Int J Clin Exp Med 2017;10(2):2419-2429
www.ijcem.com /ISSN:1940-5901/IJCEM0035689

Original Article

MiR-15a promotes phenotypic and functional maturation of LPS-induced dendritic cells through down-regulating DDX39 expression

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Received July 13, 2016; Accepted September 2, 2016; Epub February 15, 2017; Published February 28, 2017

Abstract: Chronic rejection after heart transplantation was mainly caused by adaptive T cell response activated by mature dendritic cells (DCs), is the main reason influencing the long-term survival rate which is still not satisfactory. While there is little known about the mechanism of dendritic cells maturation. In this study, we evaluated the miR-15a expression in lipopolysaccharides (LPS) induced DCs maturation, and the results showed that miR-15a was highly expressed in LPS-induced DCs. MiR-15a inhibitor inhibited the DCs activation via suppressing the surface markers expression of activated DCs (CD80, CD86, MHC-II) and cytokines levels secreted by activated DCs (IL-12, IL-6, IL-1β and TNF-α). Besides, MiR-15a inhibitor increased the endocytic activity and weakened allostimulatory activity of LPS-induced DCs. DDX39, predicted as a target gene of miR-15a, had the contrary effect on LPS-induced DCs, and overexpression of DDX39 inhibited the phenotype and functional maturation of LPS-induced DCs. Furthermore, DDX39 is the downstream effector of miR-15a in regulating DCs maturation. These findings help understanding the mechanism of DCs maturation.

Keywords: Dendritic cells, maturation, LPS, miR-15a, DDX39

Introduction

Chronic rejection after heart transplantation is the major factor causing graft inactivation and influencing the patients' long-term survival [1, 2]. The main pathological characteristics of chronic rejection after heart transplantation are cardiac allograft vasculopathy (CAV), a significant rise of infiltrates cells in myocardium mesenchyme, myocyte hypertrophy and interstitial fibrosis [3, 4]. It plays an important role on the development of CAV that activated T cells and macrophages gather around the blood vessels in the transplanted heart tissue [5]. Besides, the number of T cells and antigen presenting cells (APC) specifically increased in the transplanted tissues can stimulate and intensify inflammation development and results in graft function damage [6].

The activation of T cells not only needs the interaction between antigen specific T cell receptor (TCR) and the antigen peptide-MHC complex on the APCs, but also needs the co-stimulating signals [7]. Dendritic cells (DCs) as an important APC were the sole one activating the initial T cells, play a prominent role in recognizing and presenting antigen and starting the immune response [8, 9]. Whether in allograft immune or in mixed leucocyte reaction, DCs are effective agonists that initial T cell immune response, and only a small amount of DCs can stimulate strong T cell responses *in vivo* or *in vitro* [10].

DC precursors derived from hematopoietic stem cells in bone marrow differentiate into immature DCs under the stimulations of growth factors and differentiation factors. Immature DCs in peripheral tissues capture and process antigen to constitute the major histocompatibility complex (MHC), and then mature DCs will be produced with a range of morphological and functional changes [11]. Mature DCs will lose the activity of endocytosis but enhance the motility inducing migrating to local lymph nodes. And mature DCs are highly expressed MHC-II and co-stimulators (CD80 and CD86)

and cytokines including IL-12, IL-6, IL-1β and TNF-α [12]. The mature process of DCs also can be induced by a variety of external stimulation factors, such as lipopolysaccharide (LPS), dsRNA, CpG DNA, TNF-α and PGE2 [13, 14].

DC activation is regulated by a variety of intracellular molecules, the discovery of the molecular and the research of related mechanism attracted extensive attention currently. Discovery of new DC activation molecular and study of corresponding regulatory mechanism are the key problems in chronic rejection mechanism research and intervention target selection. MicroRNA possesses a wide range of expression profile and regulatory function, so we analyzed the different expression of microRNAs in immature and mature DCs and the effect of target gene on maturation of DCs. We found that miR-15a may regulate the phenotypic and functional maturation of DCs through controlling the DDX39 expression.

