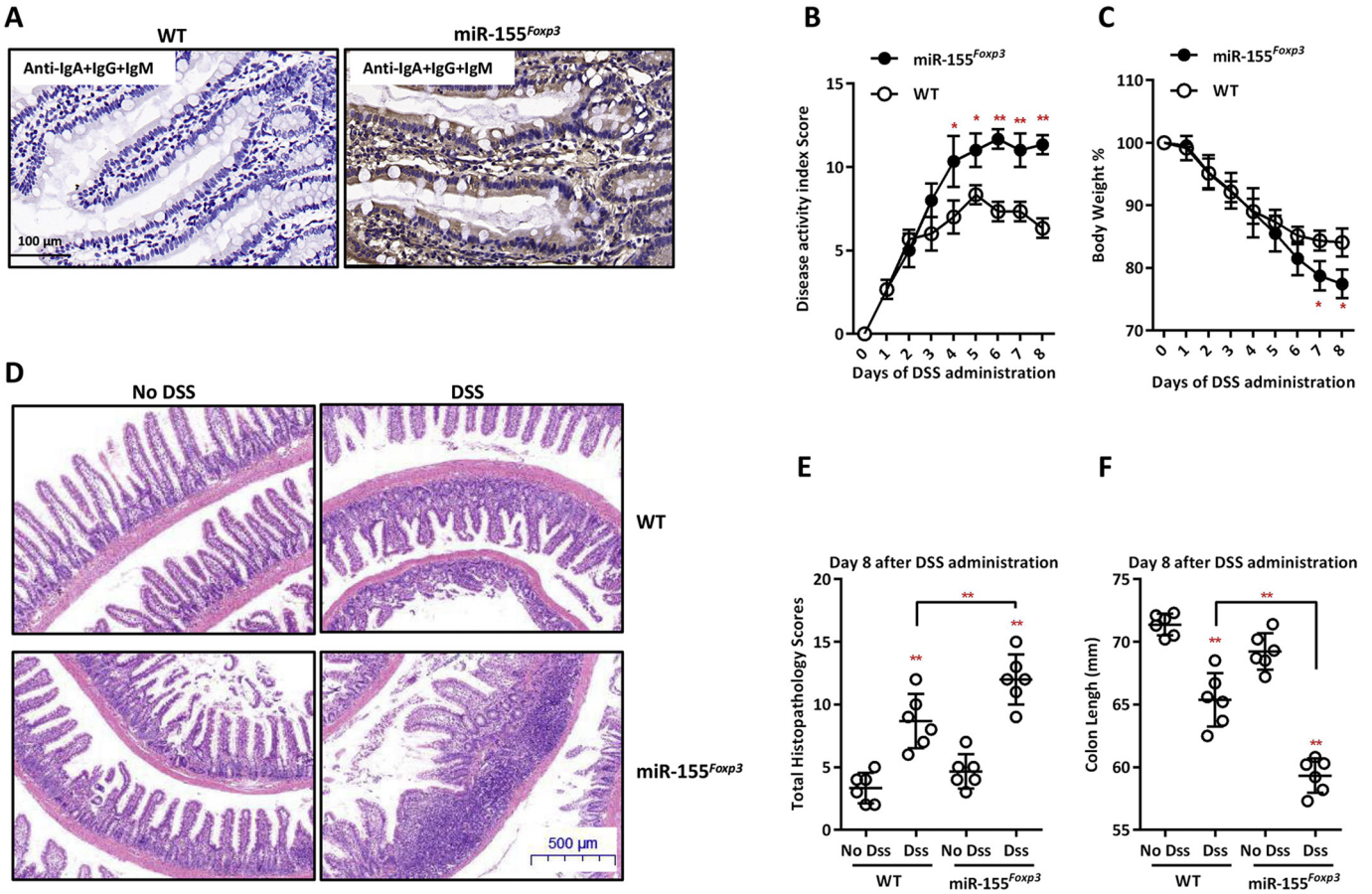

Fig. 3. miR-155^{Foxp3}causes DSS-induced intestinal damage.
Immunohistochemistry was used to observe the accumulation of IgM + IgG + IgA immune complexes in the small intestine villus epithelial cells of 20-week-old WT and miR-155^{Foxp3} mice (A). The DAI score (B) and body-mass index (C) of 20-week-old WT and miR-155^{Foxp3} mice treated with DSS. Colon HE staining images (D), histopathology scores (E) and colon lengths (F) of 20-week-old WT and miR-155^{Foxp3} mice treated with DSS. One-way ANOVA was used to compare differences between the No DSS- and DSS-treated WT or miR-155^{Foxp3} groups, *p < 0.05, **p < 0.01 vs. normal, mean ± SD, n = 6 (K-L and N-O).

2.11. Statistics and analysis

All data are shown as the mean ± S.D. One-way ANOVA and Student's paired *t*-test were used to compare differences between the groups; $p < 0.05$ was considered to represent significant differences. Spearman's correlation coefficient (R) was used to analyze the correlations of the data between the groups, $p < 0.05$ was considered to represent significant correlation. All data were calculated and plotted by SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) and Graphpad Prizm 6.

3. Results

3.1. Increased miR-155 in Treg cells in patients with autoimmune IBD

To investigate the expression levels of miR-155 in the Treg cells of IBD patients, 31 patients with UC, 37 patients with CD and 24 healthy subjects were classified according to the Montreal criteria [18]. The Montreal classification of 68 active IBD subjects is shown in Table 1. CD4 + CD25 + Foxp3 + Treg cells were sorted from the peripheral blood by flow cytometry, and the expression levels of hsa-miR-155 were assayed via qPCR. As shown in Fig. 1A, both UC and CD patients had significantly higher levels of miR-155 in their Treg cells than those in the normal subjects. MSI and CDAI were