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MicroRNA-30a controls the instability of inducible CD4+ Tregs through SOCS1

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Abstract. Inducible regulatory T cells (iTregs) are an important subset of Tregs and play a role in the maintenance of peripheral tolerance, and the occurrence of a number of diseases, including tumors and autoimmune diseases. However, the instability of iTregs is a major obstacle for their potential application in clinical trials. The underlying mechanism of iTreg instability remains largely unknown. In the present study, the expression level of microRNA (miRNA/miR)-30a in murine iTregs was evaluated using reverse transcription-quantitative PCR. miR-30a mimics and a miR-negative control (NC) were transiently transfected into iTregs using Nucleofector technology. The effects of miR-30a on the suppressive function of murine iTregs *in vitro* and *in vivo* were investigated using MTT, adoptive cell transfer (ACT) and flow cytometry assays, as well as a murine model of lung cancer. In the present study, it was identified that the expression level of miR-30a was lower in murine iTregs *in vitro* compared with natural (n)Tregs. Furthermore, compared with miR-NC, miR-30a mimics impaired the suppressive function of murine iTregs on murine CD4+ T cell proliferation *in vitro*, which was accompanied by the altered expression of cytotoxic T lymphocyte-associated antigen 4 and glucocorticoid induced tumor necrosis factor receptor, as well as transforming growth factor-β and interleukin-10. It was also observed that, compared with miR-NC, miR-30a mimics abrogated the suppressive effects of murine iTregs on murine CD8+ T cell function *in vivo*, producing an effective antitumor effect in mice bearing 3LL lung cancer cells in the ACT assay. From a mechanistic point, the expression level of suppressor of cytokine signaling 1, a putative target of miR-30a, was elevated, altering the activation of the Akt and STAT1 pathway in the miR-30a mimic transfected group compared with the miR-NC group, reducing the suppressive function of murine iTregs. The present study identified a role for miR-30a in the instability of iTregs and provided a novel insight into the development of therapeutic strategies for promoting T-cell immunity via the regulation of iTreg instability by targeting specific miRNAs.

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Abbreviations: iTregs, inducible regulatory T cells; Foxp3, forkhead box protein 3; TGF-β, transforming growth factor-β; SOCS1, suppressor of cytokine signaling 1; ACT, adoptive cell transfer

Key words: CD4+Foxp3+ Treg cells, microRNA-30a, lung cancer, SOSC1, TGF-β

Introduction

CD4+CD25+ regulatory T cells (Tregs), a subpopulation of T cells positive for CD4 that constitutively express the transcription factor forkhead box protein3 (Foxp3), account for 5-10% of the peripheral CD4+ T cells in normal humans and mice (1,2). Inducible Tregs (iTregs), a subset of Tregs, play an important role in a number of immune diseases by regulating the function of conventional CD4+ T cells and CD8+ T cells (3,4). Moreover, iTregs have also been reported to be promising targets in the development of immunotherapies against various immune related diseases (5,6). However, the instability of iTregs, including the loss of Foxp3 expression and their ability to suppress conventional CD4+ T cells and CD8+ T cells, is an obstacle for clinical research (7,8). Previous studies showed that microRNAs (miRs/miRNAs), noncoding RNAs that negatively regulate the post-transcriptional expression of their target genes and are 20-22 nucleotides in length, play an important role in the induction and instability of iTregs (9,10). It has been reported that miR-155 regulates the induction and suppressive function of iTregs through the suppressor of cytokine signaling 1 (SOCS1) pathway (11). Furthermore, the miR-17-92 cluster was reported to be involved in the induction of iTregs though Ikaros family zinc finger protein 4 (12). Similarly, our previous study showed

that miR-126 may regulate the induction of iTregs via the Akt pathway (13). However, the exact role and the mechanisms of distinct miRNAs related to the instability of iTregs have not been fully elucidated.

miR-30a is a member of the miRNA-30 family, which is associated with the inflammatory response, and the development and progression of various types of cancer (14-16). Previous studies have shown that miR-30a may be a promising new target for gene therapy against breast cancer and other types of tumors (17,18). With regards to the immune system, previous studies have suggested a potential role for miR-30a in the function of immune cells and the development of related diseases (19-21). Wu *et al (*22) found that miR-30a regulates the function of macrophages in response to *Mycobacterium tuberculosis* (MTB) stimulation by altering the expression of myeloid differentiation primary response 88. Fang *et al (*23) reported that overexpression of miR-30a in microglia promotes the apoptosis of oligodendrocyte precursor cells and inhibits their differentiation. Furthermore, the overexpression of miR-30a in transplanted microglia exacerbates the progression of experimental autoimmune encephalomyelitis (EAE), which is associated with a change in the expression of peroxisome proliferator-activated receptor-γ coactivator 1-β (23). In addition, Qu *et al* (24) reported that miR-30a regulates the differentiation of T-helper 17 (Th17) cells by targeting the interleukin (IL)-21 receptor, contributing to the development of EAE. However, the potential role of miR-30a in other immune cells types, including CD4+ Tregs remains to be explored.

In the present study, the expression pattern of miR-30a in murine CD4+ iTregs was investigated. The present study identified that the overexpression of miR-30a reduced the expression of Foxp3 in murine CD4+ iTregs and impaired their suppressive abilities *in vitro*. An adoptive cell transfer (ACT) assay showed that the overexpression of miR-30a abrogated the suppressive capacity of iTregs on the function of murine CD8+ T cells in a murine lung cancer model, which was accompanied by the altered expression of CD69, CD44 and interferon (IFN)-γ in murine CD8+ T cells. To the best of our knowledge, the data in the present study demonstrated a previously unknown role of miR-30a in the instability of iTregs and provided a novel insight into the development of therapeutic strategies for promoting T-cell immunity via the regulation of iTregs by targeting specific miRNAs.

Materials and methods

Animals. In total, 40 female B6.Cg-Foxp3^{tm2Tch}/J mice (cat. no. 006772; age, 7-10 weeks) and syngeneic recombination activating gene (RAG) 1$^{-/-}$ mice (cat. no. 002216; 7-10 weeks old) (n=120) were purchased from the Jackson ImmunoResearch Laboratories, Inc. All mice were maintained in controlled conditions at a temperature of 22-24˚C, a humidity of 55±5% with a 12 h light/dark cycle and free access to water and food in specific pathogen-free rooms in the Experimental Animal Laboratory of Zunyi Medical University. All animal experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals and all the experimental procedures were approved by the Zunyi Medical Laboratory Animal Care and Use Committee (permit no. 20150312).

Cell lines and reagents. The C57BJ/6 metastatic lung cancer cell line 3LL was a gift from Professor Chu (Shanghai Medical College, Fudan University). The cells were cultured at 37˚C with 5% CO_2 in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) containing 10% heat-inactivated FBS (Gibco; Thermo Fisher Scientific, Inc.), 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin sulfate. The miR-30 mimic (sense, UGUAAACAUCCUCGACUGGAAG and antisense, UCCAGUCGAGGAUGUUUACAUU) and a corresponding miR-negative control (NC; sense, UUCUCCGAACGUGUC ACGUTT and antisense, ACGUGACACGUUCGGAGAATT) were purchased from Ambion (Thermo Fisher Scientific, Inc.). All other reagents were purchased from Sigma-Aldrich (Merck KGaA) unless otherwise stated.

