MicroRNA let-7i Regulates Dendritic Cells Maturation Targeting Interleukin-10 via JAK1-STAT3

Signal Pathway Subsequently Induces Prolonged Cardiac Allograft Survival in Rats

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Running title: Role of MiRNA let-7i in alloimmune responses

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Abstract

BACKGROUND: In this study, we investigated whether microRNA let-7i regulates DCs maturation targeting IL-10 via JAK1-STAT3 signal pathway subsequently prolongs rat cardiac allograft survival.

METHODS: QRT-PCR, ELISA, dual-luciferase assay were performed to verify whether IL-10 was the target of let-7i, and regulatory T cells were assessed by flow cytometry and immunohistochemical study, western bolt were performed to detect JAK1, STAT3 and pSTAT3 expression. Lewis recipients of DA heart were transfused with PBS, LPS-mDCs, or let-7i-inhibitor-mDCs. Allograft survival times were recorded, and histologic studies were performed.

RESULTS: Expression of IL-10 mRNA level and production of IL-10 were increased in let-7i-inhibitor-mDCs as compared to LPS-mDCs. Luciferase activity showed that the translational level of the IL-10 luciferase reporter was decreased by let-7i-mimic, but increased by let-7i-inhibitor. MicroRNA let-7i-inhibitor suppressed DC maturation, however, pretreatment of IL-10 SiRNA attenuated the suppression. Expression of JAK1, STAT3, and pSTAT3 in mDCs were suppressed by let-7i-mimic and pretreatment of IL-10 SiRNA, however up-regulated by let-7i-inhibitor. Lewis recipients transfused with let-7i-inhibitor-mDCs significantly prolonged DA cardiac allograft survival. The allografts transfused with let-7i-inhibitor-mDCs showed slight cell infiltration and significantly preserved graft structure. Inhibition of let-7i increased CD4⁺CD25⁺Foxp3⁺ Tregs and modulated cytokine profiles both in vivo and in vitro.

CONCLUSION: MicroRNA let-7i regulated DCs maturation and function targeting the IL-10 through JAK1-STAT3 pathway. Moreover, transfusion of LPS-induced mDCs transfected with let-7i-inhibitor induced prolonged cardiac allograft survival and generated regulatory T cells.

Abbreviation

DC: dendritic cell

ELISA: enzyme-linked immunosorbent assay

FITC: fluorescein isothiocyanate

Foxp3: forkhead box P3

GAPDH: glyceraldehyde phosphate dehydrogenase

IFN: interferon

IL: interleukin

imDC: immature dendritic cell

JAK: Janus kinase

LPS: lipopolysaccharide

MCS: multiple cloning sites

mDC: mature dendritic cells

MEK: mitogen-activated protein kinase

MHC: major histocompatibility complex

MiR: microRNA

MLR: mixed lymphocyte reaction

MST: median survival time

PE: phycoerythrin

qRT-PCR: quantitative real-time reverse transcriptase polymerase chain reaction

SOCS: suppressor of cytokine signaling

STAT: signal transducer and activator of transcription

TGF: transforming growth factor

TLR: Toll-like receptor

Treg: regulatory T cell

Interleukin (IL)-10 is a key modulator with prominent anti-inflammatory effects in immune system. It is secreted by various cell types including dendritic cells (DCs), macrophages, B cells, eosinophils, and different T cell subsets, indicating its central role in many immunological reactions. IL-10 mediates maturation and activation of macrophages and DC, inhibits the production of pro-inflammatory cytokines and chemokines by inhibiting accessory CD28/B7.1 receptor interaction, and reduces the antigen-presenting capacity of DCs by down-regulating major histocompatibility complex (MHC) II molecules.^{1,2} Moreover, IL-10 promotes the generation of regulatory T cells (Tregs)³ and is required for Tregs to mediate tolerance to alloantigens.⁴ Finally, IL-10 is crucial not only for peripheral tolerance to allergens, but also in protecting the host from amplified inflammatory responses to pathogens as well as to autoimmune diseases.⁵