used to assess the correlation between the severity of UC and CD and the levels of miR-155, respectively. As shown in Fig. 1B and C, the miR-155 levels in the Treg cells were positively correlated with MSI and CDAI, which indicates that higher miR-155 expression levels in IBD patients corresponded to more severe intestinal damage. The detection of anti-dsDNA autoantibodies (serum total of IgM, IgG and IgA) and anti-Ro (SSA) and anti-La (SSB) IgG antibodies was performed in the peripheral blood of the patients with IBD. As shown in Fig. 1D–G, the levels of autoantibodies in the peripheral blood of the patients with IBD were significantly increased. Finally, a correlation between an increase in the antibodies and the levels of miR-155 was calculated. As shown in Fig. 1H, four serum autoantibodies were positively correlated with the levels of miR-155 in the Treg cells. This outcome indicates that higher miR-155 expression in the IBD patients was associated with higher levels of autoantibodies in the serum. These data suggest that miR-155 is directly related to an increase in the antibodies in the serum of patients with IBD.

3.2. miR-155^{Foxp3} causes autoimmune and DSS-induced intestinal damage in mice

The increased expression of miR-155 in Foxp3 + Tregs may contribute to the immunopathology of autoimmune IBD. To test this possibility, we used a transgenic mouse model that constitutively expressed miR-155 in the Foxp3 + Treg. Foxp3-driven Cre recombinase (Foxp3-Cre) transgenic mice were crossed with Rosa-26-flox-Stop-flox-miR-155$^{fl/-}$ (Stop-150$^{fl/-}$) transgenic mice (Fig. 2A). Overexpression of mmu-miR-155 was achieved in Foxp3 + Tregs in miR-155^{Foxp3} mice (Fig. 2B and C). The miR-155^{Foxp3} mice overexpressed miR-155 in Treg cells; the model was created to observe the effect of hypermiR-155 on self-limited intestinal injury. No apparent accumulation of the IgM, IgG