Preparation of murine CD4+ T cells. The spleens from normal B6.Cg-Foxp3^{tm2Tch}/J mice were dissected, added to PBS ready for homogenization and passed through a 70 mm cell strainer at room temperature. Erythrocytes were removed by resuspending cells in ACK lysis buffer (Beyotime Institute of Biotechnology) for 5 min at room temperature before the addition of PBS. Splenocytes were pelleted by centrifugation (600 x g at room temperature) and resuspended in PBS. CD4+ CD62L+ T cells were isolated from the splenic single cell suspensions using MACS® (cat. no. 130-106-643; Miltenyi Biotec, Inc.), according to the manufacturer's protocol, for the iTreg cell differentiation assay. In addition, CD4+ CD25- T cells were also isolated from the splenic single cell suspensions using MACS®, according to the manufacturer's protocol, for the suppressive assay *in vitro*.

iTreg cell differentiation assay. According to our previous study (13), 4x10^6 CD4+ CD62L+ T cells were cultured with 2 μg/ml anti-CD3 antibody (cat. no. MAB848-100; R&D Systems, Inc.) and 4 μg/ml anti-CD28 antibody (cat. no. MAB4832; R&D Systems, Inc.), in complete RPMI-1640 (10% FBS and 100 U of penicillin and streptomycin) containing 20 pmol/ml transforming growth factor (TGF)-β (Novus Biologicals, Ltd.) and 200 IU/ml IL-2 (Novus Biologicals, Ltd.). After 7 days of culture at 37˚C, the cells were harvested and the presence of Foxp3+ T cells was determined by flow cytometry.

Transient transfections. For transient transfections, miR-30a mimics (10 ng) or the miR-NC (10 ng) was mixed with 100 μl of T-cell Nucleofector solution (Amaxa; Lonza Group, Ltd.) and transfected into 2x10^6 iTreg cells by electroporation using a Nucleofector II instrument (program no. T-020; Amaxa; Lonza Group, Ltd.) using Nucleofector II buffer (Amaxa; Lonza Group, Ltd), according to the manufacturer' protocol. The transfection efficiency was ~65% as evaluated by flow cytometry using a fluorescently-labeled mimic control (GE Healthcare Dharmacon, Inc.). After transfection, the cells were allowed to recover for 4 h at 37˚C before use in the subsequent experiments.

Suppressive assay in vitro. To test iTregs suppressive activity, 5x10^4 CD4+CD25- cells were treated with 2 μg/ml anti-CD3 antibody and 4 μg/ml anti-CD28 antibody for 12 h as effector cells. The effector cells were incubated with or without iTregs at a ratio of 2:1 for 72 h in complete RPMI-1640 supplemented

with 5% FCS. Proliferation was analyzed using an MTT assay. DMSO was used to dissolve the formazan crystals and the absorbance was measured at a wave length of 570 nm.

Flow cytometry. Cells were incubated with blocking buffer (1% BSA and 0.1% saponin) for 30 min at 4°C and washed with PBS twice. Next, the surface markers of various immune cells were evaluated using flow cytometry performed using the Beckman Gallios flow cytometer (Beckman Coulter, Inc.) and using CellQuest Pro software (version 5.1; BD Biosciences). The following antibodies were used: CD8-allophycocyanin (cat. no. MCD0805), Ki-67-FITC (cat. no. 11-5699-42), CD69-phycoerythrin (cat. no. 12-0691-83), CD44-PE (cat. no. 12-0441-82), IFN-γ-PE (cat. no. 12-7319-82), granzyme B-FITC (cat. no. 11-8898-82) and the corresponding isotype-matched controls (cat. nos. 11-4321-42, 12-4321-80 and 17-4321-81; all from eBioscience; Thermo Fisher Scientific, Inc.). The antibodies were used at a dilution of 1:100 for 30 min at 4°C. To determine the proportion of $CD8^+$ T cells and the surface molecule expression of CD44 and CD69 on $CD8^+$ T cells, cells were stained with the aforementioned antibodies (1:100) at 4°C for 30 min and washed with PBS twice before analysis, according to the manufacturer's protocol.

To determine the expression levels of CTLA-4 and GITR on iTregs, iTregs were stained with CTLA-4-FITC (cat. no. ab24935; Abcam) and GITR-FITC (cat. no. ab72404; Abcam) at a dilution of 1:100 at 4°C for 30 min, and washed with PBS twice before analysis.

Reverse transcription-quantitative (RT-q)PCR. RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.), according to manufacturer's protocol. Primers were obtained from Sangon Biotech Co., Ltd. The TaqMan MicroRNA Reverse Transcription kit and the TaqMan Reverse Transcription kit were obtained from Takara Bio, Inc. The qPCR reactions for determining the level of miRNA or mRNA expression were performed using miScript SYBR Green PCR Kit (Exiqon; Qiagen GmbH) or QuantiFast SYBR Green PCR kit (Qiagen GmbH), respectively. RT reactions and qPCR assays were performed according to the manufacturer's protocol. The RT reactions were performed as follows: 16°C for 15 min followed by 42°C for 30 min and 85°C for 5 min. The qPCR reaction was conducted with an initial denaturation step at 95°C for 15 min, followed by 40 cycles of 94°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec. All RT reactions, including the no template and no reverse transcriptase controls, were performed in triplicate using a Bio-Rad CFX96 (Bio-Rad Laboratories, Inc.). The following primers were obtained from Exiqon (Qiagen GmbH) and used for the analysis of miRNA expression: miR-126, 5'-CAUUAUUACUUUUGGUACGCG-3' (cat. no. YP00206010); miR-31, 5'-AGGCAAGAUGCUGGCAUAGCUG-3' (cat. no. MS00001407); miR-125b, 5'-UCCCUGAGACCCUAACUUGUGA-3' (cat. no. MS00005992); miR-181a, 5'-AACAUUCAACGCUGUCGGUGAGU-3' (cat. no. YP00206081); miR-30a, 5'-UGUAAACAUCCUCGACUGGAAG-3' (cat. no. MS00011704); miR-27a, 5'-UUCACAGGGCUAAGUUCCGC-3' (cat. no. MS00001351); miR-101a, 5'-UACAGUACUGUGAUAACUGAA-3' (cat. no. MS00011011); and U6, 5'-AGAGAAGATTAGCATGGCCCCTG-3'. In addition, the following primers were also used: IL-10 forward, 5'-TACACCGGGAAGACAATAA-3' and reverse, 5'-AGGAGTCGGTTAGCAGTATG-3'; TGF-β forward, 5'-GGCGGTGCTCGCTTTGTA-3' and reverse, 5'-TCCCATGTCTGACGTATTGA-3'; SOCS1 forward, 5'-TTCGACTGCCTTTTCGAGCT-3' and reverse, 5'-GAAGAAGCAGTTCCGTTGGC-3'; and GAPDH forward, 5'-GAGCCAAACGGGTCATCATCT-3' and reverse, 5'-GAGGGGCCATCCACAGTCTT-3'. The samples were normalized to GAPDH for mRNA or U6 for miRNA expression. The relative expression was calculated using the comparative quantification cycle (Cq) method (25).