Materials and methods

Dendritic cells generation and culture

Mice bone marrow dendritic cells (BMDCs) were collected from the tibiae and femurs of 4-6 weeks old C57BL/6 (Shanghai SLAC Laboratory Animal Co. Ltd, Shanghai, China) as previously described [15] with a little improvement. First, bone marrow was flushed to a sterile petri dish with RPMI-1640 (HyClone, Rogan, USA) and the erythrocyte was lysed with ammonium chloride. Cell suspension was prepared with complete medium supplemented with 10% FBS (HyClone, Rogan, USA), 10 ng/mL rmGM-CSF (R&D systems, Minneapolis, USA) and 10 ng/mL rmIL-4 (R&D systems) and was cultured in 6-well plates for 24 h. Then, adherent cells were kept and cultured in the new same complete medium for 6 days with replacement of medium every other day. Last, Non-adherent and loose adherent cells were enrichment of BMDCs and gathered. BMDCs maturation were induced by LPS (1 μg/mL) (Sigma-Aldrich, St. Louis, MO, USA) for 24 h.

Cell surface markers analysis

Immature DCs with different treatments were harvested and incubated respectively with appropriate antibody against murine CD11c, CD80, CD86 and MHC-II for 30 min on ice followed by washing 3 times. FITC-conjugated anti-mouse CD11c, CD80, MHC-II, and PE-conjugated anti-mouse CD86 antibodies were bought from eBioscience (San Diego, USA) and used in accordance with the instructions. The expressions of these proteins were analyzed by flow cytometry.

Inflammatory cytokines detection

Cell culture supernatants of immature DCs with different treatments were collected to test the cytokine concentrations (IL-1β, IL-6, IL-12 and TNF-α) by ELISA kits (Dakawe Biotech Company, Shenzhen, China) in accordance with the instructions.

Fluorescein isothiocyanate (FITC)-dextran uptake

To detect the endocytic activity of DCs, DCs with different treatments were incubated with FITC-dextran (1 mg/mL, Sigma-Aldrich) for 1 h at 37°C. Then cells were harvested and washed twice with cold HBSS and followed by analyzing by a FACSCalibur flow cytometer.

Allogeneic mixed lymphocyte reaction assay

Splenocytes were isolated from BALB/c (4-6 weeks old, Shanghai SLAC Laboratory Animal Co. Ltd, Shanghai, China) and were used for the allogeneic T cell reaction. Immature DCs with different treatments were treated with mitomycin C (50 μg/mL, Sigma-Aldrich) for 1 h at 37°C and then added allogeneic T cells with a graded ratio (1:10, 1:25, 1:50, 1:100). After 3 days, cell proliferation was detected by CCK8 assay.

MicroRNA expression assay

MicroRNA expression of immature DCs and mature DCs induced by LPS were analyzed. Total RNA was isolated as standard method for microRNA array. MiR-15a expression was detected by qRT-PCR too.

Prediction and validation of miR-15a target gene

The target gene of miR-15a was predicted using Mirbase (http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/search.pl), and DDX39 was found as the target gene of miR-15a. Immature DCs and LPS-induced DCs were collected to detect the expression of DDX39 using western blot. Furthermore, luciferase reporter gene assay and western blot

were used to validate the miR-15a target gene. 293T cells were transfected with mimic NC, miR-15a mimic, inhibitor NC and miR-15a inhibitor for 6 h respectively, then cotransfected with the mixture of pGL3.5XDDX39wt-luciferase (pGL3.5XDDX39mut-luciferase) and pRL-TK-Renilla-luciferase for 24 h. DDX39wt luciferase and DDX39mut luciferase activities were measured using microplate reader (Infinite M1000, TECAN, Switzerland) according to the instruction. Immature DCs were cultured with mimic NC, miR-15a mimic, inhibitor NC and miR-15a inhibitor for 48 h respectively, and then total proteins were isolated to analyze the expression of DDX39 using western blot. Mimic NC, miR-15a mimic, inhibitor NC and miR-15a inhibitor were obtained from GenePharma (Shanghai, China). Antibody against DDX39 and β-actin were purchased from R&D systems and Dual-Luciferase Reporter Assay System was obtained from Promega (Madison, WI, USA).