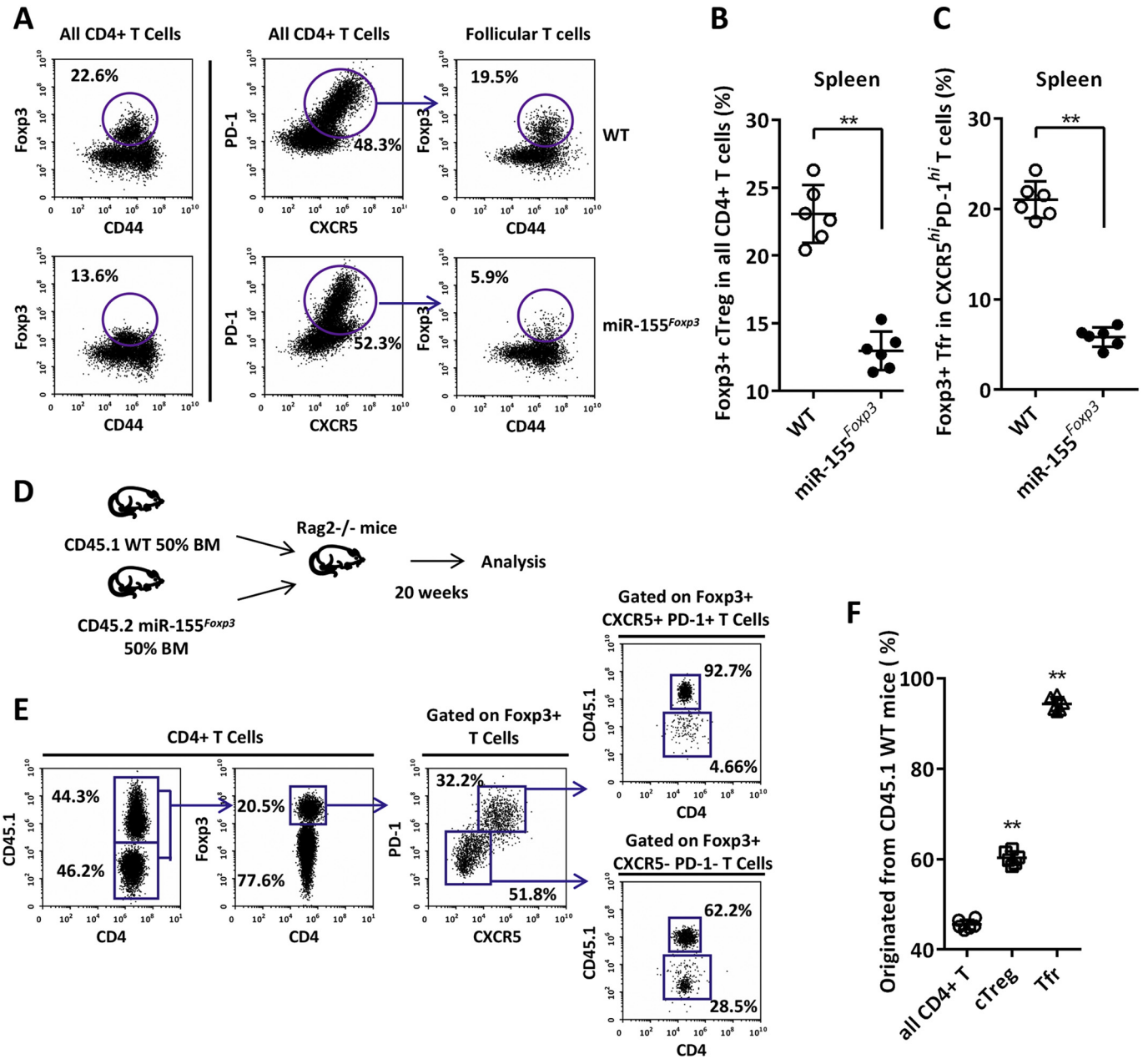

Fig. 4. miR-155^{Foxp3} leads to a lack of cTreg and Tfr cells.
Analysis of the cTreg and Tfr cells in the spleen of 20-week-old WT and miR-155^{Foxp3} mice (A–C).
Bone marrow cells of WT (CD45.1 +) and miR-155^{Foxp3} (CD45.2 +) mice were mixed at a 1:1 ratio and transplanted into Rag2 − / − immunodeficient mice to form the bone marrow (BM) chimeras (D). The proportions of Tfr cells, cTreg cells, and T cells in the spleens of CD45.1 + WT mice and chimeric mice (E and F). A Student's paired *t*-test was used to compare differences between WT and miR-155^{Foxp3} mice, *p < 0.05, **p < 0.01, mean ± SD, n = 6.

