Cellular Physiology and Biochemistry

Cell Physiol Biochem 2018;49:1221-1237
DOI: 10.1159/000493298
Published online: 6 September, 2018
Accepted: 18 August, 2018

Original Paper

Administration of Interleukin-35-Conditioned Autologous Tolerogenic Dendritic Cells Prolong Allograft Survival After Heart Transplantation

Xianglan Liu[a,b] Yong Sun[a,b] Yang Zheng[a,b] Maomao Zhang[a,b] Xiangyuan Jin[c] Kai Kang[d] Yongshun Wang[e] Shuang Li[a,b] Hanlu Zhang[a,b] Qi Zhao[a,b] Shengnan Zhang[a] Jian Wu[a,b] Bo Yu[a,b]

[a]Department of Cardiology, the Second Affiliated Hospital of Harbin Medical University, Harbin, [b]The Key Laboratory of Myocardial Ischaemia, Harbin Medical University, Ministry of Education, Harbin, [c]Department of Thoracic Surgery, the Cancer Hospital of Harbin Medical University, Harbin, [d]Department of Cardiovascular Surgery, the Second Affiliated Hospital of Harbin Medical University, Harbin, [e]Department of Biomedical Science, University of Hong Kong, Hong Kong, China

Key Words
Interleukin-35 • Dendritic cells • Regulatory T cells • Let-7i • Transplant immunity

Abstract
Background/Aims: IL-35, a powerful suppressor of inflammation and autoimmunity, is primarily secreted by regulatory T cells (Tregs) and can, in turn, promote Treg differentiation. However, the precise effect of IL-35 on dendritic cells (DCs) remains to be clarified. ***Methods:*** In this study, we investigated the expression of IL-35 in DCs after stimulation with LPS utilizing enzyme linked immunosorbent assay(ELISA), quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR) and western blotting, and the influence of IL-35 on the maturation and function of DCs by mixed lymphocyte reaction assay and flow cytometry. We further examined the regulation of IL-35 in DCs by the microRNA let-7i (let-7i) via transfected with let-7i mimic, inhibitor or suppressor of cytokine signalling 1 (SOCS1) siRNA. IL-35-overexpressing DCs were transfused into BALB/c recipients with C57BL/6 heart transplantations to verify the role of immune tolerance in transplantation. ***Results:*** The results showed that IL-35 expression was significantly up-regulated following lipopolysaccharide (LPS)-induced DC maturation. Overexpression of IL-35 suppressed DC maturation, promoted the secretion of anti-inflammatory cytokines, and subsequently affected the balance between Treg and Th17 cells. IL-35 expression in DCs was regulated by let-7i, which targets SOCS1. The transfusion of IL-35-transfected DCs induced Treg generation in mice and prolonged cardiac allograft

Jian Wu, Ph.D., M.D.
Department of Cardiology, the Second Affiliated Hospital of Harbin Medical University
Xuefu Road No. 246, Nangang District, Harbin (China)
Tel. 86-152-4500-1123, Fax +86-0451-8660-5180, E-Mail wujian780805@163.com

survival. ***Conclusion:*** Our data demonstrated that IL-35 induces tolerogenic DCs which are capable of alleviating allograft rejection. Clinical application of IL-35-treated DCs might be a promising approach for eliciting cardiac allograft immune tolerance.

Introduction

Interleukin-35 (IL-35), which is the newest identified member of the IL-12 family of heterodimeric cytokines, is composed of Ebi3, an αβ-chain subunit encoded by the Epstein-Barr virus (EBV)-induced gene 3 (IL-27B), and IL-12p35, which is encoded by IL-12A [1]. IL-35 is primarily secreted by regulatory T cells (Tregs) and promotes Treg differentiation [2, 3]. The overexpression of IL-35 has been shown to protect against various experimental autoimmune diseases [4], including experimental colitis [5, 6], collagen-induced autoimmune arthritis [7], autoimmune demyelination [8], and type 2 T helper cell (Th2)-mediated allergic asthma [9]. IL-35 can also act as a crucial regulatory cytokine to suppress the induction of inflammatory dendritic cells (DCs) at sites of inflammation in an ovalbumin-induced asthma model [10]. In addition, IL-35 has been shown to be up-regulated in non-T cells, including immature dendritic cells (imDCs), smooth muscle cells, and vascular endothelial cells [11]. According to Dixon KO, Van Der Kooij SW, Vignali DA, Van Kooten C [12], tolerogenic DCs (tolDCs), which are generated by treating DCs with dexamethasone, can maintain the mRNA expression of IL-12p35 and Ebi3. Despite these recent insights, the biological relevance of immunoregulation and the exact impact of IL-35, particularly on DCs, remain elusive [4].

Cardiac allograft rejection is a longstanding unsolved complication of heart transplantation. During 1985-1989, the 10-year survival rate estimate was only 46 percent, while during 2005-2010, the survival rate increased to 64 percent, although the 50% survival time was only 25.8 years [13]. Although advances in pharmacology have decreased, the mortality associated with acute allograft rejection, subclinical episodes and graft dysfunction continue to heavily impact medium- and long-term graft survival [14]. Pharmacological treatments are also associated with an increased risk of cancer and infections linked to an immunosuppressive state. Suppressive agents such as corticosteroids, which have been used for more than four decades, lead to side effects including weight gain, cataracts and bone loss and also enhance cardiovascular risk factors. Although the advent of triple therapy with cyclosporine, steroids and azathioprine has allowed steroid doses to be reduced, steroid-induced morbidity is still a major problem in transplant recipients [15].

Cell surface molecules such as DC-SIGN(+), cytokines (IL-6, IL-34, etc.) and enzymes controlling metabolic pathways such as the enzyme indoleamine 2, 3 dioxygenase (IDO), which is capable of inducing tolerance, have been described as new mediators of immune tolerance [16-19]. Regulatory DCs play an important role in the maintenance of immunological tolerance by inducing T cell unresponsiveness or apoptosis and generating Tregs [20-22]. IL-35 is an inhibitory cytokine that can promote the development of iTr35 cells, which constitute a population of induced Tregs with suppressive effects, but iTr35 cells do not express Foxp3 and IL-10 [23], and their *ex vivo* population expansion is limited due to their polyclonal specificity and low proliferative capacity. Our earlier experiments have shown that DC treatments elicit T cell hypo-responsiveness and expand Treg populations *in vitro* [24]. To investigate the mechanism by which IL-35-treated DCs influence T cell proliferation and differentiation, we hypothesised that IL-35-overexpressing DCs play a role in the induction of Tregs and protect against transplantation rejection.

In this study, we examined and clarified the role of IL-35 during DC maturation and subsequently induced Treg generation. Then, we examined whether DC secretion of IL-35 was regulated by the microRNA let-7i (let-7i) via the direct targeting of suppressor of cytokine signalling 1 (SOCS1). We also investigated the effect of IL-35-overexpressing DCs on transplant immunity tolerance *in vivo*.

Materials and Methods

Animals

Male BALB/c and C57BL/6 mice, aged 8 to 12 weeks, were purchased from the Second Affiliated Hospital of the Harbin Medical University Laboratory Animal Centre. All animal care was conducted in accordance with the "Principles of Animal Care" (Ethical and Animal Welfare Committee of Heilongjiang Province, China) and the "Guide for the Care and Use of Laboratory Animals" (Ethics Committee of Harbin Medical University Animal Care and Use).

Generation and stimulation of bone marrow-derived mature DCs

Immature DCs (imDCs) were generated from BALB/c mouse bone marrow mononuclear cells cultured in complete RPMI 1640 medium (HyClone, Logan, UT, USA) in the presence of 20 ng/mL recombinant murine cytokines (IL-4 and granulocyte-macrophage colony-stimulating factor (GM-CSF); PeproTech, Rocky Hill, NJ, USA) for 6 days. ImDCs were treated with lipopolysaccharide (LPS; 200 ng/ml; Sigma-Aldrich, St. Louis, MO, USA) on Day 7 for 24 h to obtain mature DCs (mDCs). Some cells were treated with recombinant murine tumour necrosis factor-α [25] (TNF-α, 50 ng/ml; PeproTech, Rocky Hill, NJ, USA), interferon-γ [12] (INF-γ, 50 ng/ml; PeproTech, Rocky Hill, NJ, USA) or recombinant mouse IL-1α [26] (10 ng/ml; R&D Systems, Minneapolis, MN, USA) for 24 h. The adherent clusters were positively selected using CD11c magnetic microbeads (Miltenyi Biotech GmBH, Bergisch Gladbach, Germany). The recovered cells that displayed more than 95% CD11c expression were used for the subsequent experiments.

Treatment with recombinant IL-35/Ebi3 or IL-35-neutralising antibodies and transfection with microRNA let-7i mimic, let-7i inhibitor, or SOCS1 siRNA

ImDCs were generated and treated with Ebi3 mouse recombinant protein (10 ng/ml; Rockland, Limerick, PA, USA) or anti-mouse IL-35 monoclonal antibody (10 ng/ml; Rockland, Limerick, PA, USA) for neutralisation on Day 6 for 24 h. Some imDCs were transfected with microRNA let-7i mimic or inhibitor (60 nmol/l; GenePharma, Shanghai, CN) by Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for 24 h on Day 6. For all transfection protocols, a control group was transfected with a mimic negative control (NC-mimic) or an inhibitor negative control (NC-inhibitor). The sequences were as follows: microRNA let-7i mimic, sense, 5'-UGAGGUAGUAGUUUGUGCUGUU-3', antisense, 5'-CAGCACAAACUACUACCUCAUU-3'; microRNA let-7i mimic negative control, sense, 5'-UUCUCCGAACGUGUCACGUTT-3', antisense, 5'-ACGUGACACGUUCGGAGAATT-3'; microRNA let-7i inhibitor, 5'-AACAGCACAAACUACUACCUCA-3'; microRNA let-7i inhibitor negative control, 5'-CAGUACUUUUGUGUAGUACAA-3'. ImDCs were also transfected with SOCS1 short interfering (si)RNA (60 nmol/l; GenePharma, Shanghai, CN) using Lipofectamine 2000 for 24 h on Day 6. After washing, the cells were cultured for 24 h in the presence of LPS (200 ng/ml) and then collected for other experiments.