The effect of miR-15a on LPS-induced DCs maturation

To estimate the relationship between miR-15a and DCs maturation, LPS-induced DCs were cultured with inhibitor NC (100 nM) and miR-15a inhibitor (100 nM) for 48 h respectively, and immature DCs were cultured as control. Then the surface markers (CD80, CD86, and MHC-II), cytokines (IL-1β, IL-6, IL-12 and TNF-α), endocytic activity and allostimulatory activity were detected as described respectively.

The effect of DDX39 on LPS-induced DCs maturation

To study the relationship between DDX39 and DCs maturation, LPS-induced DCs were transfected with pcDNA-3.1, pcDNA-DDX39, si-scramble and si-DDX39 for 48 h respectively, and immature DCs were cultured as control. The surface markers (CD80, CD86, and MHC-II), cytokines (IL-1β, IL-6, IL-12 and TNF-α), endocytic activity and allostimulatory activity were detectedas described respectively. The sequences of si-Scramble and si-DDX39 were 5'-GAAAGACATCAAGGGATCCTACGTT-3' and 5'-GAACATAACGAGGTACCATCGAGTT-3'.

Combined effect of miR-15a inhibitor and si-DDX39 on LPS-induced DCs maturation

MiR-15a inhibitor and si-DDX39 were cotransfected into LPS-induced DCs to investigate the relationship between miR-15a and DDX39 in DCs maturation, at the same time, inhibitor NC and miR-15a inhibitor were transfected into LPS-induced DCs respectively too. Immature DCs were cultured as control. The surface markers (CD80, CD86, and MHC-II), cytokines (IL-6, IL-12, IL-1β, TNF-α), endocytic activity and allostimulatory activity were detected as described respectively.

Statistical analysis

All data were obtained from three independent experiments and were analyzed using Graph Pad Prism software, the results were represented in the form of means ± SD. Statistical significance between different treatments was determined with student's t-test, when $P < 0.05$ or $P < 0.01$, the difference is statistically significant.

Results

LPS induces the DCs maturation

BMDCs were cultured and treated with 1 μg/mL LPS for 24 h, then cell surface markers of mature DCs (CD11c, CD80, CD86 and MHC-II) and cytokines secreted by mature DCs (IL-1β, IL-6, IL-12 and TNF-α) were detected using flow cytometry and ELISA respectively. Immature DCs and LPS-induced DCs were showed the typical characteristics of DCs that expression of CD11c (purified > 90%) (**Figure 1A**). CD80, CD86, MHC-II and cytokines tested were all displayed an increased expression in LPS-induced DCs ($P < 0.01$) (**Figure 1B-H**).

MiR-15a is highly expressed in LPS-induced DCs

Total RNA were isolated from immature DCs and LPS-induced DCs to analyze the micro-RNA expression using micro-RNA array. MiR-15a was elevated significantly in three LPS-induced DCs group than that in immature DCs (**Figure 1I**) as well as the qRT-PCR result ($P < 0.01$) (**Figure 1J**).

MiR-15a inhibitor suppress the DCs maturation induced by LPS

In DCs treated with miR-15a inhibitor in the presence of LPS, surface markers expression (CD80, CD86 and MHC-II) in mature DCs cell were all declined considerably and lower than