ACT. ACT was performed as previously described with some modifications (13). Syngeneic female $RAG1^{-/-}$ mice (6-8 weeks age) were divided into three groups: i) $CD8^+$ T cell transfer only; ii) $CD8^+$ T cell with iTregs transfected with miR-30a mimic; and iii) $CD8^+$ T cell with iTregs transfected with miR-NC. C57/BJ Mice (6-8 weeks old) were injected subcutaneously with 0.2 ml of a single-cell suspension in PBS containing $2x10^5$ 3LL lung cancer cells in the right lateral abdomen region. After 7 days, 3LL specific $CD8^+$ T cells, with or without iTregs transiently transfected with miR-30a mimic (10 ng) or miR-NC (10 ng) at a ratio of 2:1, were transferred into the tail vein. The size of the tumor mass in each group was determined 21 days later. The tumor volumes were measured and calculated according to the following formula: $V=(W^2 \times L)/2$, where V is the tumor volume, W is the width and L is the length. To obtain 3LL-specific $CD8^+$ T cells, C57BL/6J mice were immunized subcutaneously with $1x10^6$ mitomycin C-treated 3LL cells (10 μg/ml mitomycin C for 2 h) three times in total, every 7 days. After 10 days, the splenocytes were harvested and re-stimulated with mitomycin C-treated 3LL cells for 24 h at 37°C in the presence of IL-2 (50 U/ml) *in vitro*. Following this, $2x10^6$ 3LL specific $CD8^+$ T cells were purified by fluorescence assisted cell sorting (*MoFlo* Astrios Cell Sorter; Beckman Coulter, Inc) and adoptively transferred into syngeneic $RAG1^{-/-}$ mice bearing 3LL cells.

Intracellular staining for IFN-γ and granzyme B. Syngeneic $RAG1^{-/-}$ mice bearing 3LL cells were sacrificed by cervical dislocation following an overdose of anesthesia performed by intraperitoneal injection of 150 mg/kg pentobarbital sodium. Tumor infiltrating lymphocytes were isolated according to a previous report (13). After staining for CD8, cells were fixed and permeabilized using the Cytofix/Cytoperm and Perm/Wash buffers (both from BD Biosciences), according to the manufacturer's protocol. Cells were stained with IFN-γ-PE (1:100) or granzyme B-FITC (1:100) at 25°C for 20 min and washed twice in Perm/Wash buffer before flow cytometry analysis.

Ki-67 staining assay. Tumor infiltrating lymphocytes (TILs) were isolated as aforementioned. After staining for CD8, cells were fixed and permeabilized using the Cytofix/Cytoperm and Perm/Wash buffers (both from BD Biosciences), according to the manufacturer's protocol. Cells were stained with Ki-67-FITC (1:100) at 25°C for 20 min and washed twice in Perm/Wash buffer before flow cytometry analysis.

Plasmid construction, preparation and transfection. To identify putative targets of miR-30a for plasmid construction,

miRNA target prediction programs were used, including TargetScan (http://www.targetscan.org/vert_42/, Version 4.2), miRanda (http://www.microrna.org/microrna/getMirnaForm.do) and PicTar (https://pictar.mdc-berlin.de/). The SOCS1 coding sequence (NM_001271603) was amplified using RT-PCR from mouse cDNA from CD4+ T cells and cloned into the pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.) to generate the pcDNA3.1-SOCS1 plasmid (p-SOCS1). PrimeScriptTM RT-PCR kit was obtained from Takara Bio, Inc. The RT reaction was performed as aforementioned. The PCR reaction was conducted with an initial denaturation step at 95˚C for 5 min, followed by 40 cycles of 94˚C for 15 sec, 56˚C for 15 sec and 72˚C for 60 sec. The SOCS1 sequence was amplified from cDNA derived from CD4+ T cells using the following primers: Forward, 5'-GCGGATCCAGCAGAGAGA ACTGCGGCCGTG-3' and reverse, 5'-GGAAGCTTAGCG GCAGCCGGTCAGATCTG-3'. The product was subcloned into the *BamH*I and *Hind*III sites of the pcDNA3.1 vector. To construct p-SOCS1-WT luciferase (Luc), the 3'untranslated region (UTR; the sequence from 200 to 350 bp downstream of the stop codon) of SOCS1 was amplified from cDNA derived from CD4+ T cells using the following primers: Forward, 5'-GCCTCGAGCCTGGTTGTAGCAGCTTG-3' and reverse, 5'-GGGTCGACTTGTGCAAAGATACTG-3'. To construct p-SOCS1-mut luciferase (Luc), the following primers were used: forward, 5'-GCCTCGAGCCTGGTTGTAGCAGCT TG-3' and reverse, 5'-GGGTCGACTCGTTCGTGGAAG AGGTAG-3'. The PCR reaction was conducted with an initial denaturation step at 95˚C for 5 min, followed by 40 cycles of 94˚C for 10 sec, 56˚C for 10 sec and 72˚C for 10 sec. The PCR products were subcloned into *Xho*I and *Sal*I sites of the pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega Corporation). The identity of the clone was verified using restriction enzyme digest analysis and DNA sequencing. Endotoxin-free plasmids were purified using the Endofree plasmid mega kit (Qiagen GmbH).

For transient transfections, 10 μg of the p-SOCS1 plasmid or the control plasmid (p-Cont) plasmids were mixed with or without miR-30a mimic (10 ng) in 100 μl T-cell Nucleofector solution and used to transfect $2x10^6$ iTreg cells by electroporation using a Nucleofector II instrument as aforementioned. After transfection, the cells were allowed to recover in RPMI1640 for 4 h at 37˚C prior to subsequent experiments.

Luciferase assay. CD4+CD62L+ cells were transiently co-transfected with wild-type or mutated pSOCS1 Luc (10 μg) and miR-30 mimic (10 ng) or 10 μg the corresponding pmirGLO control (Promega Corporation) using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. The cells were cultured in the presence of 2 μg/ml anti-CD3 and 4 μg/ml CD28 antibody. After 24 h, cell were harvested and lysed in passive lysis buffer (Promega Corporation). Luciferase and Renilla activities were measured with a luminometer using luciferin (Promega Corporation) and coelenterazine (Biotium) as substrates, respectively. Luciferase activity was measured at a wavelength of 560 nm. Renilla activity was measured at a wavelength of 465 nm. Transfection efficiency was normalized using Renilla activity.

Western blotting. Cells were lysed with RIPA lysis buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml protease inhibitors and 1 μg/ml phosphatase inhibitors) on ice for 30 min. The protein concentration was determined using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc.). Equal amounts of protein (15 μg) were subjected to 12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat dry milk in PBS with 0.1% Tween-20 for 1 h at 37˚C. The membranes were then incubated overnight at 4˚C with the following antibodies at a dilution of 1:100: SOCS1 (cat. no. PA5-27239; Invitrogen; Thermo Fisher Scientific, Inc.), phosphorylated (phospho)-Akt (cat. no. 44-621G; Invitrogen; Thermo Fisher Scientific, Inc.), total Akt (cat. no. PA5-77855; Invitrogen; Thermo Fisher Scientific, Inc.), phospho-STAT1 (cat. no. 44-376G; Invitrogen; Thermo Fisher Scientific, Inc.) and total STAT1 (cat. no. PA5-95267; Invitrogen; Thermo Fisher Scientific, Inc.). The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at a dilution of 1:1,000 (cat. no. G-21234; Invitrogen; Thermo Fisher scientific, Inc.) for 1 h at room temperature. Protein bands were visualized using an ECL detection system.