Mixed lymphocyte reaction assay

Splenocytes were obtained from male BALB/c mice, and $CD3^+$ T cells were isolated from the splenocytes by magnetic cell separation using anti-CD3 microbeads (Miltenyi Biotech GmBH, Bergisch Gladbach, Germany). To analyse the effect of IL-35/Ebi3-treated DCs or IL-35-neutralising antibody-treated DCs on T cell proliferation, BALB/c mice DCs (1.0×10^5/ml) were treated with mitomycin C (10 μg/ml, Sigma-Aldrich, St. Louis, MO, USA) for 4 h and then co-cultured with BALB/c $CD3^+$ T cells (1.0×10^6/ml) in a 96-well plate for 4 days. These co-cultures were incubated with 10 mM BrdU (Biotrak 2; Amersham, Little Chalfont, UK) for 24 h, and BrdU incorporation by proliferating T cells was quantified using a BrdU enzyme-linked immunosorbent assay (ELISA). To analyse the T cell population, BALB/c DCs were pretreated with mitomycin C (10 μg/ml, Sigma-Aldrich, St. Louis, MO, USA) for 4 h and then co-cultured with BALB/c $CD3^+$ T cells at a DC/T cell ratio of 1:10. Subsequently, $CD4^+$ T cells were isolated from the co-cultured MLR cells by magnetic cell separation using anti-CD4 microbeads (Miltenyi Biotech GmBH, Bergisch Gladbach, Germany) for further flow cytology analysis.

Quantitative RT-PCR

Total RNA was isolated from different groups of DCs using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) following the protocol recommended by the manufacturer. Reverse-transcription reactions were incubated for 60 min at 37°C and 5 min at 85°C. qRT-PCR was performed according to the TaqMan real-time PCR assay kit (GenePharma, Shanghai, CN) protocol in a total volume of 20 μl using an CFX manager Real-Time System (Bio-Rad Laboratories, Hercules, California). PCR reaction mixtures contained the following components: 10 μl of Universal Master Mix, primer pairs at 10 μM, probe at 10 μM, 1 U of rTaq DNA polymerase and 10 ng of cDNA template. The PCR protocol consisted of 40 cycles of 3 min at 95°C, 12 s at 95°C, and 40 s at 62°C. Quantification of relative mRNA levels of the target genes was performed based on the $2^{-\Delta\Delta Ct}$ method described by Livak and Schmittgen [27]. The primers used for the real-time PCR reactions are shown in Table 1. Each sample was measured in triplicate.

Evaluation of cytokine production

To measure cytokine release by DCs, supernatants were collected from cultures after stimulation with LPS, IFN-γ, IL-1β, or TNF-α for 24 h. An IL-35 heterodimer ELISA kit (CUSABIO, Wuhan, CN) was used to detect the concentrations in the supernatants according to the manufacturer's instructions. Some supernatants were obtained from DCs after treatment with IL-35/Ebi3 or IL-35-neutralising antibody or after transfection with microRNA let-7i mimic, inhibitor, or RNA of si-SOCS1 and LPS stimulation for 24 h. Then, we assessed the secreted levels of IL-4, IL-10, IL-12, IL-23, IL-27, TGF-β and IFN-γ (Abcam, Cambridge, UK). In several experiments, the levels of IL-2, IFN-γ, IL-4, and IL-10 (Abcam, Cambridge, UK) were also determined in plasma from BALB/c recipient mice 14 days after transplantation. All assays were performed in triplicate.

Flow cytometry

DC purity and phenotype were analysed based on the surface expression of specific markers using flow cytometry. $CD11c^+$ DCs were selected by magnetic cell separation and incubated with 0.5 μg of fluorochrome-conjugated PE-CD80, PE-CD86 and PE-MHC-II (eBioscience, San Diego, CA, USA) for 30 min at 37°C. For each staining protocol, the appropriate isotype-matched control was included. The stained cells were assayed using a BD FACSCanto II flow cytometer. To analyse $CD4^+CD25^+Foxp3^+$ T cells, cells from MLRs or splenocytes from recipients were transfused with phosphate-buffered saline (PBS), mDCs or IL-35/Ebi3-mDCs 14 days after transplantation. Subsequently, the cells were incubated with 0.5 μg of fluorochrome-conjugated CD4-FITC or CD4-PerCP-Cy5.5 and CD25-APC (eBioscience, San Diego, CA, USA) monoclonal antibodies (mAb) for 30 min at 37°C and then fixed and permeabilised in 100 μL of eBioscience Perm-Fix solution overnight at 4°C. The cells were washed and then stained with 0.5 μg of fluorophore-conjugated anti-Foxp3-PE (eBioscience, San Diego, CA, USA) for 30 min at 4°C. After washing, the stained cells were assayed using a BD FACSCanto II flow cytometer. $CD4^+CD25^+$ T cells were identified by gating on FSC/SSC cells. The $CD4^+CD25^+Foxp3^+$ T cell population was obtained from $CD4^+CD25^+$ cells by further gating $Foxp3^+$ cells. To detect intracellular IL-17A and IFN-γ levels in the T cells, the cells were first treated with 1× cell stimulation cocktail (including Phorbol 12-Myristate 13-Acetate (40.5 μM), ionomycin (670 μM), Brefeldin

Table 1. Primers used for real-time PCR analysis

Gene	Forward	Reverse	Probe
IL-23p19	5'-ATGTGCCCCGTATCCAGTGT-3'	5'-AGGCTCCCCTTTGAAGATGTC-3'	5'-CAGTTCTGCTTGCAAAGGATCCGCC-3'
IL-27p28	5'-TGCCCCTGGGATACCATCT-3'	5'-AGGGAAGGGCCGAAGTGT-3'	5'-CCCAATGTTTCCCTGACTTTCCAGGC-3'
IL-12p40	5'-CAGTACACCTGCCACAAAGGA-3'	5'-CGGAGTAATTTGGTGCTTCAC-3'	5'-AGACTCTGAGCCACTCACATCTGCTGC-3'
IL-12p35	5'-ATCGATGAGCTGATGCAGTCT-3'	5'-GCTTCTCCCACAGGAGGTTT-3'	5'-AATCATAATGGCGAGACTCTGCGC-3'
Ebi3	5'-CAGGTGGGACCCATTGAAG -3'	5'-TGACACCTGGATGCAATACTTG-3'	5'-TCACCCTCAGGAACTCGAAACCCCA-3'
SOCS1	5'-TCCGTGACTACCTGAGTTCCTT-3'	5'-GGCATCTCACCCTCCACAC-3'	5'-ACGGGCCCAACTGCACCCAACAGCC-3'
GAPDH	5'-CCTTATTGACCTCAACTACATGG-3'	5'-CTCGCTCCTGGAAGATGGTG-3'	5'-ATGTTCCAGTATGACTCCACTCACGGCA-3'

A (5.3 mM), and monensin (1 mM) in ethanol in 500×) (eBioscience, San Diego, CA, USA) for 4-6 h at 37°C. Subsequently, the cells were surface-stained with the $CD4^+$-PerCP-Cy5.5 mAb, permeabilised and fixed in 100 µl of eBioscience Perm-Fix solution overnight at 4°C. The cells were washed twice with Perm Wash buffer and then stained with 0.3 µg of fluorophore-conjugated anti-IL-17A-PE (eBioscience, San Diego, CA, USA) or fluorophore-conjugated anti-IFN-γ-PE (eBioscience, San Diego, CA, USA) for 20 min at 4°C. After washing, the stained cells were assayed using a BD FACSCanto II flow cytometer. The TH17 cells from the $CD4^+$ cells were gated on IL-$17A^+$cells, and the TH1 cells from the $CD4^+$ cells were gated on IFN-γ^+ cells. All data were then further processed by using FlowJo 10.0.7 software.

Western blotting

Whole DCs (1×10^7) were lysed in RIPA buffer supplemented with a proteinase inhibitor cocktail (Sigma), and a total of 30 µg of protein from each group of extracts was loaded and separated on Tris-glycine SDS-PAGE gels. The following targets were probed using antibodies: IL-12p35, Ebi3, STAT1, pSTAT1, STAT3, pSTAT3, STAT4, pSTAT4, STAT5, pSTAT5, STAT6, pSTAT6, SOCS1 and β-actin (Abcam, Cambridge, UK). ECL Prime Western Blotting Detection Reagents (GE Healthcare, Piscataway, NJ, USA) and scanning densitometry (GS-710 imaging) were used to detect the signals and acquire the images. All data were obtained from three independent experiments.

Heart transplantation and treatment of recipient animals

BALB/c recipient mice were transfused with 0.1 ml of PBS only (negative control), 1.0×10^5 mDCs, or 1.0×10^5 IL-35/Ebi3-mDCs (derived from BALB/c bone marrow) via an intravenous injection into the penile vein after general anaesthesia. At 24 h after transfusion, the BALB/c recipient mice underwent a fully vascularised heterotopic heart transplantation of a C57BL/6 heart using microsurgical techniques [28]. The other BALB/c recipient mice underwent heart transplantation after receiving an oral administration of 1 mg/kg of tacrolimus (positive control). The graft function was assessed daily post-operatively by palpation for evidence of contraction. Rejection was defined as the complete cessation of the heartbeat and confirmed by direct visualisation and histological examination of the graft.