The Janus kinase (JAK) - signal transducer and activator of transcription (STAT) signaling pathway is one of the principal signaling pathways utilized by cytokine receptors. Activated JAKs eventually induce phosphorylation of STATs, followed by their translocation into the nucleus where they modulate the expression of the target genes. JAK-STAT signaling pathway is one of the most important pleiotropic cascades employed by cells to transduce signals for hormones, growth factors, and cytokines.⁶ STAT3 plays a critical role in the immune system which includes controlling DC production,⁷ inhibiting macrophage inflammatory signaling,⁸ and associating with forkhead box P3 (Foxp3), which is relevant to the function of Tregs.⁹

MicroRNA (MiR), which is a class of small non-coding RNA, has been implicated in the regulation of gene expression essential for organ development, cellular differentiation, homeostasis, and functioning. MiR targets degradation of mRNA or translational control, thereby regulating the expression of genes.¹⁰ Moreover, several evidences have demonstrated the critical role of MiR in immune responses which includes MiR-181-induced B lymphocytes differentiation from hematopoietic stem cells,¹¹ negative regulation of innate immune response by interfering Toll-like receptor (TLR) signaling pathway by MiR-146a,¹² and

involvement of MiR-155 in both innate and adaptive immune responses.¹³

In previous study,¹⁴ we have demonstrated that expression of MiR let-7i was up-regulated in lipopolysaccharide (LPS)-induced DC maturation. Additionally, let-7i inhibition depresses the maturation and functional state of DCs. Moreover, DCs treated with let-7i-inhibitor induced T cell hyporesponsiveness and expanded Tregs in vitro. In the current study, we provided evidences suggesting that let-7i targeting IL-10 via JAK1-STAT3 signal pathway regulated DCs maturation, and subsequently induced cardiac allograft tolerance in rat heart transplantation model.

Methods

Animals

Male Lewis (RT1^l) and Dark Agouti (DA, RT1^{av1}) rats (220 - 250 g) were purchased from Shanghai Lab Animal Research Center (China). All animal care and procedures were in compliance with the 'Principles of Animal Care' (National Society for Medical Research, USA) and the 'Guide for the Care and Use of Laboratory Animals' (Institute of Laboratory Animal Resources/NIH, USA).

Isolation of DCs, transfection of MiR let-7i-mimic, inhibitor or IL-10 Si-RNA, and LPS stimulation

DCs were isolated from the bone marrow of Lewis rats. Bone marrow was flushed from femurs and tibia, and was passed through a 200 pore size mesh to remove fibrous tissues. The red blood cells were lysed and the remaining cells were cultured at 2×10^6 cells/ml in RPMI 1640 medium (HyClone, Logan, UT) supplemented with cytokines (10ng/ml GM-CSF and IL-4 for each rat; PeproTech, Rocky Hill, NJ).

Immature DCs (imDCs) were generated and were transfected with MiR let-7i mimic or inhibitor (60 nM, GenePharma, Shanghai, China) using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 5 h at 6th day. One part of imDCs transfected with IL-10 Si-RNA (60nM, GenePharma, Shanghai, China) also using Lipofectamine 2000 for 5 h at 5th day before transfection with let-7i-inhibitor. Most experiments used

let-7i-mimic or inhibitor at 60 nM for transfection. To induce DC maturation, cells at 6th day in vitro were treated with the TLR ligand-LPS (200 ng/ml; Sigma-Aldrich, St. Louis, MO) for 12 h.

After culturing in LPS, mature DCs (mDCs) were subsequently collected for co-culture with T cells or for in vivo injection.

Mixed lymphocyte reaction (MLR) assay

DCs used at different densities ($0\text{--}15 \times 10^4$) as stimulators for T cells (2×10^5), were isolated from Lewis rat spleens. To assess the impact of DCs in different functional states on T cell proliferation and function, a co-culture system was developed. Isolated rat T cells were cultured for 8 days in 96-well round bottom microplates in the presence of 10 µg/ml mitomycin C-pretreated DCs at DC:T cell ratio of 1:10.

In another MLR study, the responder cells were splenocytes from Lewis recipients transfused with phosphate buffer saline (PBS), LPS-mDCs, and let-7i-inhibitor-mDCs 14 days after transplantation. The stimulator cells were DA (allogeneic) splenocytes treated with 100 µg/ml mitomycin C for 30 minutes at 37°C. The responder cells ($2.5 \times 10^6/\text{mL}$) were co-cultured with the stimulator cells ($5.0 \times 10^6/\text{mL}$) at 37°C in 96-well, round-bottomed, tissue-culture plates for 4 days. Proliferation was assessed by using an Enzyme-linked Immunosorbent Assays (ELISA) for bromodeoxyuridine incorporation (Biotrak, version 2, Amersham, Little Chalfont, United Kingdom [UK]) according to the manufacturer's instructions.

Quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was isolated from each DC subset (LPS, let-7i inhibitor) with Trizol reagents (Invitrogen, USA), reverse-transcribed to cDNA with specific stem-loop primers, and amplified using SYBR Premix Ex Taq II (Bioneer). The reverse transcription reactions were incubated for 30 min at 16°C, 42 min at 42°C, and 5 min at 85°C. The PCR protocol consisted of 40 cycles of 10 s at 95°C and 1 min at 60°C. The primers used for RT-PCR were as follows: IL-10 (forward: 5'-CCTGCTCTTACTGGCTGGAG-3'; reverse: 5'-TCCAGCTGGTCCTTCTTTTG-3') and glyceraldehyde phosphate dehydrogenase (GAPDH) (forward:

5'-GCTTCGGCAGCACATATACTAAAAT-3'; reverse: 5'-CGCTTCACGAATTTGCGTGTCAT-3'). Each sample was measured in triplicate.

ELISA

To measure the release of cytokines by DCs, supernatants were collected from the cultures following let-7i mimic or inhibitor transfection and LPS stimulation. The levels of IL-4, IL-10, IL-12, IL-17, IL-35 and transforming growth factor- β (TGF- β) were determined by ELISA according to the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). In some experiments, the levels of interferon (IFN)- γ , IL-4, IL-10, IL-12 and TGF- β in Lewis recipient's plasma 14 days after transplantation were also determined. All the assays were performed in triplicate.

Dual-luciferase assay

The vector consists of pGL3-IL-10-3'UTR-WT and pGL3-IL-10-3'UTR-Mut. On the basis of pGL3-control-multiple cloning sites (MCS), the wild-type and mutant variants of IL-10 3'UTR sequence were inserted into vector. The template of wild-type insert fragment was rat IL-10 3'UTR, and that of mutant was based on the wild-type carrier. Both the templates were produced by a series of PCR amplification. MiR let-7i mimic, inhibitor, and negative control with Lipofectamine RNAi MAX (RNA 10nmol/L) were transfected into the DCs before the transfection of the vectors with Lipofectamine 2000 (firefly luciferase plasmid 10 ng, Renilla luciferase plasmid 2ng). The luciferase activity was assessed at 24h following the transfection.

Flow cytometry

Purity and phenotype of the DCs were analyzed by surface expression of specific markers using flow cytometry. The following antibodies were used: phycoerythrin (PE)-CD80, PE-CD86, and PE-MHC-II (eBioscience, San Diego, CA, USA). For each staining protocol, the appropriate isotype-matched control was included.

For Treg analysis, expressions of CD4⁺, CD25⁺, and Foxp3⁺ were also determined. The cells in mixed lymphocyte reactions (MLRs) supernatant were stained with fluorochrome-conjugated CD4- fluorescein isothiocyanate (FITC), CD25-APC and Foxp3-PE mAb, as well as their isotype controls (all from eBioscience, USA). In some of the experiments, the splenocytes from the recipients transfused PBS, LPS-mDCs or let-7i inhibitor-mDCs 14 days after transplantation were also stained. The stained cells were analyzed using FACS Canto II system (BD Biosciences, USA) to determine the percentage of CD4⁺CD25⁺Foxp3⁺ in CD4⁺ cells.

Western blotting

Western blotting was used to assess the expression of JAK1-STAT3 in DCs. Briefly, total cell lysates were obtained after exposure to LPS and were blotted to determine JAK1, STAT3, STAT3 phosphorylation (pSTAT3) and β -actin (loading control) using anti- JAK1, anti-STAT3, anti-pSTAT3 and anti- β -actin (Abcam, USA). The JAK1, STAT3, pSTAT3 and β -actin protein levels were quantified using scanning densitometry (GS-710 imaging). The data was obtained in triplicate for independent experiments.