Statistical analysis. All experiments were performed three times independently. The data were analyzed using GraphPad Prism 5.0 (GraphPad Software, Inc.) and are presented as the mean ± SD. Student's t-test was used when two conditions were compared and ANOVA with Bonferroni or Newman-Keuls correction was used for multiple comparisons. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of miR-30a in iTregs. To investigate the possible role of miR-30a in the instability of CD4+ iTregs, iTregs were generated *in vitro* according to a previous report13). The relative expression levels of several miRNAs, including miR-181, miR-126, miR-31 and miR-30a were determined. These miRNAs were selected according to previous studies (13,26,27). The expression level of miR-126 in iTregs was higher than that in natural (n)Tregs (Fig. 1A), which was consistent with our previous study (13). The expression levels of miR-31 and miR-125b were also higher in iTregs compared with nTregs. However, the expression levels of miR181a, miR-30a, miR-27a and miR-101a were significantly lower in iTregs (Fig. 1A; $P<0.05$).

To analyze the potential role of these four downregulated miRNAs in the instability of iTregs, re-stimulated iTregs were analyzed for change in the expression of these miRNAs. The suppressive capacity of iTregs decreased during the re-stimulation time course (Fig. 1B). It was found that only the expression of miR-30a increased during the time course (Fig. 1B), which was negatively associated with the suppressive capacity of the iTregs. Therefore, the present data suggested that miR-30a may be involved in the instability of the suppressive function of iTregs.

miR-30a reduces the suppressive function of iTregs in vitro. The possible effect of miR-30a on the suppressive capacity

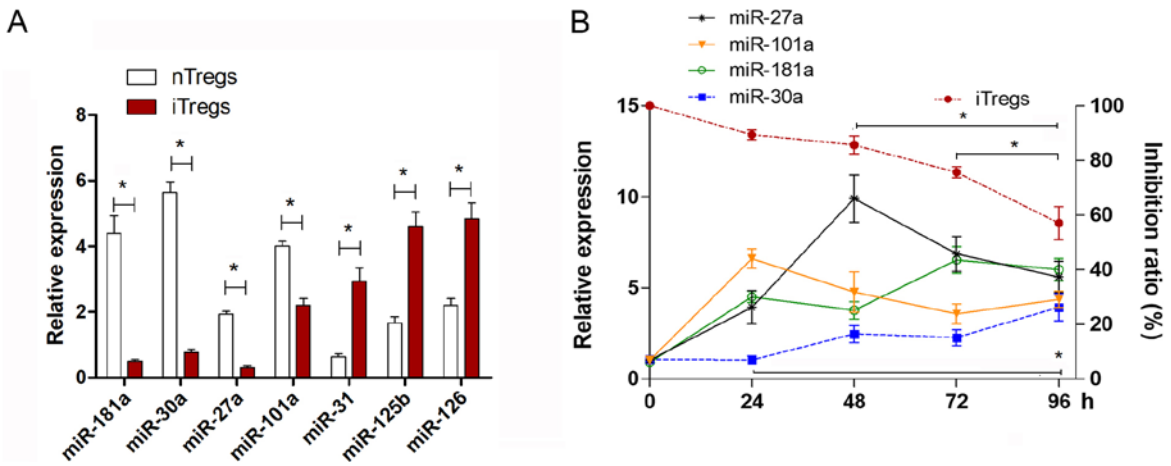

Figure 1. Relative expression of miR-101, miR-30a, miR-27a and miR-181a in iTregs. (A) Expression levels of the indicated miRNAs in iTregs and nTregs were analyzed using reverse transcription-quantitative PCR. (B) iTregs were re-stimulated in the presence of anti-CD3 and anti-CD28 antibodies plus interleukin-2 (200 IU/ml) at the indicated times. The relative expression of the miRNAs and the suppressive capacity of these cells on the proliferation of $CD4^+CD25^-$ T cells were analyzed. *P<0.05. miR, microRNA; iTregs, inducible regulatory T cells; nTregs, natural regulatory T cells.

of iTregs was investigated. The expression level of miR-30a in iTregs in the miR-30a mimic transfected group increased significantly compared with the miR-NC transfected group (Fig. 2A; P<0.05). Compared with the miR-NC transfected group, the expression levels of cytotoxic T lymphocyte-associated antigen 4 and glucocorticoid induced tumor necrosis factor receptor, functional surface molecules, were significantly reduced on the iTregs in the miR-30a mimic group (Fig. 2B and C; P<0.05). The expression of IL-10 and TGF-β in iTregs also decreased significantly following miR-30a overexpression (Fig. 2D; P<0.05). To investigate this finding, changes to the suppressive activity of iTregs *in vitro* were also examined. The present data suggested that the suppressive activity of iTregs on $CD4^+CD25^-$ T cell proliferation in the miR-30a mimic group was lower than in the miR-NC group (Fig. 2E; P<0.05). These data suggested that miR-30a regulated the suppressive capacity of iTregs *in vitro*.

miR-30a reduces the suppressive function of iTregs in vivo. To investigate the effect of miR-30a on the suppressive capacity of iTregs *in vivo*, an ACT assay was performed. Tumor size increased significantly in the $CD8^+$ T cells + iTregs co-transferred group compared with the group transferred with only $CD8^+$ T (Fig. 3A; P<0.05). However, it was found that the size of the tumor was significantly smaller in the miR-30a mimic-transfected iTregs group compared with the miR-NC group (Fig. 3A; P<0.05). The change in the function of $CD8^+$ T cells *in vivo* was further analyzed. The expression levels of CD69 and CD44 on $CD8^+$ T cells increased significantly in the miR-30a mimic-transfected iTregs + $CD8^+$ T cells co-transferred group compared with the miR-NC-transfected iTregs + $CD8^+$ T cells co-transferred group (Fig. 3B; P<0.05). In addition, the proliferation and IFN-γ secretion of $CD8^+$ T cells increased significantly in the miR-30a mimics-transfected iTregs + $CD8^+$ T cells co-transferred group (Fig. 3C-E; P<0.05). In $CD8^+$ T cells, the level of secreted granzyme B, a type of molecule associated with cytotoxicity, increased significantly in the miR-30a mimic-transfected iTregs group compared with the miR-NC group (Fig. 3F and G; P<0.05). These findings suggested that miR-30a regulated the suppressive activity of iTregs *in vivo*.