Histological studies of harvested grafts

The cardiac allografts were removed from BALB/c recipient mice that were transfused with PBS, mDCs, or IL-35/Ebi3-mDCs 14 days after heart transplantation and were examined histologically and immunohistochemically. Histologic and immunohistochemical staining were performed as previously described [29]. The haematoxylin and eosin staining was assessed by grading according to the 2005 classification of the International Society for Heart and Lung Transplantation [30] to detect acute cellular rejection as follows: Grade 0, no rejection; Grade 1 (mild), interstitial and/or perivascular infiltrate with up to 1 focus of myocyte damage; Grade 2 (moderate), 2 or more foci of infiltrate with associated myocyte damage; and Grade 3 (severe), diffuse infiltrate with multifocal myocyte damage with or without oedema, haemorrhage, and/or vasculitis. Five microscopic fields were examined in each section under a microscope at an original magnification of 40×. For the immunohistochemical staining, the sections were fixed, pre-incubated in Block Ace and incubated with anti-Foxp3 (Abcam, Cambridge, UK), followed by an incubation with alkaline phosphatase-conjugated anti-rabbit Ig for anti-Foxp3 and Vector Blue (resulting in a blue colour). The sections were then incubated with a rabbit anti-mouse type IV collagen polyclonal antibody (LB1403; Cosmo Bio, Tokyo), peroxidase-conjugated anti-rabbit Ig and diaminobenzidine (resulting in a brown colour).

Statistical analysis

The results are expressed as the means ± SDs. Statistical significance was determined by performing unpaired t-tests or analysis of variance (ANOVA) using GraphPad Prism software. The cardiac allograft survival in the groups of mice was compared using Mann-Whitney U testing. P-values < 0.05 were considered statistically significant.

Results

IL-35 expression was up-regulated during LPS-induced DC maturation

Previous studies have demonstrated that IL-35 was mainly secreted by Tregs or tolDCs [31, 32]. However, the expression and regulation of IL-35 on DCs remain to be clarified. In this study, LPS, TNF-α, IFN-γ and IL-1 were used to evaluate the IL-35 production by DCs in response to Toll-like receptor stimulation. The IL-35 expression level was markedly higher after LPS, TNF-α, IFN-γ and IL-1 stimulation than that observed for the imDCs group, and expression was highest with LPS treatment (Fig. 1A). To further evaluate the expression of IL-35 following LPS stimulation, we activated DCs with LPS for different durations and found that the expression of IL-35 was time-dependent up to 4 h following LPS stimulation and reached a peak at 4 h (Fig. 1B) compared with the control group treated with PBS. To determine whether other IL-12 family cytokines were affected by LPS, we detected the expression levels of IL-12, IL-23 and IL-27 in the cell supernatants and found that all cytokines were significantly increased in mDCs (Fig. 1C). LPS-treated DCs exhibited up-regulation of IL-12p35 and Ebi3 transcripts at much higher levels than those in untreated DCs (Fig. 1D). IL-12p35 and Ebi3 protein levels were detected via Western blotting and were significantly increased after LPS treatment (Fig. 1E). These results suggested that IL-35 was highly increased in DCs after LPS stimulation.

IL-35 suppressed DC maturation and promoted Treg generation

To determine whether IL-35 regulates DC maturation and function, the expression patterns of maturation-specific proteins in DCs were determined. After 24 h of stimulation

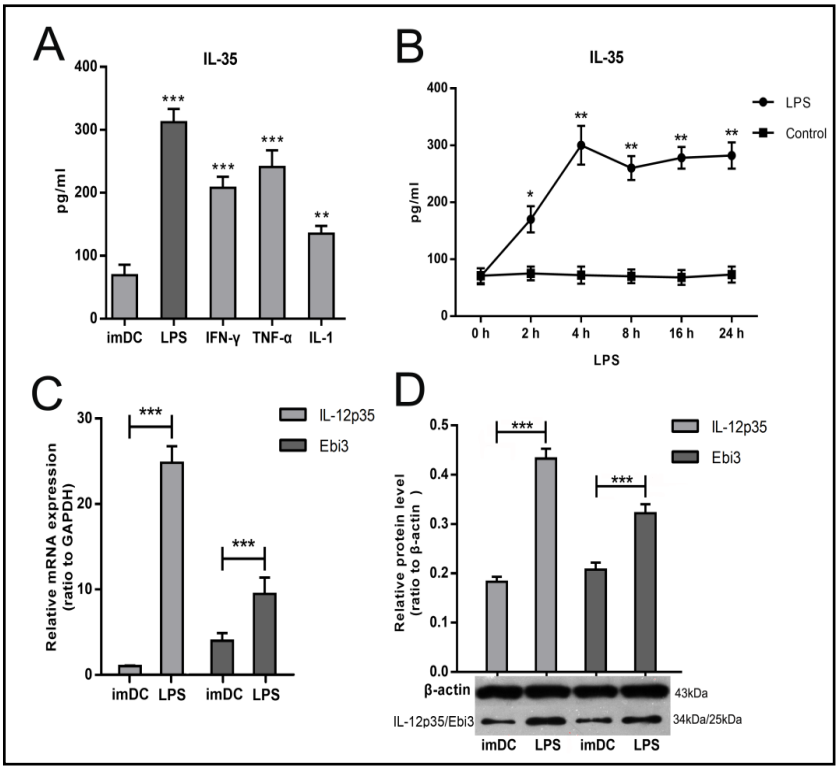

Fig. 1. Interleukin-35 expression was up-regulated during lipopolysaccharide-induced dendritic cell maturation. (A) Bone marrow-derived dendritic cell (DCs) were cultured for 6 days in the presence of IL-4 and GM-CSF to obtain immature DCs (imDCs) and then treated with lipopolysaccharide (LPS), tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ) or IL-1 for 24 h. The IL-35 expression levels in the DC supernatants were analysed using an ELISA kit. (B) The DC supernatants were harvested at different times (0, 2, 4, 8, 16, or 24 h) after the treatment with LPS. An ELISA kit was used to detect the expression levels of IL-35. (C) The levels of IL-12 cytokine family members were analysed in DC supernatants after 24 h of treatment with LPS using ELISA kits. The mRNA (D) and protein (E) expression levels of IL-12p35 and Ebi3 were detected in DCs after 24 h of LPS stimulation. The data shown are derived from three independent experiments and are expressed as means ± SD. *P<0.05, **P<0.01, ***P<0.001.