Heart transplantation

After general anesthesia, Lewis recipients were transfused with 0.3 ml of PBS only (negative control), 1.0×10^6 of LPS-mDCs or let-7i-inhibitor-mDCs (Lewis bone marrow derived) by means of intravenous injection into the penile vein. 24 h after transfusion, Lewis recipients underwent fully vascularized heterotopic heart transplantation of a DA heart by using microsurgical techniques.¹⁵ Other Lewis recipients underwent heart transplantation after giving oral administration of 1 mg/kg of Tacrolimus (positive control). Post-operatively, graft function was assessed daily by palpation for evidence of contraction. Rejection was defined as complete cessation of the heartbeat, and was confirmed by direct visualization and histologic examination of the graft.

Immunohistochemical and histologic studies of harvested grafts

Cardiac allografts in Lewis recipients, which were transfused with PBS, LPS-mDCs or let-7i-inhibitor-mDCs, were removed 14 days after heart transplantation, and were studied histologically and immunohistochemically.

Hematoxylin and Eosin (HE) staining was assessed by grading according to the classification of International Society for Heart and Lung Transplantation 2005 (ISHLT 2005)¹⁶ for acute cellular rejection (Grade 0, No rejection; 1 (mild), Interstitial and/or perivascular infiltrate with up to 1 focus of myocyte damage; 2 (moderate), Two or more foci of infiltrate with associated myocyte damage; 3 (severe), Diffuse infiltrate with multifocal myocyte damage $\pm$ edema, $\pm$ hemorrhage $\pm$ vasculitis). In each section, five microscopic fields were examined under a microscope at a magnification of $\times 40$.

For immunohistochemical staining, the sections were dewaxed, underwent antigen retrieval, and were incubated with anti-Foxp3 (FJK-16s, eBioscience) followed by incubation with the secondary antibody. Staining was performed by the immunoperoxidase method and visualized with DAB (ZSGB-BIO, China).

Statistical analysis

Data were expressed as means $\pm$ SD. Cardiac allograft survival in groups of rats was compared using Mann-Whitney U testing (Graphpad Prism; Graphpad, CA, USA). Other data were compared using analysis of variance (ANOVA) with Ryan method. A value of $p < 0.05$ was considered as statistically significant.

Results

Dendritic cells maturation

To investigate the effect of let-7i targeting IL-10 during DC maturation, we first determined the IL-10 mRNA expression levels and production of IL-10 in bone marrow-derived DCs that were transfected with the let-7i-inhibitor (60nM, 90 nM, 120nM, 150nM, 180nM) by qRT-PCR and ELISA. The IL-10 level was increased in let-7i-inhibitor-mDCs as compared to LPS-mDCs, (peaks were both observed in 150nM

let-7i-inhibitor groups; Figure 1A-B). IL-10 mRNA expression levels and production of IL-10 in DCs in response to LPS with or without let-7i-inhibitor were also analyzed by qRT-PCR and ELISA at different time points. Results have demonstrated that IL-10 mRNA expression levels increased up to 8 h and then declined (Figure 1C), whereas the production of IL-10 was increased continuously (Figure 1D).

Using biological information database (TargetScan, PicTar, and KEGG PATHWAY), it was found that let-7i was predicted to target rat IL-10. Luciferase activity showed that the translational level of IL-10 luciferase reporter was significantly decreased by let-7i-mimic, whereas markedly increased by let-7i-inhibitor. However, the contrary result was observed in cells transfected with mutant IL-10 3'-UTR (Figure 1E). These results suggested that IL-10 was direct target of let-7i.

After 12h of LPS stimulation, the levels of CD80, CD86, and MHC-II were significantly increased in mDCs and let-7i-mimic-mDCs as compared to imDCs (Figure 1F). Moreover, mDCs transfected with let-7i-inhibitor expressed lower levels of CD80, CD86, and MHC-II, however, pretreatment with IL-10 SiRNA resulted in a slight elevation of CD80, CD86, and MHC-II expression.

Effect of DCs on inducing Tregs

To determine whether MiR let-7i targeting IL-10 regulates DCs function in inducing Tregs, we transfected let-7i-mimic, let-7i-inhibitor or IL-10 SiRNA (before transfection of let-7i-inhibitor) to DCs in MLR, and determined CD4⁺CD25⁺Foxp3⁺ T cells. Results showed that the CD4⁺CD25⁺Foxp3⁺ T cells decreased by mDCs and let-7i-mimic-mDCs, whereas significantly increased in MiR let-7i-inhibitor-mDCs (Figure 2). Moreover, pretreatment of IL-10 SiRNA attenuated the induction of Tregs.