Expression of SOCS1 in iTregs. Previous studies showed that SOCS1 is important for the expression of Foxp3 in Tregs (28,29). To investigate the potential molecular mechanism through which miR-30a regulates the suppressive activity of iTregs, putative targets of miR-30a were identified using computer-aided miRNA target prediction programs, including TargetScan (http://www.targetscan.org/vert_42/, Version 4.2), miRanda (http://www.microrna.org/microrna/getMirnaForm.do) and PicTar (https://pictar.mdc-berlin.de/), and found a 'matched seed sequence' of miR-30a in the 3'UTR of SOCS1 at position 285-292 (Fig. 4A). The expression level of SOCS1 was determined in iTregs. The present data suggested that the expression level of SOCS1 was significantly downregulated by >5-fold in iTregs transfected with the miR-30a mimic compared with the miR-NC group (Fig. 4B; P<0.05). The regulation effect of miR-30a on SOCS1 expression was further examined. The protein expression levels of SOCS1 decreased significantly in iTregs transfected with miR-30a mimic compared with miR-NC (P<0.05; Fig. 4C). The luciferase assay showed that miR-30a could bind to the putative sites of wild-type SOCS1 (Fig 4D; P<0.05). The present data suggested that SOCS1 may be a novel target of miR-30a in iTregs.

miR-30a regulates the suppressive function of iTregs by regulating SOCS1. To further elucidate the role of SOCS1 in the miR-30a-mediated regulation of iTregs suppressive capacity, a eukaryotic expression vector encoding SOCS1 was constructed. It was found that the expression level of SOCS1 increased significantly in the p-SOCS1 transfected group compared with the control (Fig. S1; P<0.05), indicating the effective transfection of p-SOCS1. Compared with the miR-30a mimic transfected group, the expression level of Foxp3 in iTregs in the miR-30a mimic and p-SOCS1 co-transfected group did not change significantly (data not shown). It was found that the expression levels of IL-10 and TGF-β in iTregs in the miR-30a mimic and p-SOCS1 co-transfected

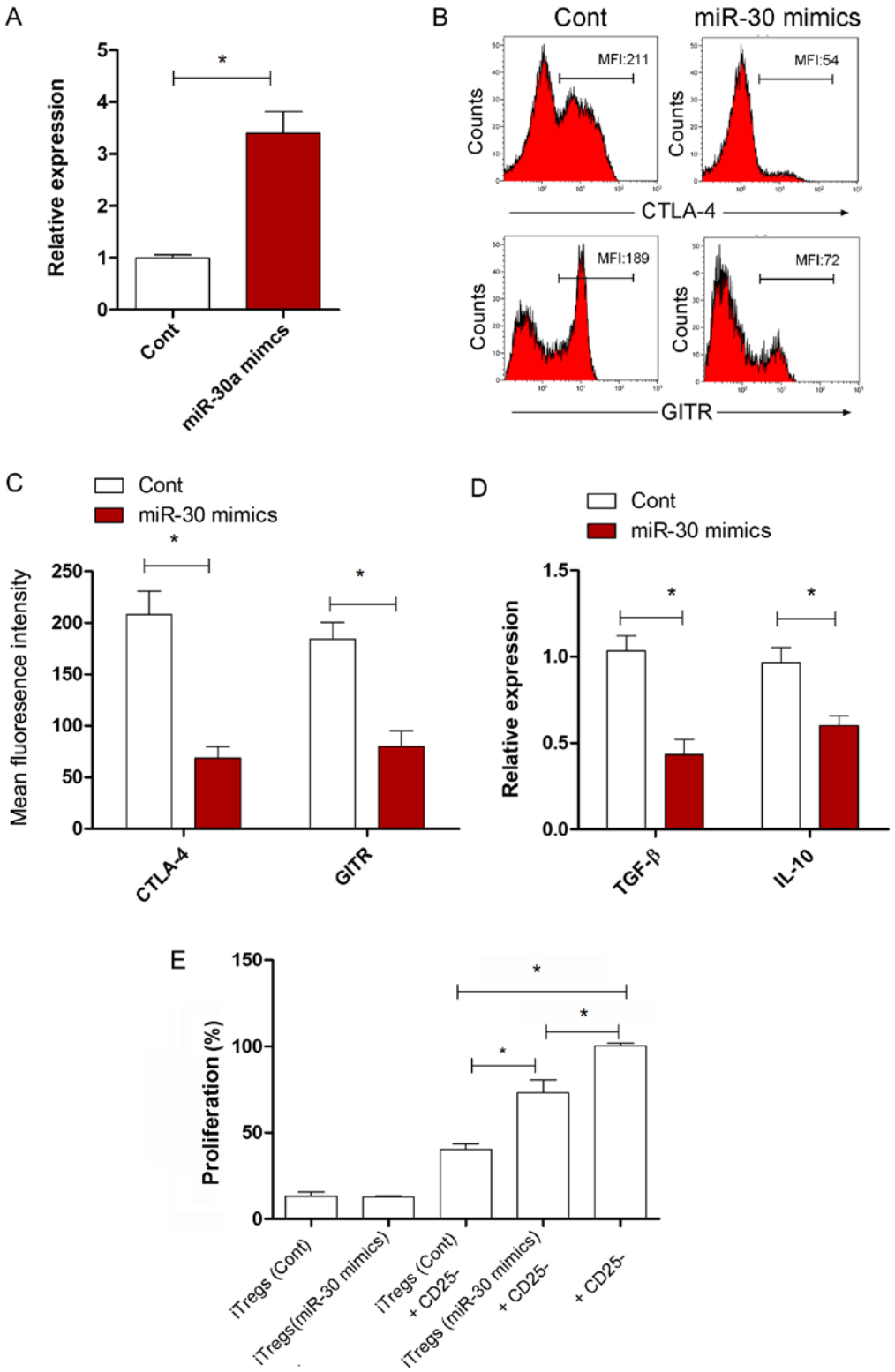

Figure 2. miR-30a reduces the suppressive activity of iTregs *in vitro*. (A) The expression level of miR-30a was analyzed using RT-qPCR. (B) The expression levels of CTLA-4 and GITR were analyzed using flow cytometry and (C) quantified. (D) The relative expression levels of TGF-β and IL-10 were determined using RT-qPCR. (E) The suppressive activity of iTregs on the proliferation of $CD4^+CD25^-$ T cells was analyzed. A representative of three independent experiments is shown. *P<0.05. miR, microRNA; iTregs, inducible regulatory T cells; IL, interleukin; Cont, control; CTLA-4 cytotoxic T lymphocyte-associated antigen 4; GITR, glucocorticoid induced tumor necrosis factor receptor; TGF-β, transforming growth factor-β; RT-qPCR, reverse transcription-quantitative PCR; MACS, MFI.

group increased significantly compared with the miR-30a mimic and p-Cont co-transfected group (Fig. 5A; P<0.05). The suppressive activity of iTregs in the miR-30a mimic and p-SOCS1 co-transfected group was higher than the miR-30a mimic and p-Cont co-transfected group *in vitro* (Fig. 5B; P<0.05), indicating that the altered expression of SOCS1 could