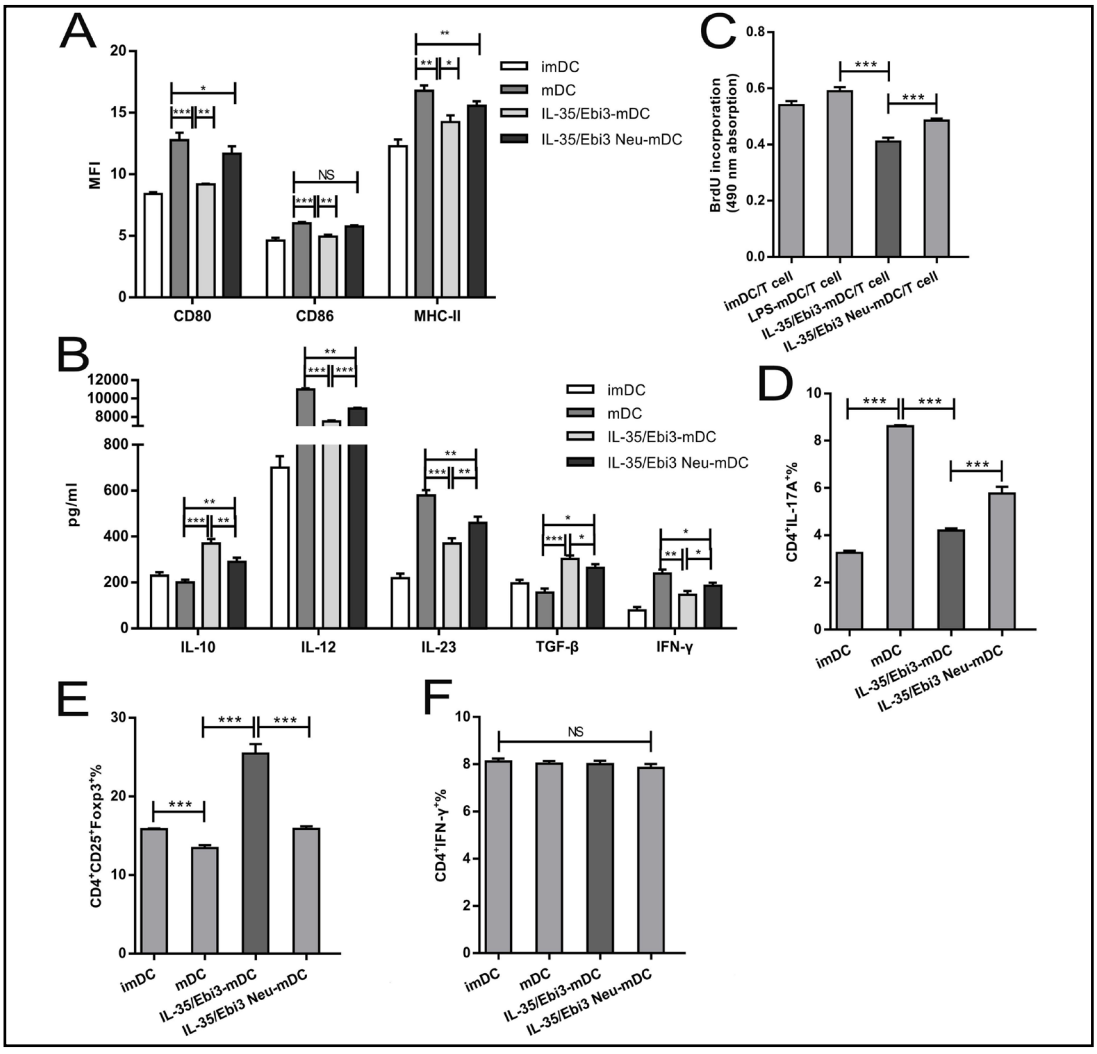

Fig. 2. Interleukin-35 suppressed dendritic cell maturation promoted regulatory T cell generation. Bone marrow-derived dendritic cell (DCs) were cultured for 6 days in the presence of IL-4 and GM-CSF to obtain immature DCs (imDCS) and treated with lipopolysaccharide (LPS) for 24 h to obtain mature DCs (mDCS). The imDCs were treated with IL-35/Ebi3 or IL-35/Ebi3-neutralising antibody for 24 h before LPS stimulation. (A) The DC subtypes (CD80, CD86, and MHC-II) were analysed using flow cytometry. (B) Cytokine expression levels in DC supernatants were analysed using ELISA kits. (C) BALB/c DCs (1.0×10^5/ml) were treated with mitomycin C (10 μg/ml) for 4 h, co-cultured with BALB/c mice $CD3^+$ T cells (1.0×10^6/ml) in a 96-well plate for 4 days and then incubated with BrdU (10 mM, 24 h) to quantify proliferation. T cell proliferation was assessed by BrdU ELISA. The proliferation of T lymphocytes was significantly lower following treatment with IL-35/Ebi3-mDCs than that observed in the control group. (D-F) Co-cultured T cells from MLRs were incubated with $CD4^+$, $CD25^+$, and $Foxp3^+$ antibodies or $CD4^+$ and $IL\text{-}17A^+$ or $CD4^+$ and IFN-γ^+ antibodies. T cells were detected using flow cytometry to analyse the regulatory T cell (Treg) (D), Th17 (E) and Th1 (F) subtypes. The data shown are derived from three independent experiments and are expressed as means ± SD. *P<0.05, **P<0.01, ***P<0.001, NS denotes not significant.

with LPS, the DCs expressed comparable levels of CD80, a key characteristic of the mature state, CD86 and MHC-II, while treatment with mouse recombinant protein IL-35/Ebi3 before LPS stimulation resulted in the generation of immature DCs as determined by lower expression levels of CD80, CD86 and MHC-II (Fig. 2A). After treatment with the IL-35-neutralising antibody, the ability of IL-35 to induce lower expression levels of CD80, CD86

and MHC-II on DCs was hampered. Thus, IL-35 could functionally inhibit LPS-triggered DC maturation. ImDCs can preserve the production of immunoregulatory cytokines while maintaining a lower production of inflammatory cytokines. Following IL-35 activation, the DCs exhibited a strong production of IL-10 and TGF-β, whereas the secretion of IL-12, IL-23 and IFN-γ was decreased (Fig. 2B).

To ascertain the allostimulatory capabilities of the IL-35-treated DCs, IL-35/Ebi3 or IL-35-neutralising antibody-triggered DCs were co-cultured with allogenic $CD3^+$ T lymphocytes, and then, T cell proliferation was analysed. The results show that the proliferation of T cells was not induced by IL-35/Ebi3-treated DCs. Furthermore, the co-stimulatory capabilities of DCs after stimulation with IL-35/Ebi3 were decreased (Fig. 2C). To analyse the T cell population, $CD4^+$ T cells were isolated from co-cultured MLR cells and analysed by using flow cytometry. IL-35/Ebi3-induced DCs promoted $CD4^+CD25^+Foxp3^+$ Treg differentiation (Fig. 2D) and suppressed $CD4^+IL\text{-}17A^+$ Th17 cell differentiation (Fig. 2E), suggesting that IL-35/Ebi3-induced DCs exhibit suppressive activity. The blockade of IL-35 with IL-35-neutralising antibody abrogated the inhibitory effect of IL-35 on the DCs. T cells that simultaneously expressed CD4 and IFN-γ were measured, and there was no significant difference between IL-35/Ebi3-treated DCs and the other groups (Fig. 2F). Thus, IL-35 treatment caused DCs to remain immature, which then affected the balance between Treg and Th17 cells.

IL-35 could activate the STAT family to regulate DC maturation

STATs are classic transcription factors that directly engage DNA regulatory elements and thereby control the transcription of associated genes [33]. STATs have been found to intrinsically regulate DC function and importantly participate in DC maturation and the production of inflammation cytokines. Insight into one possible mechanism has been provided by an analysis of signalling via the receptor for IL-35 [34]. IL-35 signalling via STAT1 and STAT4 drive the inhibitory programme in T cells [35, 36]. We investigated whether IL-35 also affects DCs via the STAT1 and STAT4 pathways. As shown in Fig. 3, the expression of phosphorylated STAT signalling pathway proteins in differentially treated DCs was analysed by Western blotting. We found that the expression levels of phosphorylated STAT1, STAT3 and STAT4 were significantly higher in IL-35/Ebi3-treated DCs than those in the other groups, but changes in the expression of phosphorylated STAT5 and STAT6 were not significant. Thus, IL-35 might activate the STAT1, STAT3 and STAT4 pathways to perform its biological function in DCs.

The secretion of IL-35 in DCs was regulated by let-7i via targeting SOCS1

In our previous study, we found that let-7i expression was up-regulated and could regulate the secretion of inflammatory cytokines by DCs [24]. To determine whether the expression of IL-35 was also regulated by let-7i in DCs, we transfected DCs with let-7i mimic or inhibitor and compared the effects with those produced by an NC-mimic or NC-inhibitor following stimulation with LPS. Expectedly, the transfection with the let-7i mimic resulted in the overexpression of let-7i, whereas the let-7i inhibitor suppressed the expression of let-7i in the DCs as determined by qRT-PCR. IL-35 expression was significantly higher in the let-7i inhibitor-treated DCs than in the other groups (Fig. 4A), and the IL-35 level peaked at the 150 nmol/L concentration of the let-7i inhibitor (Fig. 4B). According to a qRT-PCR analysis of mRNA expression, the expression levels of IL-12p35 and Ebi3 were also substantially higher in the DCs treated with the let-7i inhibitor (Fig. 4C). We also detected the secretion levels of IL-4, IL-10, IL-12, IL-23, TGF-β and IFN-γ in the DCs transfected with the let-7i mimic or inhibitor. The transfection of DCs with the let-7i inhibitor promoted secretion of the anti-inflammatory cytokines IL-10 and TGF-β and had an inverse effect on the secretion of the pro-inflammatory cytokines IL-12 and IFN-γ (Fig. 4D), which was consistent with the results observed in DCs treated with IL-35/Ebi3. Collectively, these results suggest that the expression of IL-35 is regulated by let-7i. The down-regulation of let-7i expression can induce DCs to secrete IL-35, resulting in immune tolerance.