Modulation of cytokine profile by let-7i in DCs

Levels of IL-12 and IL-17 were increased in let-7i-mimic group whereas decreased in let-7i-inhibitor group. Pretreatment of IL-10 SiRNA before MiR let-7i-inhibitor transfection increased the induction of IL-12 and IL-17 (Figure 3). On the contrary, levels of IL-4, IL-35 and TGF- β were decreased in let-7i-mimic groups

whereas increased in let-7i-inhibitor groups. Pretreatment of IL-10 SiRNA before let-7i-inhibitor transfection decreased IL-4, IL-35 and TGF- β inductions.

Effect of MiR let-7i on the expression of JAK-STAT3

To investigate whether MiR let-7i regulated DCs function through JAK1-STAT3 signal pathway, expression of JAK1, STAT3, and STAT3 phosphorylation (pSTAT3) in LPS-mDCs was estimated by western blotting. Expression of JAK1, STAT3, and pSTAT3 decreased in mDCs and MiR let-7i-mimic-mDCs as compared to imDCs. However, the expression was increased in MiR let-7i-inhibitor-mDCs (Figure 4). Moreover, pretreatment of IL-10 SiRNA before MiR let-7i-inhibitor transfection significantly attenuated the expression of JAK1, STAT3, and pSTAT3.

Survival of cardiac allografts in rats

Lewis recipients transfused with PBS and LPS-mDCs rejected DA grafts acutely (median survival times [MSTs]: 8 and 9 days, respectively; Figure 5A), whereas those transfused with let-7i-inhibitor-mDCs had significantly prolonged allograft survival (MST: 40 days). Lewis recipients, which were treated with Tacrolimus (1 mg/kg/day) for 7 days, prolonged the allograft survival slightly (MST: 14 days).

Histologic features of cardiac allografts

Histological examination of cardiac allografts showed slight cell infiltration and preserved graft structure in let-7i-inhibitor-mDCs transfused transplant recipients. However, allografts from PBS transfused recipients showed severe myocyte damage, edema, and moderate inflammatory infiltration, and LPS-mDCs transfused allografts showed aggressive diffused infiltration with myocyte damage (Figure 5B, C, D). We observed a significant difference by grading among PBS, LPS-mDCs and let-7i-inhibitor-mDCs injected groups (Figure 5E).

Generation of Tregs in rats treated with MiR let-7i-inhibitor-mDCs

Results of flow cytometry study showed that the percentage of CD4⁺CD25⁺Foxp3⁺ cells in CD4⁺ cells was

significantly increased in the spleens of let-7i-inhibitor-mDCs transfused recipients (Figure 6A).

Maximum proliferation of Lewis splenocytes (responder cells) against DA splenocytes (stimulator cells) occurred on day 4 of the MLRs. Proliferation of splenocytes from let-7i-inhibitor-mDCs transfused recipients was significantly suppressed compared with those of splenocytes from PBS and LPS-mDCs transfused recipients ($p < 0.001$ and $p < 0.0001$; Figure 6B), suggested that the suppressive cells were induced in let-7i-inhibitor-mDCs transfused recipients.

Immunohistochemical study showed that cardiac allografts from let-7i-inhibitor-mDCs transfused recipients had more Foxp3⁺ cells than those from PBS and LPS-mDCs transfused recipients (Figure 6C, D, E, F), suggested that the CD4⁺CD25⁺Foxp3⁺ regulatory cells were induced in let-7i-inhibitor-mDCs transfused recipients.

Cytokine production in Lewis recipients

In the plasma of Lewis recipients transfused with let-7i-inhibitor-mDCs, levels of IL-12 and IFN- γ were decreased, whereas IL-4, IL-10 and TGF- β were increased (Figure 7) as compared to PBS and LPS-mDCs transfused recipients.