and IgA immune complexes was observed in 4 week old miR-155^{Foxp3} mice in the colon (Fig. S1A). Anti-dsDNA IgM, IgG, IgA anti-nuclear (Fig. 2D–F), anti-Ro (SSA) and anti-La (SSB) IgG antibodies (Fig. 2G–I) were spontaneously increased in serum of 20-week-old miR-155^{Foxp3} mice, and significant accumulations of the IgM, IgG and IgA immune complexes were observed in mouse colonic villus epithelial cells (Fig. 3A). There were no significant differences in body-mass index, DAI score, or intestinal pathological damage in the 20-week-old miR-155^{Foxp3} mice compared to the WT mice. However, the DSS-induced miR-155^{Foxp3} mice showed more severe signs of intestinal disease than the WT mice; the severity of disease was defined as an increased overall disease activity index (DAI) measured as weight loss, fecal blood and soft stool/diarrhea (Fig. 3B and C). Histological analysis of the intestine showed more severe tissue damage in the colon of the DSS-treated miR-155^{Foxp3} mice, a shorter colon length and more severe inflammatory cell infiltration (Fig. 3D–F). The expression and release of four representative pro-inflammatory cytokines TNF-α, IL-6, PGE2 and MCP-1 in colon homogenate were detected using the Elisa method. There was no significant change in the pro-inflammatory factor levels in the colons of the 4- or 20-week-old mice compared to the WT mice (Fig. S1B–I). When treated with DSS, the inflammatory cytokines of the miR-155^{Foxp3} mice were significantly higher than those of the WT mice (Fig. S1F–I). These data indicate that miR-155 exacerbates intestinal damage in acute enteritis.