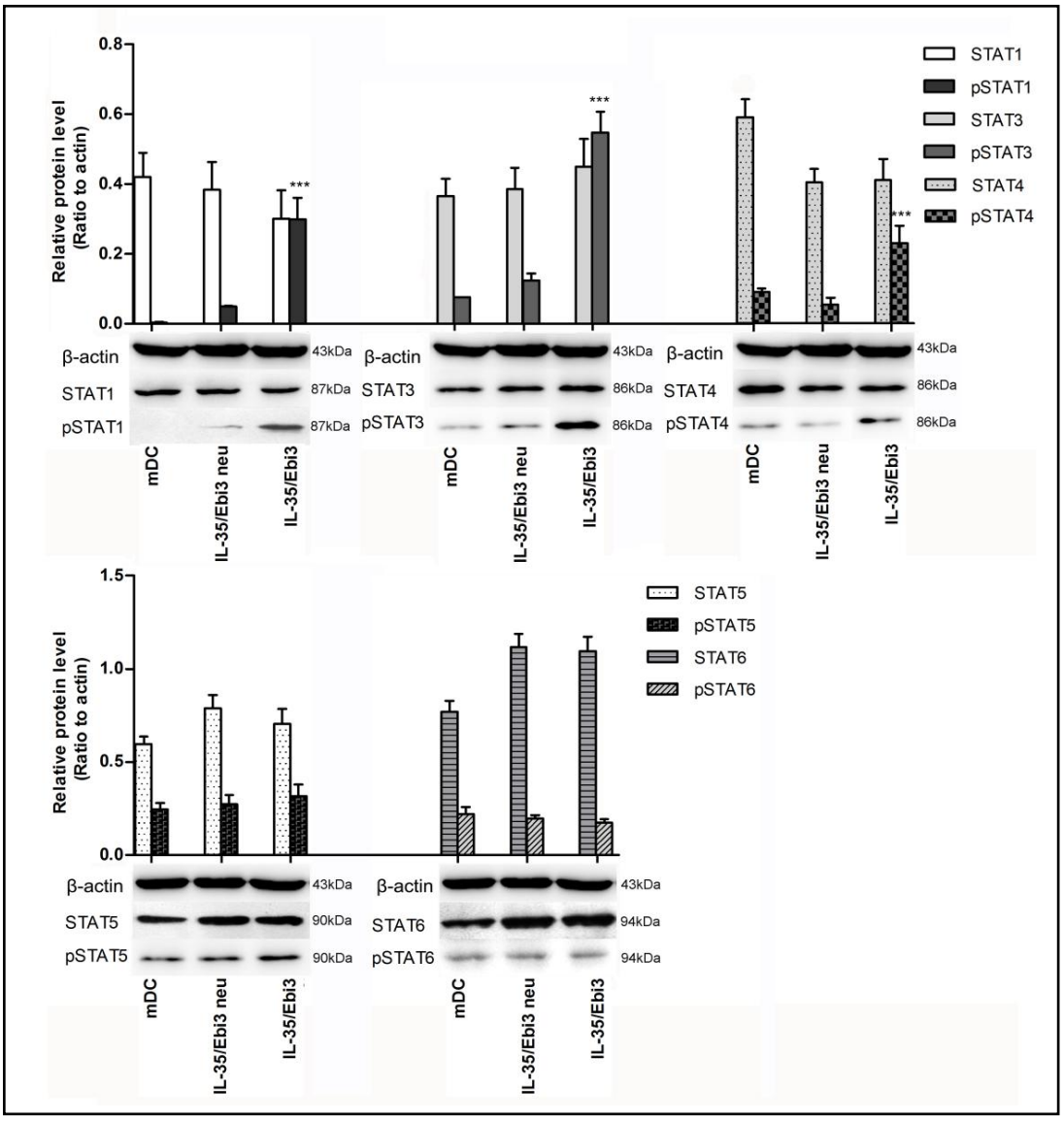

Fig. 3. Interleukin-35 activated the signal transducer and activator of transcription family to regulate dendritic cell maturation. imDCs were treated with IL-35/Ebi3 or IL-35/Ebi3-neutralising antibody for 24 h and then stimulated with LPS. Whole dendritic cell (DCs) (1×10^7) were lysed in RIPA buffer supplemented with a proteinase inhibitor cocktail, and a total of 30 μg of protein from each group extract was loaded and separated on Tris-glycine SDS-PAGE gels. Western blotting was performed using the indicated primary antibodies, and β-actin was included as a loading control. The prefix "p" indicates phosphorylation. The blots are representative of three independent experiments. ***P<0.001.

SOCS1 is a negative regulator and plays an essential role in suppressing systemic autoimmunity mediated by DCs [37, 38] . The silencing of SOCS1 enhances antigen presentation by DCs and antigen-specific anti-tumour immunity [38]. SOCS1 functions as an attenuator of antigen presentation for the control of self-tolerance through restricted pro-inflammatory cytokine signalling, such as IL-12 signalling, in mature DCs [39]. In our previous study, we investigated whether let-7i targets SOCS1 and concluded that the let-7i-mediated regulation of SOCS1 protein expression was regulated by post-transcriptional effects [24]. Our qRT-PCR and Western blot results were consistent with those obtained in previous studies, which showed that the down-regulation of let-7i causes an increase in SOCS1 expression (Fig. 5A-B). To investigate possible molecular restrictions in DCs affecting

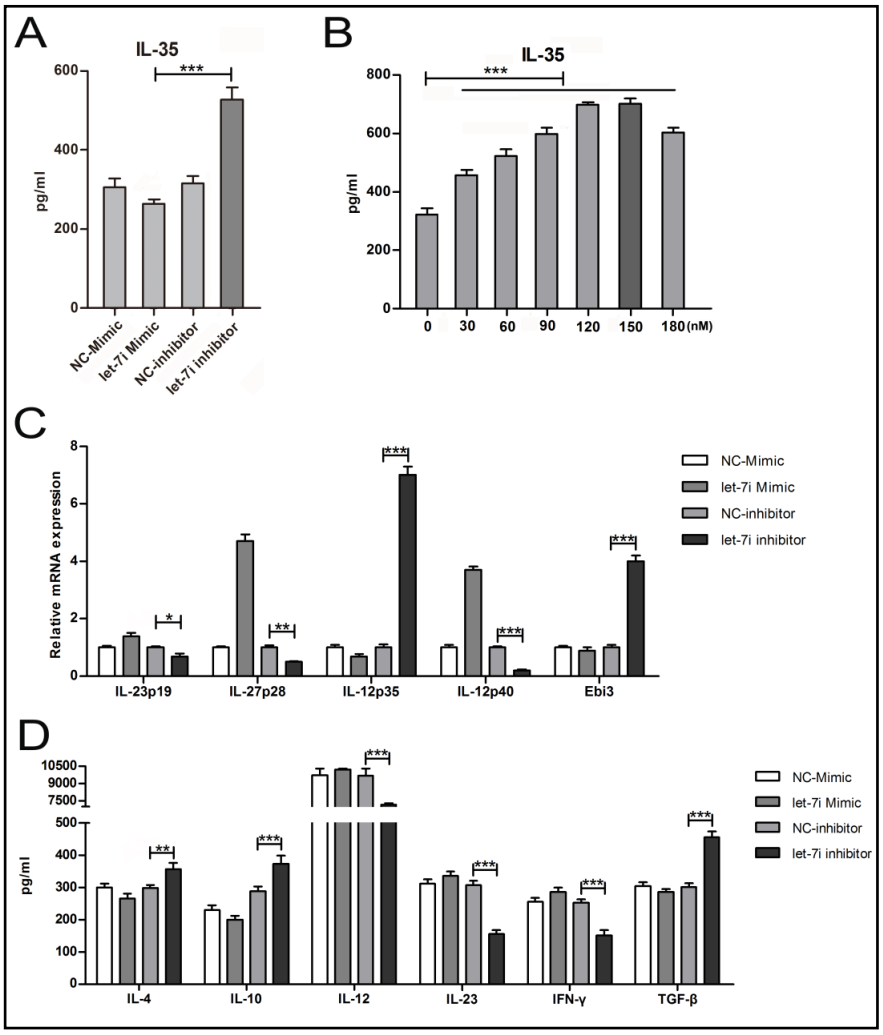

Fig. 4. Secretion of interleukin-35 in dendritic cells was regulated by microRNA let-7i. (A) Dendritic cells (DCs) were transfected with a let-7i mimic or inhibitor or negative control (NC) for 24 h before lipopolysaccharide (LPS) treatment, and the expression levels of IL-35 in the supernatant were detected via ELISA. (B) DCs were transfected with different doses of the let-7i inhibitor, and the expression levels of IL-35 were then detected. (C) Changes in the mRNA expression of IL-12p35 and Ebi3 in let-7i-treated DCs were examined by qRT-PCR. GAPDH mRNA expression in the same samples was used as an endogenous reference to determine the relative mRNA expression. (D) ELISA was used to detect changes in the secreted cytokine levels in the DC supernatant after transfection with let-7i. The data are expressed as the means ± SDs of six independent experiments. *P<0.05, **P<0.01, ***P<0.001.

the secretion of IL-35 in response to the down-regulation of let-7i, we tested the effect of silencing SOCS1 in DCs on the expression of IL-35. The mRNA (Fig. 5C) and protein (Fig. 5D) levels of IL-12p35 and Ebi3 and the production (Fig. 5E) of IL-35 in the supernatant all decreased. The down-regulation of let-7i observed after SOCS1 silencing did not promote DC secretion of a higher level of IL-35. This finding confirmed that let-7i might target SOCS1 to regulate the expression of IL-35 in DCs.

Transfusion of IL-35/Ebi3-treated DCs generated Tregs and prolonged cardiac allograft survival

BALB/c recipient mice transfused with PBS and mDCs rejected C57BL/6 grafts (median survival times [MSTs]: 6 days, 7 days, Fig. 6A), whereas those transfused with IL-35/Ebi3-mDCs had significantly prolonged allograft survival (MST: 21 days). The allograft survival was slightly prolonged (MST: 11 days) in the BALB/c recipient mice that were treated with tacrolimus (1 mg/kg/day) for 7 days. The histological examinations of the cardiac allografts obtained 14 days after transplantation showed the infiltration of inflammatory cells but significantly preserved structures with a few myocardial injuries in the transplant recipients