Discussion

In this study, we investigated whether IL-10 is the target of let-7i, and the association between let-7i and JAK/STAT pathway. Moreover, we explored the indirect effect of let-7i on the production of IL-35 and TGF- β , examined whether mDCs transfected with let-7i-inhibitor could induce prolongation of allograft survival in rat heart transplantation. Our results showed that let-7i regulates DCs maturation targeting IL-10, through JAK1-STAT3 pathway. Furthermore, in vivo experiments demonstrated that transfusion of mDCs, which were transfected with let-7i-inhibitor, induces prolonged survival of cardiac allografts and generates Tregs. These results were consistent with the findings of in vitro experiments.

The distribution of DCs is dynamic, and it became clear recently that steady-state DCs do not remain immature and tolerogenic by default.¹⁷ Therefore, dynamic control of DC maturation is essential for influencing the generation and/or peripheral expansion of Tregs. Huang G et al¹⁸ demonstrated that mitogen-activated protein kinase (MAPK) p38 α activity in DCs plays an important role in mediating oral tolerance and induced Treg (iTreg) generation in vivo. Otherwise, MiRs (such as MiR 142-3p, let-7i, 155, and 148/152) also participates in regulating the maturation of DCs.¹⁹ Several studies have demonstrated that transfusion of imDCs prolongs the organ allograft survival in animal models,^{20,21,22} and expression of MiR is relevant to the outcomes of organ transplantation.^{23,24,25} In our study, we have demonstrated that inhibition of let-7i in mDCs decreased the expression levels of CD80, CD86 and MHC-II (Figure 1F), however, increased CD4⁺CD25⁺Foxp3⁺ T cells (Figure 2). Moreover, Lewis recipients transfused with let-7i-inhibitor-mDCs had significantly prolonged allograft survival (Figure 5A). These results indicated that inhibition of let-7i impedes maturation of LPS-stimulated mDC and generated Tregs.

MiR let-7 family is a regulator of developmental timing and cellular proliferation, and comprises of 12 members which are expressed from eight distinct loci.^{26,27} MiR let-7i, a member of let-7 family, plays an important role in anticancer drug resistance in vivo and in vitro.²⁸ In our previous study,¹⁴ we have demonstrated that DCs' maturation and functional state were regulated by let-7i by targeting suppressor of cytokine signaling 1 (SOCS1). In dilated cardiomyopathy, let-7i targets TLR4 at the post-transcriptional stage and limits its expression. The negative relationship between let-7i and TLR4 was confirmed by in vivo study on dilated cardiomyopathy patients.^{29,30} In the present study, we also demonstrated that let-7i targets IL-10 in the regulation of DCs' maturation.

IL-10 is an important immunosuppressive factor that affects the main function of DCs in antigen presentation, and the release of immune mediators' function³¹ and enhances the release of anti-inflammatory factor, TGF- β and IL-35. Recently, many studies have been conducted on regulation of IL-10 by MiRs, such

as MiR-17-92, 98, 106a, 126, 155 and 466l, in various immunological diseases.^{32,33,34,35,36,37} IL-10-mediated JAK-STAT pathway plays an important role in maintaining immune homeostasis and tolerance.³⁸ Yeganegi M et al.³⁹ have demonstrated that activation of STAT3 by myeloid DCs via IL-10 receptor is a key step in the anti-inflammatory effect of IL-10. Hirata N et al.⁴⁰ have demonstrated that blocking the JAK pathway by JAK inhibitor I significantly inhibited the production of IL-10 by DCs. STAT3 was observed for its potential role as cell-intrinsic negative regulator of DC activity.⁴¹ Hyperactivation of STAT3 was found to be associated with abnormal differentiation of DCs.⁴² These studies have demonstrated that JAK/STAT3 pathway negatively regulates the activation of DC. In the present study, the expression of mDC surface markers, when the DCs were pretreated with IL-10 siRNA before transfection with let-7i-inhibitor, were increased (Figure 1F), whereas decreased for Tregs in MLR (Figure 2) as compared to let-7i-inhibitor-mDCs. Moreover, JAK1/STAT3/pSTAT3 proteins were significantly up-regulated in transfection with let-7i-inhibitor, whereas those transfected with let-7i-mimic and pretreatment of IL-10 SiRNA before the transfection with let-7i-inhibitor were down-regulated (Figure 4). Therefore, we hypothesized that MiR let-7i regulates DC' maturation and function targeting IL-10 via JAK1-STAT3 pathway.