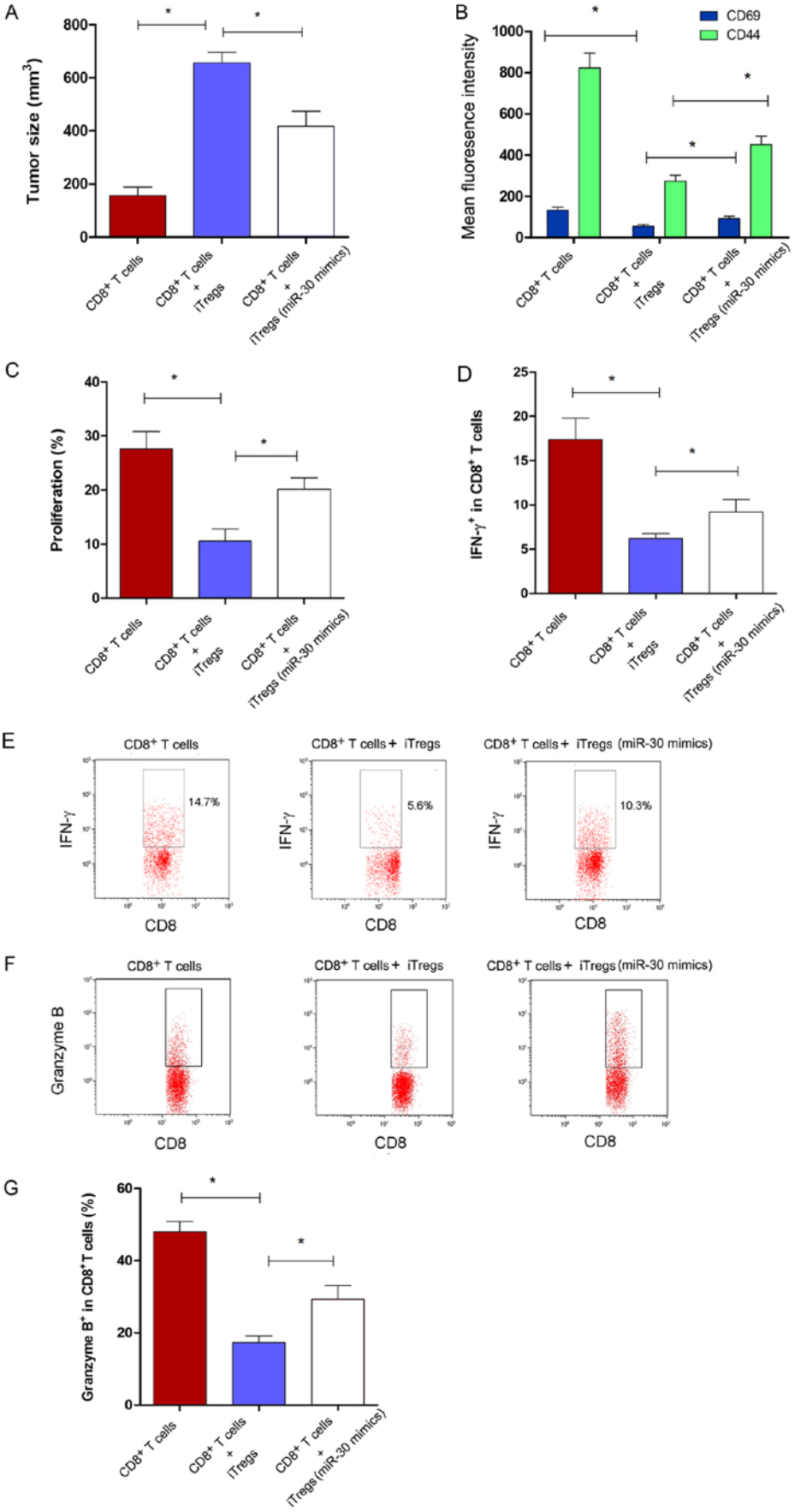

Figure 3. miR-30a reduces the suppressive activity of iTregs *in vivo* (A) The tumor weight was determined on day 21. (B) After 10 days, tumor infiltrating lymphocytes were obtained. The expression levels of the surface molecules CD69 and CD44 on $CD8^+$ T cells were determined by flow cytometry. (C) Proliferation of $CD8^+$ T cells was determined using the Ki-67 assay. (D) Percentage of $CD8^+$ T cells secreting IFN-γ was analyzed by flow cytometry. (E) Flow cytometry results for IFN-γ. (F) The expression of granzyme B on $CD8^+$ T cells was analyzed by flow cytometry and (G) quantified. A representative of three independent experiments is shown. n=6 in each group. $^*P<0.05$. miR, microRNA; iTregs, inducible regulatory T cells; IFN-γ, interferon-γ.