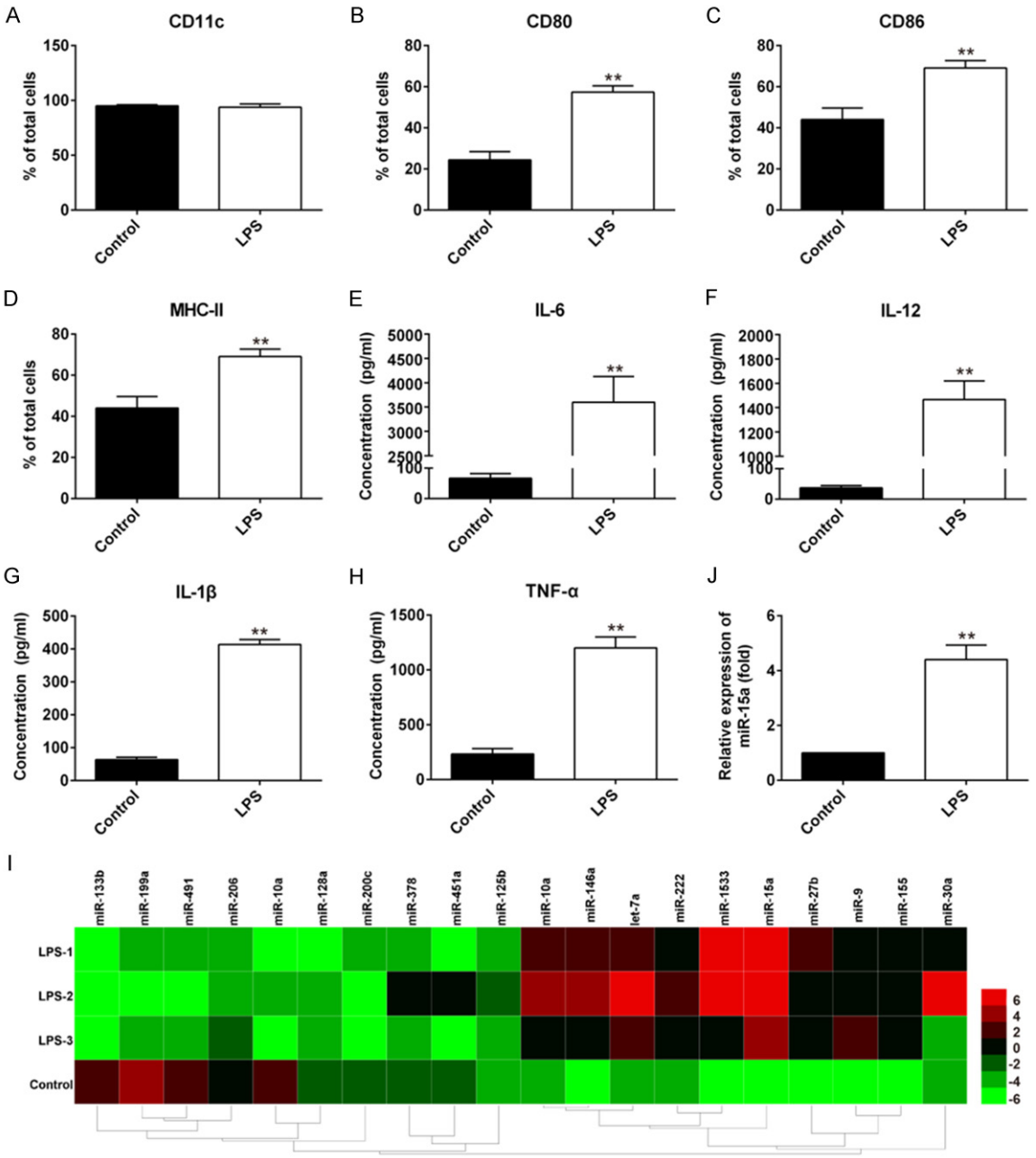

Figure 1. Expression of maturation markers in BMDCs induced by LPS and screened microRNA with differential expression in mature DCs. A-D: Detection of surface markers (CD11c, CD80, CD86 and MHC-II) of mature DCs using flow cytometry, $N = 3$, $^{**}P < 0.01$ versus control; E-H: Detection of cytokines (IL-6, IL-12, IL-1β, TNF-α) in cell culture supernatants using ELISA, $N = 3$, $^{**}P < 0.01$ versus control; I: Part of heat map of microRNA array in LPS-induced DCs and control DCs; J: Confirmation of miR-15a expression using qRT-PCR in LPS-induced DCs and control DCs. $N = 3$, $^{**}P < 0.01$ versus control.