3.3. miR-155^{Foxp3} leads to a lack of Tfr cells

To explore the role of miR-155 in acute intestinal injury, we

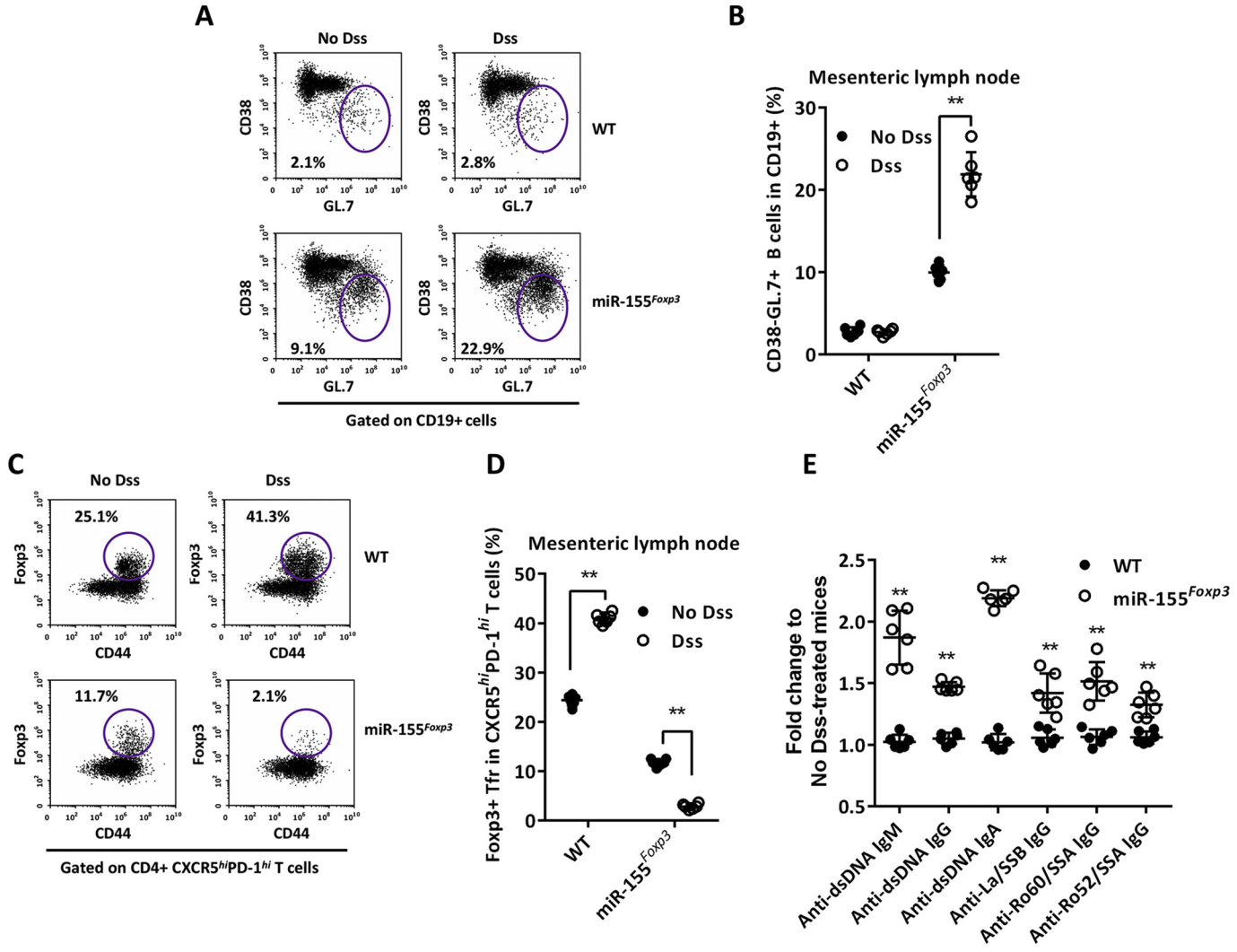

Fig. 5. miR-155^{Foxp3} causes aggravated DSS-induced humoral autoimmunity.
The number of GC B cells (A and B) and the number of Tfr cells (C and D) in WT and miR-155^{Foxp3} mice mesenteric lymph nodes in the presence or absence of DSS treatment. The production of autoantibodies in WT and miR-155^{Foxp3} mice induced by DSS treatment (E). One-way ANOVA was used to compare differences between the No DSS- and DSS-treated WT or miR-155^{Foxp3} groups, *p < 0.05, **p < 0.01 vs. normal, mean ± SD, n = 6.

evaluated the effects of miR-155 on the central Treg (cTreg) and T follicular regulatory (Tfr) cells. As shown in Fig. 4A, the proportion of cTreg (CD4$^+$ Foxp3$^+$) cells in the spleens of the miR-155^{Foxp3} mice was reduced by approximately 40% (Fig. 4B), while the proportion of Tfr (CXCR5^{hi}PD-1^{hi}Foxp3$^+$) cells was reduced by 70% (Fig. 4C). To determine whether miR-155 is an essential regulator of the formation of cTreg and Tfr cells, bone marrow (BM) cells from the WT (CD45.1+) and miR-155^{Foxp3} (CD45.2+) mice were mixed at a 1:1 ratio and transplanted into the Rag2$^{-/-}$ immunodeficient mice creating the BM chimeras; the Tfr cell sources in the chimera host mice were analyzed after 20 weeks (Fig. 4D). As shown in Fig. 3C, the population of Tfr cells in the chimeric mice was almost exclusively from the CD45.1+ WT mice with > 60% of the cTreg cells originating from the CD45.1+ WT mice; the proportions of the T cells derived from the WT or miR-155^{Foxp3} BM were similar (Fig. 4E and F). This outcome indicates that miR-155-overexpression leads to a lack of cTreg and Tfr cells. Due to the lack of Tfr, the fraction of the CD19+ GL.7+ CD38- GC B cells was significantly increased in the mesenteric lymph nodes of the miR-155^{Foxp3} mice (Fig. 5A and B); this outcome may lead to spontaneous autoimmunity of the miR-155^{Foxp3} mice. Furthermore, there was no significant increase in the number of B cells in the mesenteric lymph nodes of the WT mice induced with DSS (Fig. 5A and B); only Tfr cells were rapidly depleted from the spleen of the miR-155^{Foxp3} mice (Fig. 5C and D), thus producing an excess of the GC B cells (Fig. 5A and B) and nonspecific antibodies (Fig. 5E). This finding may explain the potential exacerbation of acute inflammatory bowel injury by miR-155.