IL-35 is a member of the IL-12 family which includes Ebi3 (IL-27 β) and IL-12 α /p35 (IL-12 β),⁴³ has been found to contribute to Treg function.⁴⁴ It is secreted by Treg, but not conventional T cells, and is required for maximal Treg function in vitro and in vivo.^{45,46} IL-35 induces Breg cells to suppress experimental autoimmune uveitis in mice,⁴⁷ and suppresses the antibody responses.⁴³ Moreover, the induction of IL-35 production has been shown to ameliorate the disease symptoms in the experimental colitis and collagen-induced arthritis.^{48,49} Furthermore, allergic airway inflammation was ameliorated by IL-35 through the suppression of circulating specific and total IgE.⁵⁰ In our study, secretion of IL-35 by DCs in vitro was decreased in let-7i-mimic group whereas increased in the let-7i-inhibitor group. However, pretreatment with IL-10 SiRNA before transfection of let-7i-inhibitor decreased the production of IL-35. These results have

suggested that let-7i indirectly regulates IL-35 secretion targeting IL-10.

In conclusion, we have demonstrated that let-7i regulates DCs' maturation targeting the IL-10 through JAK1-STAT3 pathway. Moreover, transfusion of LPS-mDCs transfected with let-7i-inhibitor induced prolonged survival of cardiac allografts, and generated Tregs in our model.

Disclosure statement

The first two authors contributed equally to the manuscript. None of the authors has a financial relationship with a commercial entity that has an interest in the subject of the presented manuscript or other conflicts of interest to disclose.

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Figure legends

Figure 1. MicroRNA (MiR) let-7i regulates the DCs maturation targeting the IL-10. The expression of IL-10 mRNA levels and productions of IL-10 in bone marrow-derived DCs transfected with different concentration of MiR let-7i-inhibitor detected by (A) qRT-PCR and (B) ELISA. $*p < 0.05$ for difference compared with LPS group. The expression of IL-10 mRNA levels and productions of IL-10 of DCs in response to LPS with or without transfection of MiR let-7i-inhibitor detected by (C) qRT-PCR and (D) ELISA at different time points. $*p < 0.05$ for the difference compared with 0h group. (E) Targeting of let-7i to 3'UTR region of IL-10 mRNA. $*p < 0.05$ for the difference compared with cells transfected with the reporter construct. (F) LPS-stimulated CD80, CD86 and MHC II expressions on DCs. $*p < 0.05$ for the difference compared with LPS group, and $\#p < 0.05$ for difference compared with MiR let-7i-inhibitor group [LPS⁽⁻⁾ group (imDC) was used as positive control, and only LPS⁽⁺⁾ group (mDC) was used as negative control). Data are shown as means $\pm$ SD values from three independent experiments, and were compared using analysis of variance (ANOVA) with Ryan method.

Figure 2. Expression of CD4⁺, CD25⁺, and Foxp3⁺ in T cells co-cultured with DCs, which were transfected with MicroRNA (MiR) let-7i-mimic, inhibitor, or pretreated with IL-10 SiRNA before transfection of let-7i-inhibitor, as determined by flow cytometry. After 4 days of co-culture, the cells in MLR supernatant were stained with fluorochrome-conjugated CD4- fluorescein isothiocyanate (FITC), CD25-APC and Foxp3-PE mAb, as well as their isotype controls (all from eBioscience, USA). Data are shown as means $\pm$ SD values from three independent experiments, and were compared using analysis of variance (ANOVA) with Ryan method. $*p < 0.05$ for the difference compared with imDC group, and $\#p < 0.05$ for the difference compared with MiR let-7i-inhibitor group (IL-10 siRNA⁽⁻⁾MiR-let 7i mimic⁽⁻⁾MiR-let 7i inhibitor⁽⁻⁾LPS⁽⁻⁾ group was used as positive control and IL-10 siRNA⁽⁻⁾MiR-let 7i mimic⁽⁻⁾MiR-let 7i inhibitor⁽⁻⁾LPS⁽⁺⁾ group was used as negative control).