in DCs treated with inhibitor NC in presence of LPS ($P < 0.01$); These markers in DCs treated with inhibitor NC in presence of LPS were obviously higher than those in control group ($P < 0.01$) (**Figure 2A-C**). Cytokines in the cell culture supernatants were also detected by ELISA, and cytokines in DCs treated with inhibitor NC and miR-15a inhibitor in presence of LPS were all much higher than those in control DCs, but cytokines in DCs treated with miR-15a inhibitor were significantly lower than those in DCs treated with inhibitor NC ($P < 0.01$) (**Figure 2D-G**).

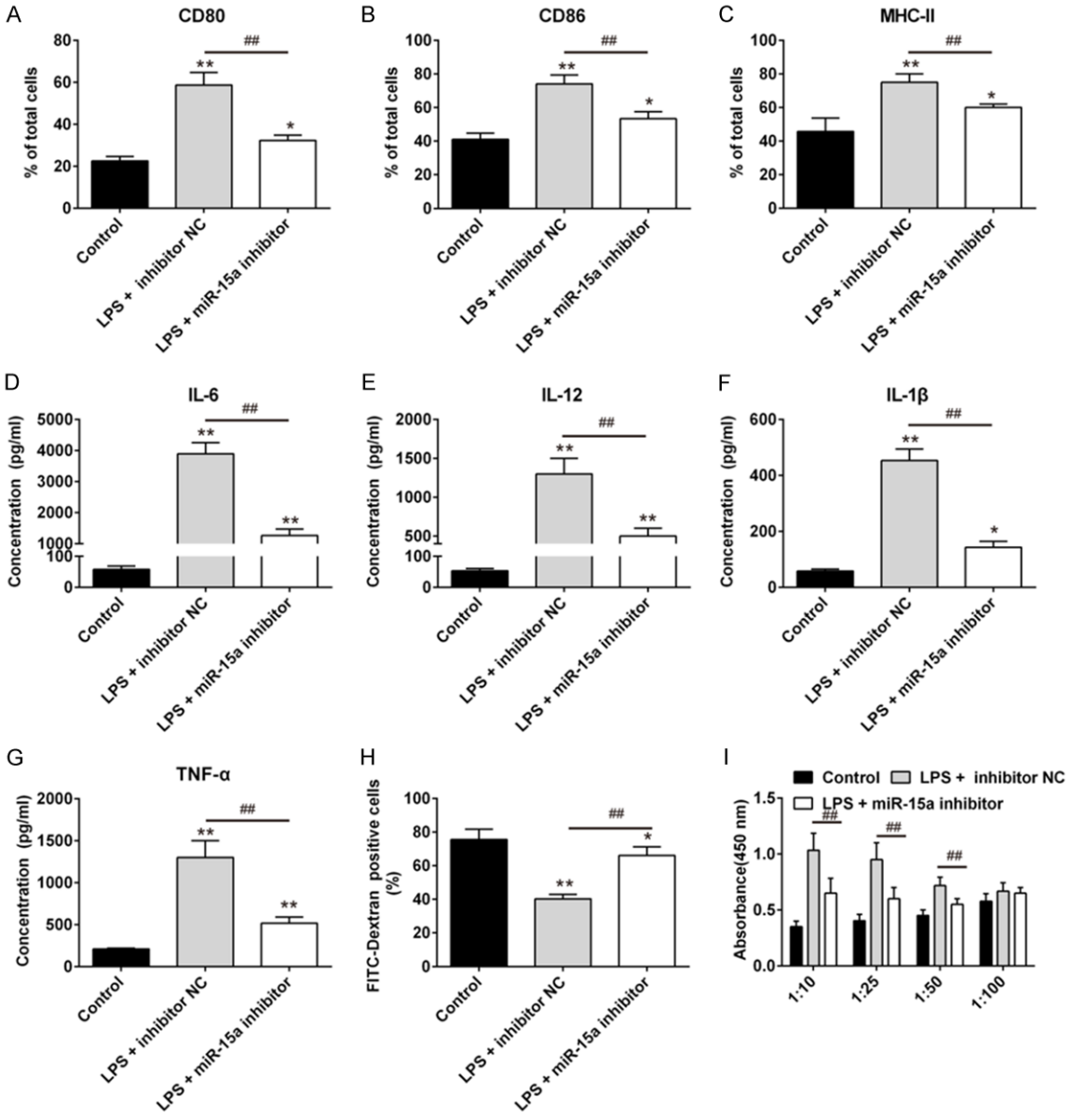

Figure 2. Downregulation of miR-15a suppressed LPS-induced DCs maturation. To evaluate the role of miR-15a in LPS-induced DCs maturation, miR-15a inhibitor and NC inhibitor were respectively transfected into LPS-induced DCs, and immature DCs were cultured as control. A-C: Detection of surface markers of mature DCs using flow cytometry, $N = 3$, $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control, $^{\#\#}P < 0.01$; D-G: Detection of cytokines secreted by mature DCs in cell culture supernatants using ELISA, $N = 3$, $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control, $^{\#\#}P < 0.01$; H: The endocytic activity of the DCs was detected using flow cytometer and the comparisons were did between the proportion of FITC-dextran positive cells, $N = 3$, $^{*}P < 0.05$ and $^{**}P<0.01$ versus control, $^{\#\#}P < 0.01$; I: Allogeneic mixed lymphocyte reaction were did using allogeneic spleen T cells with the different ratios of DC/T cell, $N = 3$, $^{\#\#}P < 0.01$.

Furthermore, DCs treated with inhibitor NC significantly decreased the endocytic activity of capturing FITC-dextran ($P < 0.01$), which would be rescued by treating with miR-15a inhibitor ($P < 0.01$) even if still lower than control DCs ($P < 0.05$) (**Figure 2H**); the results of CCK8 assay indicated that treatment of LPS and inhibitor NC could increase the allostimulatory capacity and it was the strongest when the ratio of DCs and T cells was 1:10, and allostimulatory capacity would significantly drop when DCs treated with LPS and miR-15a inhibitor ($P <$