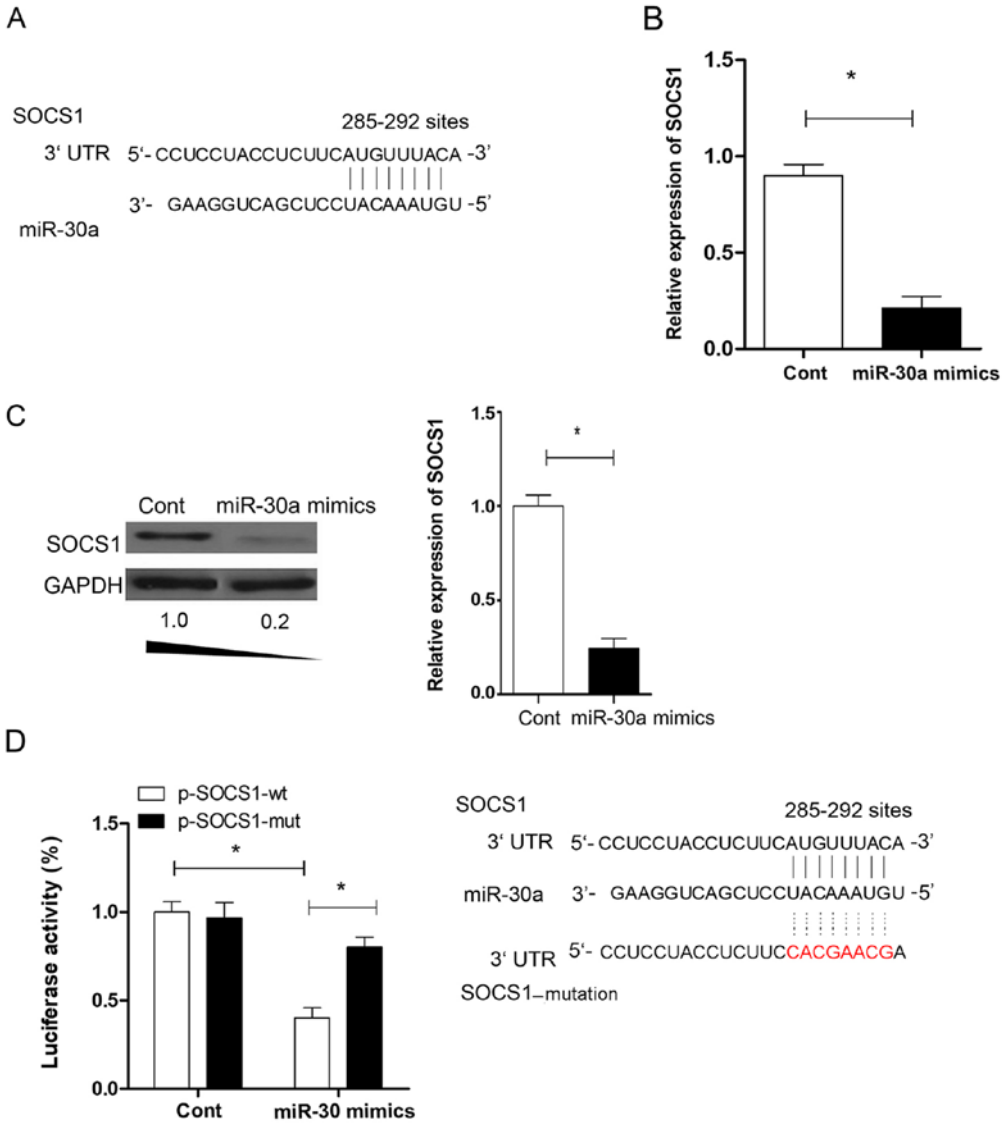

Figure 4. miR-30a regulates the expression of SOCS1. (A) Analysis of miR-30a and the SOCS1 sequence. (B) $CD4^+CD62L^+$ T cells were purified from splenocytes of B6.Cg-Foxp3^{tm2Tch}/J mice using magnetic-activated cell sorting (MACS) and were cultured with anti-CD3, anti-CD28, transforming growth factor-β (20 pmol/ml) and interleukin-2 (200 IU/ml). After 7 days, $Foxp3^+$ T cells were purified using fluorescence activated cell sorting. Cells were transiently transfected with 10 ng miR-30 mimic or Cont and cultured *in vitro*. After 24 h, the relative expression of SOCS1 was determined using reverse transcription-quantitative PCR. (C) The protein level of SOCS1 was determined using western blot analysis. (D) A luciferase assay was performed. A representative of three independent experiments was shown. *P<0.05. SOCS1, suppressor of cytokine signaling 1; miR, microRNA; WT, wild-type; mut, mutated; UTR, untranslated region.

reverse the effect of miR-30a on the suppressive capacity of iTregs *in vitro*.

Previous studies have reported that the STAT1 and the Akt pathways are involved in the induction and function of iTregs (28,30). Therefore, the present study analyzed the changes in these pathways. The present data showed that, compared with the miR-30a mimic and p-Cont co-transfection group, the expression levels of total Akt and STAT1 did not change significantly in the iTregs in the miR-30a mimic and p-SOCS1 co-transfection group (Fig. 5C; P>0.05). However, the expression levels of phospho-STAT1 and phospho-Akt increased significantly in iTregs co-transfected with miR-30a mimic and p-SOCS1 compared with iTregs co-transfected with the miR-30a mimic and p-Cont (Fig. 5C; P<0.05).

To further examine the role of SOCS1 overexpression and the effect of miR-30a on the suppressive capacity of iTregs, ACT was performed. The present data suggested that the proliferation of $CD8^+$ T cells decreased significantly following co-transfer of iTregs transfected with p-SOCS1 and miR-30a mimic compared with $CD8^+$ T cells transferred alone (Fig. 5D; P<0.05), which was consistent with the aforementioned data (Fig. 5B). The proliferation of $CD8^+$ T cells decreased significantly in the miR-30a mimic + p-SOCS1 iTregs co-transferred group compared with the miR-30a mimic + p-Cont iTregs co-transferred group (Fig. 5D; P<0.05). The expression of IFN-γ and granzyme B in $CD8^+$ T cells decreased significantly in the miR-30a mimic and p-SOCS1 iTregs co-transferred group (Fig. 5E and F; P<0.05). Consistent with this, the tumor size increased significantly following co-transfer of $CD8^+$ T and iTregs co-transfected with miR-30a and p-SOCS1 compared with cells transfected with miR-30a and p-Cont (Fig. 5G; P<0.05). The present data