3.4. miR-155 inhibits the expression of CTLA-4 in Treg cells

The high expression of miR-155 in the Treg cells of the miR-155^{Foxp3} mice may result in the degradation of CTLA-4 mRNA. As a next step, we verified the targeted inhibition of CTLA-4 by miR-155 in Treg. CTLA-4 protein and mRNA are downregulated in the miR-155^{Foxp3} mice in the cTreg and Tfr cells (Fig. 6A–B). Possible targeted binding of miR-155 to the CTLA-4 3′UTR was predicted by the miRNA binding target prediction website (http://www.targetscan.org/) (Fig. 6C). The pGL3-luc-CTLA-4 3′UTR luciferase reporter gene was constructed and transfected into the cTreg and Tfr cells. At the same time, 100 nM miR-155 mimics or inhibitors were cotransfected to the cTreg and Tfr cells as shown in Fig. 6D. The miR-155 mimics and inhibitor upregulate and downregulate the fluorescence intensity of the CTLA-4 3′UTR luciferase reporter plasmid in the cTreg and Tfr cells, respectively. After the PAR-CLIP experiments, qPCR was used to detect the levels of CTLA-4 mRNA in the coprecipitated samples, and the levels of GAPDH, B2M and

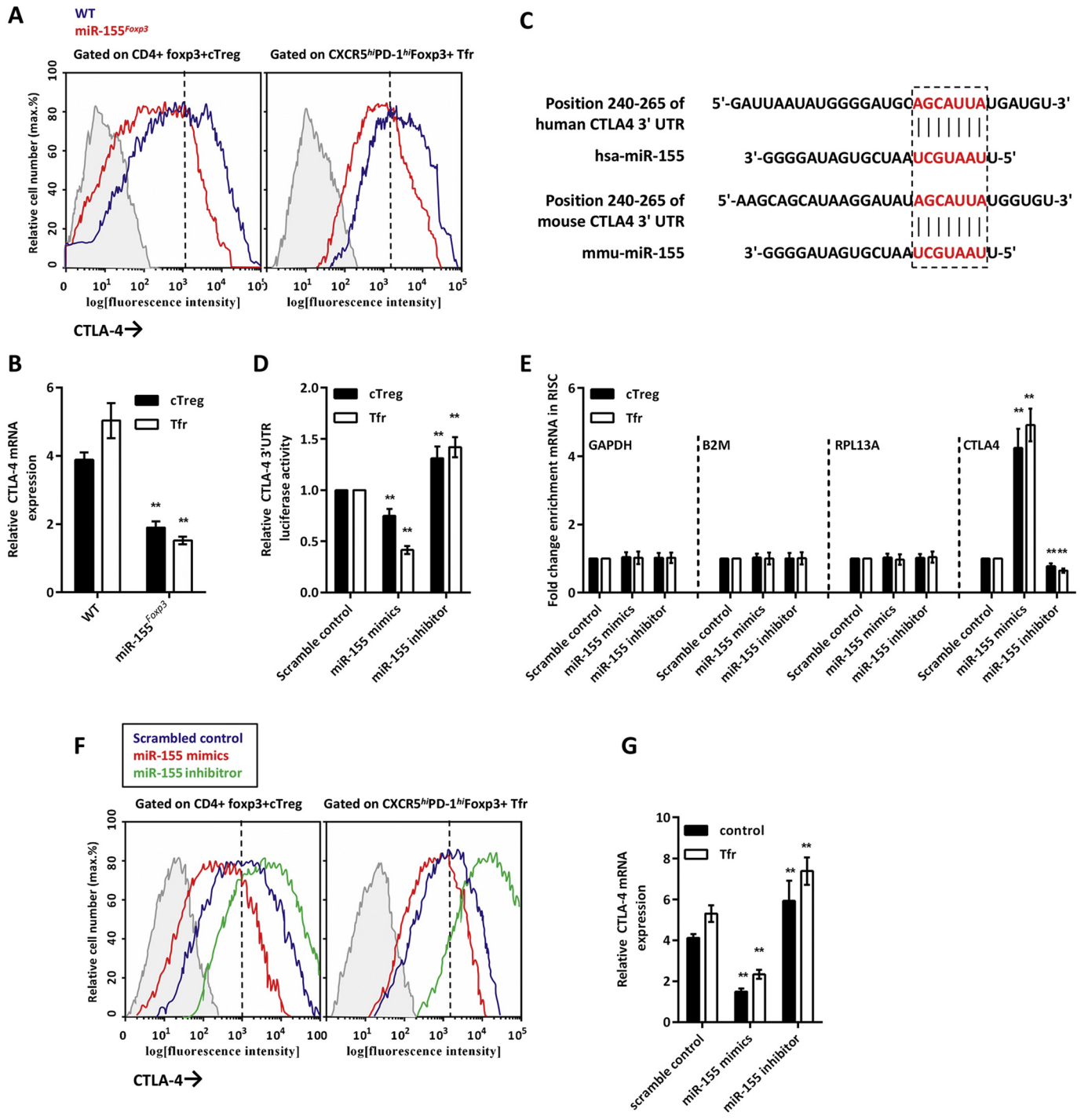

Fig. 6. miR-155 inhibits the expression of CTLA-4 in Treg.
The expression levels of CTLA-4 protein (A) and mRNA (B) in WT and miR-155^{Foxp3} mice. Predicted binding site of miR-155 in the CTLA-4 3′UTR (C). The mimics or inhibitors of miR-155 and the pGL3-luc-CTLA-4 3′UTR luciferase reporter gene were cotransfected into cTreg and Tfr cells cultured in vitro, and the fluorescence expression intensity (D) was detected. PAR-CLIP experiments were performed to observe the enrichment in the CTLA-4 mRNA in RISC (E). The mimics or inhibitors