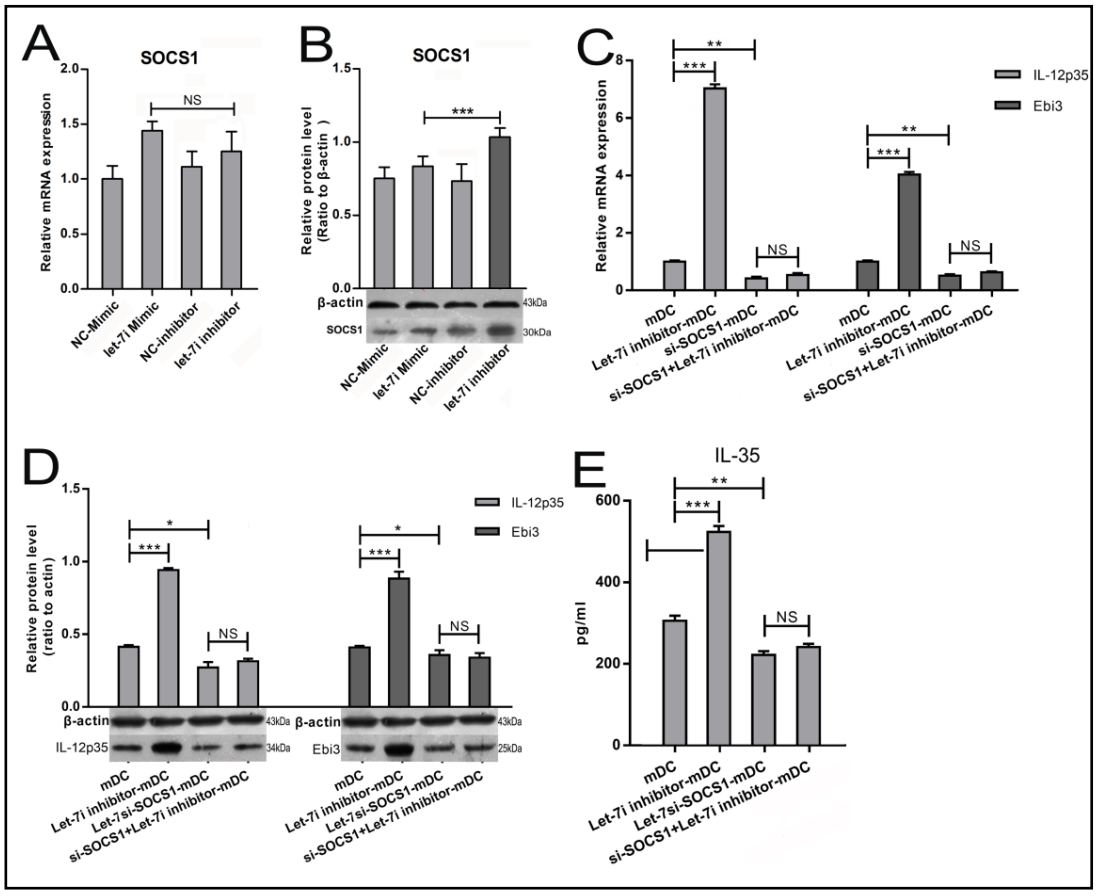

Fig. 5. The secretion of interleukin-35 from dendritic cells was regulated by microRNA let-7i via suppressor of cytokine signalling 1 targeting. Dendritic cells (DCs) were transfected with a let-7i mimic, inhibitor or a negative control for 24 h and then treated with lipopolysaccharide (LPS), and the expression levels of suppressor of cytokine signalling 1(SOCS1) mRNA (A) and protein (B) were detected by qRT-PCR and Western blotting, respectively. The data are expressed as the means ± SDs of n = 3 samples pooled from three independent experiments. The cells were transfected with SOCS1 short interfering (si)RNA (60 nM) for 24 h before transfection with the let-7i inhibitor, treated overnight with LPS on Day 7 and then harvested. IL-12p35 and Ebi3 mRNA (C) and protein (D) expression levels were determined, and IL-35 expression levels were detected via ELISA (E). The abbreviation "NC" indicates negative control compared to let-7i. The data are expressed as the means ± SDs. "NS" denotes not significant. *P<0.05, **P<0.01, ***P<0.001.

receiving IL-35/Ebi3-mDCs, whereas allografts from the PBS recipients showed severe myocyte damage and oedema, which were characteristic of the acute rejection process (Fig. 6B). In each HE-stained section, a significant difference was observed by grading using a semiquantitative scale. IL-35/Ebi3-mDCs could prolong allograft survival and resulted in less histological damage.

The immunohistochemical results showed that the cardiac allografts from recipients transfused with IL-35/Ebi3-mDCs exhibited a high frequency of $Foxp3^+$ cell infiltration (Fig. 6D) and expression of the anti-inflammatory cytokine IL-10 in the blood serum (Fig. 6E). Then, we examined the $CD4^+CD25^+Foxp3^+$ Treg immune response in grafted splenocytes from recipients transfused with IL-35/Ebi3-mDCs. According to the flow cytometry analysis, the proportion of $CD4^+CD25^+Foxp3^+$ Tregs was significantly higher in the spleens of recipients transfused with IL-35/Ebi3-mDCs (Fig. 6C) than in recipients transfused with PBS and mDCs. This finding was further supported by the observation that IL-35, as an inhibitory cytokine, induced DC immunosuppressive activities, suggesting that the IL-35-induced DC-driven suppressor response is meaningful *in vivo*.

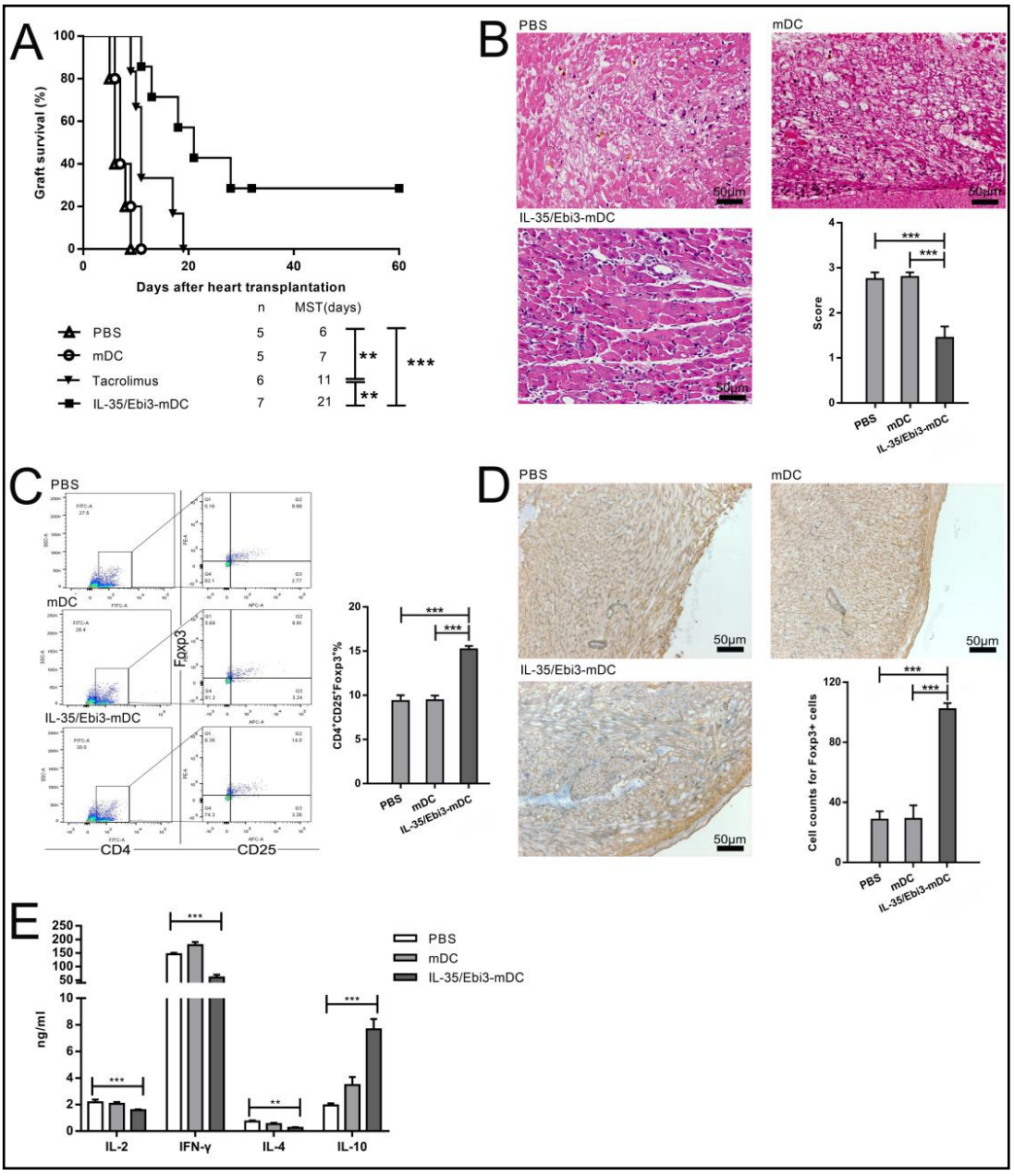

Fig. 6. Transfusion of IL-35/Ebi3-treated dendritic cells generated regulatory T cells and prolonged cardiac allograft survival. (A) Graft survival times in BALB/c recipient mice that were transfused once with phosphate buffered saline (PBS), mature DCs (mDCs) or IL-35/Ebi3-mDCs or received an oral administration of tacrolimus at 1 mg/kg/day for 7 days. (B) Histological studies of harvested cardiac allografts stained with haematoxylin and eosin 14 days after transplantation (original magnification, 20×). Assessment of acute cellular rejection using haematoxylin and eosin staining by grading according to the 2005 classification of the International Society for Heart and Lung Transplantation. (C) Expression of $CD4^+$ $CD25^+Foxp3^+$ T cells in BALB/c splenocytes as determined by flow cytometry. (D) Cell count of infiltrating Foxp3+ cells in cardiac allografts in each group 14 days after grafting. (E) Levels of cytokines in the plasma from BALB/c recipient mice 14 days after transplantation. The data are expressed as the means ± SDs of samples from six mice from each group. MST, median survival time, "NS" denotes not significant, **P<0.01, ***P<0.0001.

Discussion

In this study, the expression of IL-35 was significantly increased in DCs after LPS stimulation. IL-35 could suppress DC maturation, thereby inhibiting pro-inflammatory cytokine secretion and promoting the secretion of anti-inflammatory cytokines by targeting the STAT1/STAT3/STAT4 signalling pathway. IL-35/Ebi3-treated DCs suppressed the proliferation of T cells and affected the balance between Tregs and Th17 cells. The expression of IL-35 in DCs was regulated by let-7i via targeting SOCS1. Transfusion of IL-35/Ebi3-treated DCs generated Tregs *in vivo* and prolonged cardiac allograft survival.