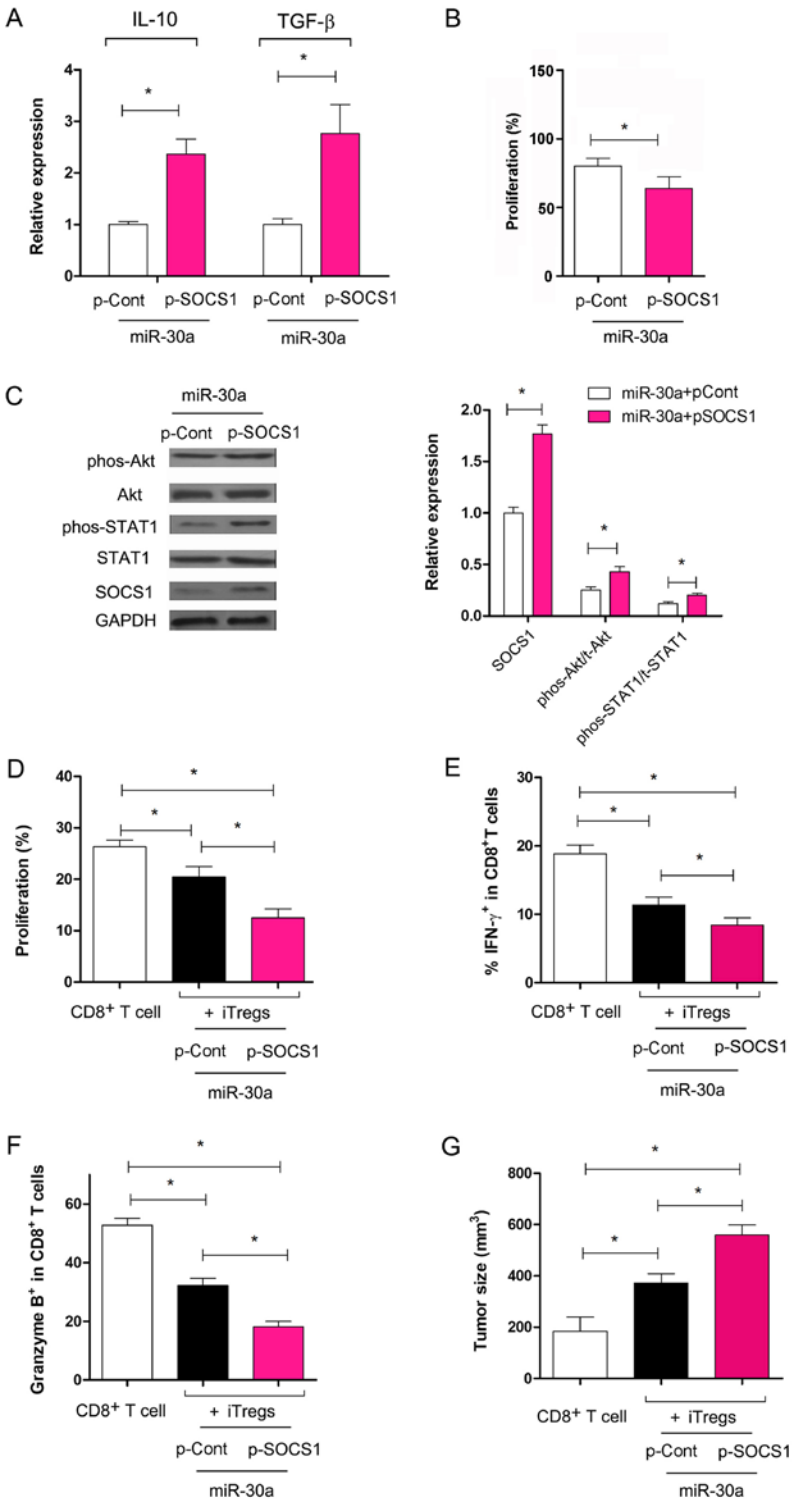

Figure 5. Overexpression of SOCS1 reverses the effect of miR-30a on the suppressive function of iTregs. (A) Relative expression levels of TGF-β and IL-10 were analyzed using reverse transcription-quantitative PCR. (B) The suppressive capacity of iTregs was determined. (C) Expression levels of SOCS1, Akt, phos-Akt, STAT1 and phos-STAT1 were analyzed using western blot analysis and quantified. (D) C57BL/6J mice were immunized with inactivated 3LL lung cancer cells three times every 7 days. After 10 days, splenocytes were harvested and re-stimulated using mitomycin C-treated 3LL cells for 24 h in the presence of IL-2 (50 U/ml) *in vitro*. Following this, $2x10^6$ 3LL specific $CD8^+$ T cells were purified by fluorescence assisted cell sorting and transferred with or without iTregs at a ratio of 2:1 into syngeneic recombination activating gene $1^{-/-}$ mice (n=6). After 10 days, tumor infiltrating lymphocytes were obtained. The expression level of Ki-67 in $CD8^+$ T cells was determined using flow cytometry. (E) The percentage of IFN-γ secreting cells and (F) the expression of granzyme B in $CD8^+$ T cells was analyzed using flow cytometry. (G) The tumor size was determined for each group. $^*P<0.05$. TGF-β, transforming growth-β; SOCS1, suppressor of cytokine signaling 1; miR, micro RNA; IL, interleukin; iTregs, inducible regulatory T cells; p-, plasmid; phos-, phosphorylated; Cont, control; IFN, interferon.

suggested that miR-30a regulated the suppressive capacity of iTregs through the expression of SOCS1, which was accompanied by an altered activity of the Akt and STAT1 pathways.

Discussion

In the present study, it was found that miR-30a regulated the suppressive function of iTregs *in vitro*. ACT assays showed that miR-30a abrogated the suppressive function of iTregs on $CD8^+$ T cells activity in a murine lung cancer model. This effect was accompanied by the altered expression of CD69, CD44 and IFN-γ in $CD8^+$ T cells. The overexpression of SOCS1, a target of miR-30a, reversed the effect of miR-30a on the suppressive function of iTregs.

In total, >700 miRNAs have been identified in mammals and have been implicated in a wide range of biological functions (31). Previous studies have shown that different subsets of Tregs express unique miRNA signatures (32,33). These miRNAs have been reported to play important roles in the development and function of Tregs (34-36). iTregs, a distinct subset of Tregs, were reported to play an important role in peripheral immune tolerance and may be a promising target in immune therapy against related clinical diseases; therefore, investigating the molecular mechanism of the regulation of induction and the functional maintenance of iTregs is required. For example, miR-155 was reported to modify the induction and suppressive function of Tregs (37,38) and miR-21 was found to regulate the induction of iTregs *in vitro* (9). A recent study showed that miR-27 contributed to the induction and function of iTregs (26). Similarly, in our previous study, it was found that miR-126 may be a novel regulator involved in the induction and function of iTregs (13). Collectively, these previous studies suggested that distinct miRNAs play different roles in the induction and maintenance of the suppressive function of iTregs. However, knowledge about the distinct miRNAs involved in the regulation of iTreg induction and their suppressive functions is limited. Therefore, it is important to explore the underlying molecular mechanism of iTreg development and function, and also to develop strategies for iTreg-based immune therapy.

Recently, miR-30a, a member of the miRNA-30 family, was reported to play an important role in the biological function of a number of immune cells (9,16-18). Wu *et al* (22) found that miR-30a regulated the function of macrophages in response to MTB stimulation. The overexpression of miR-30a in transplanted microglia exacerbated the progression of EAE (23). In the present study, miR-30a was identified to regulate the suppressive capacity of iTregs. Consistent with this, Qu *et al* (24) reported that miR-30 regulated the differentiation of Th17 cells by targeting the IL-21 receptor, contributing to the development of EAE. The present study found that miR-30a regulated the suppressive functions of iTregs on $CD8^+$ T cells, which provided a robust antitumor effect of $CD8^+$ T cells *in vivo*, indicating that miR-30a may be a potential novel target in iTreg-based immunotherapy against tumors. Consistent with our previous study, it has been reported that the depletion of Tregs enhances the antitumor function of TILs *in vivo* (39). Collectively, the present and previous results suggested that immunotherapy targeting Tregs may represent an alternative to ACT immunotherapy to enhance antitumor efficacy.

Previous studies suggested that the SOCS1 protein is an important factor in the development of various diseases (40,41). SOCS1 overexpression was reported to decrease the proliferation and induce the apoptosis of cancer cells, which involved the Akt and STAT1 pathways (42). The dysregulation of SOCS1 signaling participates in various pathological processes in systemic lupus erythematosus, such as hematological abnormalities and autoantibody generation, which is associated with the JNK-STAT pathway (43). SOCS1 was also reported to be a key regulator of Treg development (44,45). A deficiency in SOCS1 impairs the suppressive functions of iTregs *in vivo* (29). In the present study, it was found that miR-30a regulated the expression of SOCS1 in iTregs. It was further shown that the overexpression of SOCS1 reversed the effects of miR-30a on the maintenance of the suppressive capacity of iTregs. These data may provide new evidence for the important role of the miR-30a/SOCS1 axis in the suppressive function of iTregs. Takahashi *et al* (28) reported that, in the absence of SOCS1, the suppressive function of iTregs did not change significantly *in vitro*, which may be related to the hypermethylation of the Foxp3 promoter. However, in the present study, it was found that miR-30 regulated the suppressive capacity of iTregs *in vitro* and *in vivo*. Moreover, the overexpression of SOCS1 reversed the effects of miR-30a on the suppressive capacity of iTregs. In total, two main factors may explain the different findings between the present study and the study by Takahashi *et al* (28). The experimental settings in the study by Takahashi *et al* (28) were different from those in the present study. Moreover, in the present study, the overexpression of SOCS1 not only altered the activity of the STAT1 pathway, but also affected the activity of the Akt pathway. Consistent with this, previous studies have reported that the Akt pathway is important for the induction and suppressive functions of Tregs (46,47). The different experimental settings may have contributed to the different findings in the present study compared with the study by Takahashi *et al* (28). Additionally, other potent targets of miR-30a, not investigated in the present study, may also contribute to the effect of miR-30a on the function of iTregs, which remain to be explored in future studies.

In conclusion, the present study revealed that miR-30a served an important role in the suppressive capacity of iTregs through the regulation of the SOCS1 pathway. To the best of our knowledge, these results have identified a previously unknown role of miR-30a in the instability of iTregs that may facilitate the development of therapeutic strategies for promoting T-cell immunity by regulating iTregs by targeting specific miRNAs.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YZ and YL performed the experiments, analyzed the data and wrote the paper. JL performed the experiments. XH designed and wrote the paper. LX conceived and designed the experiments, analyzed the data and wrote the paper. All authors reviewed the paper.

Ethics approval and consent to participate

All the experimental procedures were approved by the Ethical Committee of Zunyi Medical Laboratory Animal Care and Use Committee.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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