Figure 3. Effect of MicroRNA (MiR) let-7i on the cytokine profile secreted by imDCs, LPS-stimulated DCs, MiR let-7i-mimic-transfected DCs, MiR let-7i-inhibitor-transfected DCs, and IL-10 SiRNA-treated MiR let-7i-inhibitor-transfected DCs. Data are shown as means $\pm$ SD values from three independent experiments, and were compared using analysis of variance (ANOVA) with Ryan method. $*p < 0.05$ for the difference compared with imDC group, and $\#p < 0.05$ for the difference compared with MiR let-7i-inhibitor group (IL-10 siRNA⁽⁻⁾MiR-let 7i mimic⁽⁻⁾MiR-let 7i inhibitor⁽⁻⁾LPS⁽⁻⁾ group was used as positive control and IL-10 siRNA⁽⁻⁾MiR-let 7i mimic⁽⁻⁾MiR-let 7i inhibitor⁽⁻⁾LPS⁽⁺⁾ group was used as negative control).

Figure 4. Effect of MicroRNA (MiR) let-7i on the expression of JAK1, STAT3, and STAT3 phosphorylation (pSTAT3) in mDCs. Data are shown as means $\pm$ SD values from three independent experiments, and were compared using analysis of variance (ANOVA) with Ryan method. $*p < 0.05$ for the difference compared with imDC group, and $\#p < 0.05$ for the difference compared with MiR let-7i-inhibitor group (IL-10 siRNA⁽⁻⁾MiR-let 7i mimic⁽⁻⁾MiR-let 7i inhibitor⁽⁻⁾LPS⁽⁻⁾ group was used as positive control and IL-10 siRNA⁽⁻⁾MiR-let 7i mimic⁽⁻⁾MiR-let 7i inhibitor⁽⁻⁾LPS⁽⁺⁾ group was used as negative control).

Figure 5. Graft survival of Lewis recipients and histologic assessment. (A) Graft survival times of Lewis recipients transfused with PBS, Lewis bone marrow derived LPS-mDCs or MiR let-7i-inhibitor-mDCs for one time, or oral administration of 1 mg/kg/day of Tacrolimus for 7 days. MST, median survival time; $\#p < 0.05$ and $***p < 0.0001$ for difference between two groups. Cardiac allograft survival in groups of rats was compared using Mann-Whitney U testing (Graphpad Prism; Graphpad, CA, USA). (B, C, D) Histologic studies of harvested cardiac allografts, which were stained with hematoxylin-eosin (HE), 14 days after transplantation (magnification $\times 20$). (E) Assessment of HE staining by grading according to the classification of International Society for Heart and Lung Transplantation 2005 (ISHLT 2005) for acute cellular rejection. Data are shown as means $\pm$ SD values, and were compared using analysis of variance (ANOVA) with Ryan method. $***p < 0.0001$ for difference between two groups.

Figure 6. Evidence of generation of regulatory cells in Lewis allograft recipients treated with MiR let-7i-inhibitor-mDCs. (A) Expression of CD4⁺, CD25⁺, and Foxp3⁺ in Lewis splenocytes, as determined by flow cytometry. Data are means $\pm$ SD (n=5 rats in each group) for the percentage of CD4⁺CD25⁺Foxp3⁺ in CD4⁺ cells. (B) Results of cell-proliferation assays in mixed lymphocyte reaction (MLRs). The stimulator cells were DA (allogeneic) splenocytes treated with mitomycin C. Data are means $\pm$ SD values derived from samples from 6 rats in each group. (C) Cell count of infiltrating Foxp3⁺ cells in cardiac allografts from each group 14 days after grafting. Data are means $\pm$ SD values derived from samples from 5 rats in each group. Data were compared using analysis of variance (ANOVA) with Ryan method * $p < 0.01$, ** $p < 0.001$ and *** $p < 0.0001$ for difference between two groups. (D, E, F) Infiltration of Foxp3⁺ cells in cardiac grafts 14 days after grafting (magnification $\times 20$) (The PBS group was used as negative control).

Figure 7. Levels of cytokines in Lewis recipient's plasma 14 days after transplantation. Data are shown as means $\pm$ SD values from three independent experiments, and were compared using analysis of variance (ANOVA) with Ryan method. ** $p < 0.001$ and *** $p < 0.0001$ for difference between two groups (The PBS group was used as negative control).

	n	MST(days)		
● PBS	7	8	***	#
■ LPS-mDC	7	9		
▼ Tacrolimus	6	14		
▲ let-7i-Inhibitor-mDC	9	40