IL-35, which is an inhibitory cytokine produced by thymus-derived natural regulatory T cells, is the most recently identified member of the IL-12 family. The cytokine IL-35 has attracted increasing attention as a key regulator of immunity and immunological tolerance. Recent studies indicate that IL-35 also regulates the effector functions of plasma cells and monocyte-derived DCs [10]. Although IL-35 appears to be essential to the maintenance of immune tolerance and represents a promising candidate for immunotherapy, its functionality and tolerogenic potential have been poorly investigated [4]. Immature or induced tolDCs play an essential role in the maintenance of peripheral tolerance [4]. TolDCs are generated *ex vivo* by culturing precursors with culture medium containing GM-CSF (mainly at a low dose) and eventually with IL-4, IL-10, or TGF-β or drugs such as vitamin-D3 (VitD3), rapamycin, tacrolimus, dexamethasone or mitomycin C [21, 40]. TolDCs are characterised by low levels of MHC-II and co-stimulatory markers, reduced secretion of pro-inflammatory cytokines, and high expression of tolerogenic molecules, such as IL-10 and TGF-β [41]. In this study, we observed consistent down-regulation of MHC-II and co-stimulatory markers following treatment of DCs with IL-35/Ebi3. In addition, IL-35 promoted the secretion of anti-inflammatory cytokines, such as IL-10 and TGF-β, and decreased the secretion of IL-12 and IFN-γ in DCs. Our results demonstrated that IL-35-induced DCs tend to become immature and exhibit immune suppression functions, potentially providing a new mechanism for the regulation of DC immunity.

T cell-based approaches for treating inflammatory conditions, such as allergies, autoimmune diseases and graft-versus-host responses, have great potential and notable limitations [23, 42]. The therapeutic potential of human T cells is limited by their polyclonal specificity, poorly defined markers for enrichment and low proliferative capacity, which limits *ex vivo* population expansion. TolDCs have the capacity to induce tolerance in the peripheral tissue via the expansion or induction of several subtypes of regulatory lymphocytes, which are mainly classical induced Tregs. VitD3-treated DCs prevent T cell priming and induce the apoptosis of effector T cells. TolDCs can also induce naïve and memory T cell apoptosis [21]. Exogenous IL-35 has been shown to promote the development of Tregs, but these cells do not express Foxp3, IL-10 or TGF-β [23]. Exogenous IL-35 also has no effect on the proliferation of Th17 cells [43]. In this study, DCs treated with IL-35 potently inhibited T cell proliferation, and we also observed that IL-35-treated mDCs, but not IL-35 or mDCs alone, induced an increase in the proportion of $CD4^+CD25^+Foxp3^+$ Tregs and significantly suppressed increases in the Th17 cell population. Therefore, the treatment of DCs with IL-35 can further regulate T cell proliferation and function. In the context of IL-35-mediated propagation of immune tolerance, IL-35-conditioned DCs might exert a similar function on Tregs. These findings provide further insight into the ability of IL-35 to induce immature DCs, indirectly affecting T cell proliferation and expanding the $Foxp3^+$ Treg population *in vitro*.

Therapeutic strategies using cell products that are composed of immunoregulatory cell populations are being tested [44]. Pluripotent mesenchymal stem cells have been postulated to have immunosuppressive properties. Tan and colleagues demonstrated that autologous MSC infusion as an induction agent for living-donor kidney transplantation increased Treg generation [44, 45]. DCs with tolerogenic properties have been explored extensively in small animal models, and a meta-analysis demonstrated their potency in prolonging allograft

survival in multiple transplantation models [21]. For human injection, the feasibility of generating *ex vivo* DCs with tolerogenic properties has now been proven. The main challenge in bringing tolDCs into the clinic is the requirement of preserving their tolerogenic properties upon transfusion into the patient. However, there are two major concerns regarding the use of tolDCs in transplantation: inflammatory mediators and immunosuppressive drugs. Organ transplantation induces inflammation in the graft microenvironment that can gradually lead to tolDC maturation, and treatment with immunosuppressive drugs can affect the ability of tolDCs to induce tolerance. In this study, we transfused IL-35/Ebi3-mDCs into BALB/c heart transplant recipient mice. The mice transfused with IL-35/Ebi3-mDCs exhibited significantly prolonged allograft survival. Allograft survival was slightly prolonged in BALB/c recipients that were treated with mDCs, which can be partially attributed to the systemic delivery of IL-35/Ebi3-mDCs but not the transfer of mDCs. The IL-35-induced immature DC phenotype contributes to the Treg infiltration of cardiac allografts. IL-35 treatment of imDCs and then stimulation with LPS induces a DC hypotype that displays tolerogenic features, remains stable and is able to prolong organ graft survival. Thus, DC transfer, along with the delivery or overexpression of IL-35, may represent a potential preventive and therapeutic tool for the regulation of allograft rejection [4]. Considering the particular tolerance mechanisms employed by IL-35, its combination with other tolerogenic molecules, such as TGF-β or IL-10, might potentiate immunosuppressive effects in therapeutic settings.

Unlike other diseases or conditions, transplantation involves allorecognition between two parts, the graft and the host. Autologous tolerogenic DCs may be more effective than donor tolerogenic DCs for transplantation, and the safety and efficiency of DCs have been discussed. Using donor bone marrow DCs (stimulated with low doses of GM-CSF) in a rat heart transplant model, DePaz et al. showed that tolDC therapy was able to increase rat cardiac allograft survival [46]. However, a later study using donor tolDCs or apoptotic bodies from donor tolDCs showed that tolerance was mediated by the presentation of donor peptides (from donor cells or apoptotic bodies) by recipient DCs, which inhibits $CD4^+$ T cell activation and favours Treg expansion [22, 47]. Both therapies have been shown to be partially efficient, but on the other hand, the risk of sensitisation (including the development of alloantibodies) remains. Therefore, the use of autologous tolDCs appears to be a better alternative, at least in terms of safety, because it avoids the risk of sensitisation. In this study, autologous DCs treated with IL-35/Ebi3 displayed an immature phenotype and, upon injection the day before transplantation, were able to prolong cardiac allograft survival. We also demonstrated that rats receiving heart allografts and treated with autologous DCs transfected with let-7i achieved remarkable prolonged survival of cardiac allografts and generated Tregs [29]. These combined results demonstrated that autologous tolDCs are even more efficient than donor tolDCs. Finally, results confirming the efficacy and safety of autologous tolDCs in humans in transplantation will be evaluated in the coming years.

Conclusion

Overexpression of IL-35 regulated DC maturation and function and subsequently affected the balance between Tregs and Th17 cells. The transfusion of IL-35-transfected DCs induced the generation of Tregs in mice and prolonged cardiac allograft survival. In conclusion, our data demonstrated that IL-35 can induce DC immune tolerance and that IL-35-overexpressing DCs might be a promising approach for regulating immune tolerance and immunological disorders.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (Grants NO. 81330033, 81670373, 81771946 and 81670459) and the Natural Science Foundation

of Heilongjiang Province of China (Grant NO. H2015048). This study was also supported by the Graduate Student Innovation Foundation of Heilongjiang Province (YJSCX2017-39HYD).

Abbreviations

DCs (dendritic cells); imDC (immature dendritic cells); IL-35 (interleukin-35); LPS (lipopolysaccharide); mDC (mature dendritic cells); MST (median survival times); PBS (phosphate-buffered saline); SOCS1 (suppressor of cytokine signalling 1); Th2 (type 2 T helper cell); tolDCs (tolerogenic DCs); Tregs (regulatory T cells).

Disclosure Statement

The authors declare that they have no competing interests.

References

1 Li X, Mai J, Virtue A, Yin Y, Gong R, Sha X, Gutchigian S, Frisch A, Hodge I, Jiang X, Wang H, Yang XF: IL-35 is a novel responsive anti-inflammatory cytokine--a new system of categorizing anti-inflammatory cytokines. PLoS One 2012;7:e33628.
2 Wang RX, Yu CR, Dambuza IM, Mahdi RM, Dolinska MB, Sergeev YV, Wingfield PT, Kim SH, Egwuagu CE: Interleukin-35 induces regulatory B cells that suppress autoimmune disease. Nat Med 2014;20:633-641.
3 Jones LL, Chaturvedi V, Uyttenhove C, Van Snick J, Vignali DA: Distinct subunit pairing criteria within the heterodimeric IL-12 cytokine family. Mol Immunol 2012;51:234-244.
4 Haller S, Duval A, Migliorini R, Stevanin M, Mack V, Acha-Orbea H: Interleukin-35-producing CD8alpha+ dendritic cells acquire a tolerogenic state and regulate T cell function. Front Immunol 2017;8:98.
5 Wirtz S, Billmeier U, Mchedlidze T, Blumberg RS, Neurath MF: Interleukin-35 mediates mucosal immune responses that protect against t-cell–dependent colitis. Gastroenterology 2011;141:1875-1886.
6 Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, Cross R, Sehy D, Blumberg RS, Vignali DAA: The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature 2007;450:566-569.
7 Niedbala W, Wei XQ, Cai B, Hueber AJ, Leung BP, McInnes IB, Liew FY: IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells. Eur J Immunol 2007;37:3021-3029.
8 Zandian M, Mott KR, Allen SJ, Dumitrascu O, Kuo JZ, Ghiasi H: Use of cytokine immunotherapy to block CNS demyelination induced by a recombinant HSV-1 expressing IL-2. Gene Ther 2011;18:734-742.
9 Huang CH, Loo EX, Kuo IC, Soh GH, Goh DL, Lee BW, Chua KY: Airway inflammation and IgE production induced by dust mite allergen-specific memory/effector Th2 cell line can be effectively attenuated by IL-35. J Immunol 2011;187:462-471.
10 Dong J, Wong CK, Cai Z, Jiao D, Chu M, Lam CW: Amelioration of allergic airway inflammation in mice by regulatory IL-35 through dampening inflammatory dendritic cells. Allergy 2015;70:921-932.
11 Shao Y, Cheng Z, Li X, Chernaya V, Wang H, Yang XF: Immunosuppressive/anti-inflammatory cytokines directly and indirectly inhibit endothelial dysfunction--a novel mechanism for maintaining vascular function. J Hematol Oncol 2014;7:80.
12 Dixon KO, van der Kooij SW, Vignali DA, van Kooten C: Human tolerogenic dendritic cells produce IL-35 in the absence of other IL-12 family members. Eur J Immunol 2015;45:1736-1747.
13 Opelz G: President's Address. The Transplantation Society--Berlin 2012. Transplantation 2013;95:4-7.
14 Benza RL, Coffey CS, Pekarek DM, Barchue JP, Tallaj JA, Passineau MJ, Grenett HE: Transforming growth factor-beta polymorphisms and cardiac allograft rejection. J Heart Lung Transpl 2009;28:1057-1062.
15 Opelz G, Dohler B, Laux G, Collaborative Transplant S: Long-term prospective study of steroid withdrawal in kidney and heart transplant recipients. Am J Transplant 2005;5:720-728.
16 Munn DH, Mellor AL: Indoleamine 2, 3 dioxygenase and metabolic control of immune responses. Trends Immunol 2013;34:137-143.