of miR-155 were cotransfected into cTreg and Tfr cells to detect the expression of CTLA-4 protein (F) and mRNA (G). One-way ANOVA was used to compare differences between the No DSS- and DSS-treated WT or miR-155^{Foxp3} groups, *p < 0.05, **p < 0.01 vs. normal, mean ± SD, n = 6.

RPL13A were measured as internal references. As shown in Fig. 6E, the miR-155 mimics and inhibitors upregulated and downregulated the CTLA-4 mRNA enrichment in RISC, respectively. Moreover, the miR-155 mimics and inhibitor upregulated and downregulated the expression of the CTLA-4 protein and mRNA, respectively (Fig. 6F–G). These results indicate that miR-155 can directly target the inhibition of CTLA-4 expression.

4. Discussion

During infection, inflammation and immune responses, Treg cells migrate from the blood to the draining lymph nodes and tissues to inhibit the activation and proliferation of antigen-specific T cells [19]. cTregs are located in the germinal centers (GC) of the secondary lymphoid tissues. Tfr cells differentiated from cTregs are the inhibitory cells that regulate the GC B cell clonal selection and antibody affinity

maturation and prevent excessive nonspecific immune responses and excessive generation of autoantibodies [20]. Deregulation of Tfr cells and GC B cells can cause severe autoimmune diseases [21]. Foxp3 is an important specific Treg lymphocyte regulator expressed in cTreg and cTreg-derived differentiated Tfr cells [22]. In the current study, the overexpression of miR-155 in mouse Foxp3+ Treg cells severely depleted the Tfr cells, potentially causing miR-155 elevation in the DSS-induction experimental mice with autoimmune intestinal injury.