17 Conde P, Rodriguez M, van der Touw W, Jimenez A, Burns M, Miller J, Brahmachary M, Chen HM, Boros P, Rausell-Palamos F, Yun TJ, Riquelme P, Rastrojo A, Aguado B, Stein-Streilein J, Tanaka M, Zhou L, Zhang J, Lowary TL, Ginhoux F et al.: DC-SIGN(+) Macrophages Control the Induction of Transplantation Tolerance. Immunity 2015;42:1143-1158.
18 Zhao X, Boenisch O, Yeung M, Mfarrej B, Yang S, Turka LA, Sayegh MH, Iacomini J, Yuan X: Critical role of proinflammatory cytokine IL-6 in allograft rejection and tolerance. Am J Transplant 2012;12:90-101.
19 Bezie S, Picarda E, Ossart J, Tesson L, Usal C, Renaudin K, Anegon I, Guillonneau C: IL-34 is a Treg-specific cytokine and mediates transplant tolerance. J. Clin. Invest. 2015;125:3952-3964.
20 Steinman RM, Hawiger D, Nussenzweig MC: Tolerogenic dendritic cells. Annu Rev Immunol 2003;21:685-711.
21 Moreau A, Alliot-Licht B, Cuturi MC, Blancho G: Tolerogenic dendritic cell therapy in organ transplantation. Transpl Int 2017;30:754-764.
22 Marin E, Cuturi MC, Moreau A: Tolerogenic Dendritic Cells in Solid Organ Transplantation: Where Do We Stand? Front Immunol 2018;9:274.
23 Collison LW, Chaturvedi V, Henderson AL, Giacomin PR, Guy C, Bankoti J, Finkelstein D, Forbes K, Workman CJ, Brown SA, Rehg JE, Jones ML, Ni HT, Artis D, Turk MJ, Vignali DA: IL-35-mediated induction of a potent regulatory T cell population. Nat Immunol 2010;11:1093-1101.
24 Zhang M, Liu F, Jia H, Zhang Q, Yin L, Liu W, Li H, Yu B, Wu J: Inhibition of microRNA let-7i depresses maturation and functional state of dendritic cells in response to lipopolysaccharide stimulation via targeting suppressor of cytokine signaling 1. J Immunol 2011;187:1674-1683.
25 Liu S, Yu Y, Zhang M, Wang W, Cao X: The involvement of TNF-α-related apoptosis-inducing ligand in the enhanced cytotoxicity of IFN-β-stimulated human dendritic cells to tumor cells. J Immunol 2001;166:5407-5415.
26 Guo Z, Zhang M, An H, Chen W, Liu S, Guo J, Yu Y, Cao X: Fas ligation induces IL-1beta-dependent maturation and IL-1beta-independent survival of dendritic cells: different roles of ERK and NF-kappaB signaling pathways. Blood 2003;102:4441-4447.
27 Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402-408.
28 Niimi M: The technique for heterotopic cardiac transplantation in mice: experience of 3000 operations by one surgeon. J Heart Lung Transpl 2001;20:1123-1128.
29 Sun Y, Jin X, Liu X, Zhang M, Liu W, Li Z, Han N, Tan M, Chi D, Zhu J, Zhang B, Yu B, Wu J: MicroRNA let-7i regulates dendritic cells maturation targeting interleukin-10 via the Janus kinase 1-signal transducer and activator of transcription 3 signal pathway subsequently induces prolonged cardiac allograft survival in rats. J Heart Lung Transplant 2016;35:378-388.
30 Stewart S, Winters GL, Fishbein MC, Tazelaar HD, Kobashigawa J, Abrams J, Andersen CB, Angelini A, Berry GJ, Burke MM, Demetris AJ, Hammond E, Itescu S, Marboe CC, McManus B, Reed EF, Reinsmoen NL, Rodriguez ER, Rose AG, Rose M et al.: Revision of the 1990 working formulation for the standardization of nomenclature in the diagnosis of heart rejection. J Heart Lung Transpl 2005;24:1710-1720.
31 Dixon KO, van der Kooij SW, Vignali DA, van Kooten C: Human tolerogenic dendritic cells produce IL-35 in the absence of other IL-12 family members. Eur J Immunol 2015;45:1736-1747.
32 Collison LW, Delgoffe GM, Guy CS, Vignali KM, Chaturvedi V, Fairweather D, Satoskar AR, Garcia KC, Hunter CA, Drake CG, Murray PJ, Vignali DA: The composition and signaling of the IL-35 receptor are unconventional. Nat Immunol 2012;13:290-299.
33 Villarino AV, Kanno Y, O'Shea JJ: Mechanisms and consequences of Jak-STAT signaling in the immune system. Nat Immunol 2017;18:374-384.
34 Zheng Y, Wang Z, Deng L, Zhang G, Yuan X, Huang L, Xu W, Shen L: Modulation of STAT3 and STAT5 activity rectifies the imbalance of Th17 and Treg cells in patients with acute coronary syndrome. Clin Immunol 2015;157:65-77.
35 Shuai K, Liu B: Regulation of JAK-STAT signalling in the immune system. Nat Rev Immunol 2003;3:900-911.
36 Mertens C, Darnell JE, Jr.: SnapShot: JAK-STAT signaling. Cell 2007;131:612.
37 Nakagawa R, Naka T, Tsutsui H, Fujimoto M, Kimura A, Abe T, Seki E, Sato S, Takeuchi O, Takeda K, Akira S, Yamanishi K, Kawase I, Nakanishi K, Kishimoto T: SOCS-1 participates in negative regulation of LPS responses. Immunity 2002;17:677-687.

38 Hanada T, Yoshida H, Kato S, Tanaka K, Masutani K, Tsukada J, Nomura Y, Mimata H, Kubo M, Yoshimura A: Suppressor of cytokine signaling-1 is essential for suppressing dendritic cell activation and systemic autoimmunity. Immunity 2003;19:437-450.
39 Evel-Kabler K, Song XT, Aldrich M, Huang XF, Chen SY: SOCS1 restricts dendritic cells' ability to break self tolerance and induce antitumor immunity by regulating IL-12 production and signaling. J Clin Invest 2006;116:90-100.
40 Jiga LP, Ehser S, Kleist C, Opelz G, Terness P: Inhibition of heart allograft rejection with mitomycin C-treated donor dendritic cells. Transplantation 2007;83:347-350.
41 Rutella S, Danese S, Leone G: Tolerogenic dendritic cells: cytokine modulation comes of age. Blood 2006;108:1435-1440.
42 Verbsky JW, Chatila TA: T-regulatory cells in primary immune deficiencies. Curr Opin Allergy Cl 2011;11:539-544.
43 Vignali DA, Kuchroo VK: IL-12 family cytokines: immunological playmakers. Nat Immunol 2012;13:722-728.
44 Morath C, Schmitt A, Kalble F, Zeier M, Schmitt M, Sandra-Petrescu F, Opelz G, Terness P, Schaier M, Kleist C: Cell therapeutic approaches to immunosuppression after clinical kidney transplantation. Pediatr Nephrol 2018;33:199-213.
45 Tan J, Wu W, Xu X, Liao L, Zheng F, Messinger S, Sun X, Chen J, Yang S, Cai J, Gao X, Pileggi A, Ricordi C: Induction therapy with autologous mesenchymal stem cells in living-related kidney transplants: a randomized controlled trial. JAMA 2012;307:1169-1177.
46 Collison LW, Delgoffe GM, Guy CS, Vignali KM, Chaturvedi V, Fairweather D, Satoskar AR, Garcia KC, Hunter CA, Drake CG, Murray PJ, Vignali DA: The composition and signaling of the IL-35 receptor are unconventional. Nat Immunol 2012;13:290-299.
47 Divito SJ, Wang Z, Shufesky WJ, Liu Q, Tkacheva OA, Montecalvo A, Erdos G, Larregina AT, Morelli AE: Endogenous dendritic cells mediate the effects of intravenously injected therapeutic immunosuppressive dendritic cells in transplantation. Blood 2010;116:2694-2705.