The increase in miR-155 can be observed in the peripheral blood of patients with IBD and the colons of DSS-treated mice [9,10]; however, these data are not sufficient to explain the tissue cell source of miR-155. In this study, a significant increase in miR-155 was observed in the peripheral Treg cells of 68 patients with confirmed IBD; the increase was proportional to the severity of IBD. It is noteworthy that the level of miR-155 in the Treg cells is also proportional to the levels of peripheral anti-dsDNA autoantibodies (including IgM, IgG and IgA) and anti-Ro (SSA) and anti-La (SSB) IgGs. This outcome suggests that a high expression of miR-155 in Treg cells is directly related to autoimmune IBD. Mice that conditionally overexpressed miR-155 in Treg cells also showed excessive levels of autoantibodies in the serum and deposition of IgM, IgG and IgA observed in the colonic goblet epithelial cells. However, compared to the WT mice, the miR-155^{Foxp3} mice did not develop significant IBD disease characteristics, including DAI, body mass index, and pathological damage, and had only mild colonic inflammation and mild colon shortening. However, miR-155^{Foxp3} mice showed more severe acute intestinal injury after treatment with the experimental IBD inducer DSS. This outcome indicates that hypermir-155 in Treg cells can aggravate the extent of the damage in acute colitis.

The overexpression of miR-155 in Treg cells severely reduces the numbers of Tfr cells. Although the expression of cTreg cells was also reduced in the miR-155^{Foxp3} mice, the Tfr cells from the miR-155^{Foxp3} donor mice were barely detectable in the Rag2−/− host mice that received bone marrow transplantation. Furthermore, the WT mouse Tfr cells did not show significant changes after DSS induction, whereas the Tfr cells were completely depleted from the miR-155^{Foxp3} mice. We hypothesize that DSS-induced colonic injury triggers a mechanism of autoimmune enhancement; however, it is not known why DSS induction leads to the depletion of Tfr cells. The depletion of Tfr cells by DSS increased the number of GC B cells in the intestinal lymph nodes of the miR-155^{Foxp3} mice and significantly increased the levels of autoantibodies in the serum. The role of miR-155 in the control of helper T cells has been extensively studied [23], including helper T cell proliferation, T cell development [24], GC response [25], immunoglobulin class switch and plasma cell maturation [26]. However, there is limited knowledge regarding the significance of miR-155 in regulatory T cells. miR-155 can target several immune-related molecules, such as SOCS1 [27], κB-Ras1 [28], and CTLA-4 [29]. CTLA-4 is a coinhibitory molecule that is expressed by T cells, and it is an important immunoregulatory factor and an inhibitor of inflammation [30]. Decreased CTLA-4 in Treg leads to spontaneous autoimmunity [31]. Moreover, CTLA-4 is also directly related to the pathological progression of autoimmune IBD. In our previous studies, we verified that CTLA-4 may be required for Tfr cell differentiation and production [15]. Tfr cells inhibit B cell responses and prevent humoural autoimmune-mediated intestinal damage by regulating Tfh-dependent GC responses. The lack of Tfr cells may be responsible for the immunosuppressive disorder of inflammatory bowel disease caused by CTLA-4 deficiency. We observed a targeted inhibition of CTLA-4 by miR-155 in both Treg and Tfr cells, which may be a direct cause of the Tfr cell depletion induced by miR-155.

Thus, our study found that miR-155 enhances upregulated inflammation by inhibiting the production of Tfr cells, while its excessive elevation reduces the number of Tfr cells and enhances the nonspecific immune response. A lack of Tfr cells leads to an enhanced GC response, the activation of GC B cells, and the overproduction of autoantibodies. This spontaneous autoimmunity does not damage intestinal epithelial cells at a steady state and only worsens the damage during acute colon injury induced by DSS.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.03.009.

Acknowledgements

This study was supported by the Natural Science Foundation of Shaanxi Province (Grant No. 2017JM8139) and the National Natural Science Foundation of China (Grant No. 81800082).

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