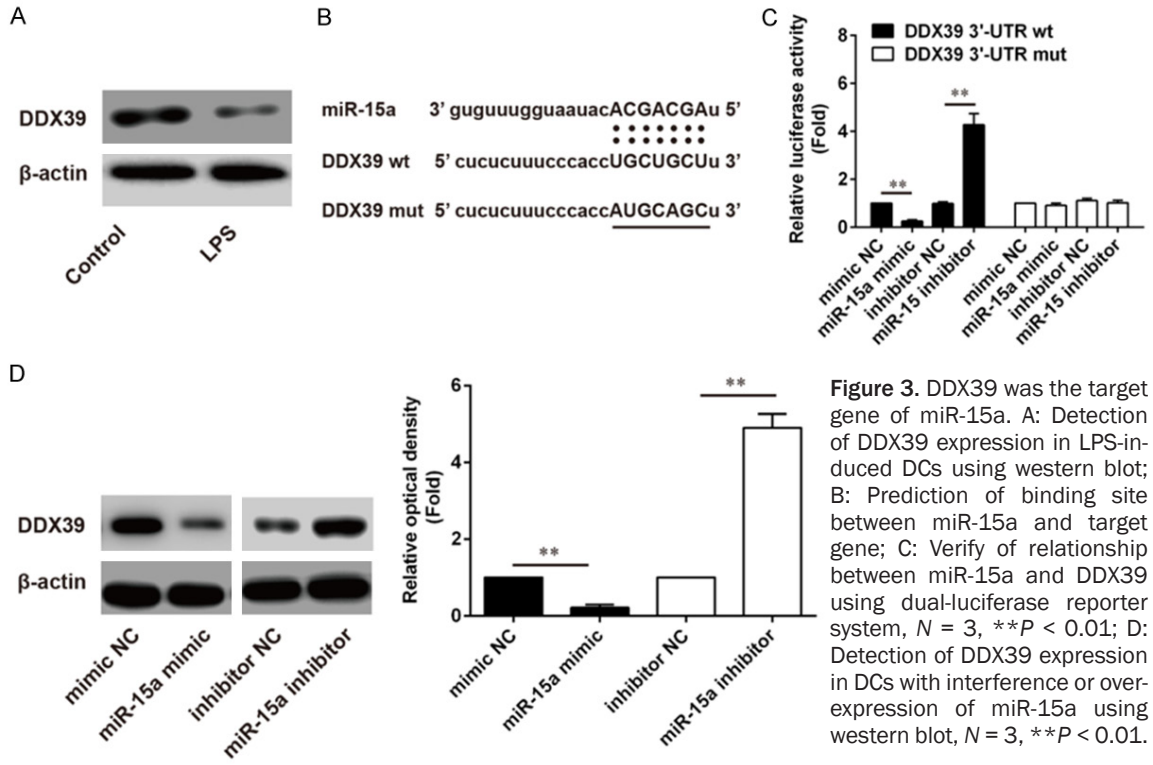

Figure 3. DDX39 was the target gene of miR-15a. A: Detection of DDX39 expression in LPS-induced DCs using western blot; B: Prediction of binding site between miR-15a and target gene; C: Verify of relationship between miR-15a and DDX39 using dual-luciferase reporter system, $N = 3$, $^{**}P < 0.01$; D: Detection of DDX39 expression in DCs with interference or overexpression of miR-15a using western blot, $N = 3$, $^{**}P < 0.01$.

0.01) (**Figure 2I**). These indicate that miR-15a inhibitor can suppress phenotype and functional maturation of LPS-induced DCs.

MiR-15a inhibits the DDX39 expression in DCs

According to the result of target gene prediction, DDX39 might be the target gene of miR-15a, so the binding site in DDX39 mRNA sequence was mutated as a mutant (**Figure 3B**). The relationship between miR-15a and DDX39 3'-UTRwt/DDX39 3'-UTRmut were verified by luciferase reporter gene assay, and luciferase activity was increased dramatically in DDX393'-UTRwt group when treated with miR-15a inhibitor, that was lowest when treated with miR-15a mimic; but the luciferase activities in DDX39 3'-UTRmut with different treatments were low and no significant differences between them (**Figure 3C**). Western blot results showed that DDX39 expression in LPS-induced DCs was lower than that in immature DCs (**Figure 3A**), and DDX39 expression was changing with different treatments, DCs showed a highest level of DDX39 when treated with miR-15a inhibitor and a lowest level of DDX39 when treated with miR-15a mimic (**Figure 3D**).

Overexpression of DDX39 inhibits the DCs maturation induced by LPS

Cell surface markers and cytokines were increased when DCs were treated with pcDNA-3.1 in presence of LPS, and these increases would be dipped in LPS-induced DCs transfected with pcDNA-DDX39 ($P < 0.01$); on the contrary, cell surface markers expressions and cytokines levels in LPS-induced DCs treated with si-DDX39 were higher than LPS-induced DCs transfected with pcDNA-DDX39 and si-Scramble ($P < 0.05$) (**Figure 4A-G**). Endocytic activity of LPS-induced DCs transfected with pcDNA-DDX39 was higher than that of LPS-induced DCs transfected with pcDNA-3.1; and endocytic activity of LPS-induced DCs transfected with si-Scramble was higher than that of LPS-induced DCs treated with si-DDX39 (**Figure 4H**). The changes of allostimulatory capacity of LPS-induced DCs with these different treatments were contrary to the changes of endocytic activity (**Figure 4I**). These indicate that overexpression of DDX39 can suppress phenotype and functional maturation of LPS-induced DCs.

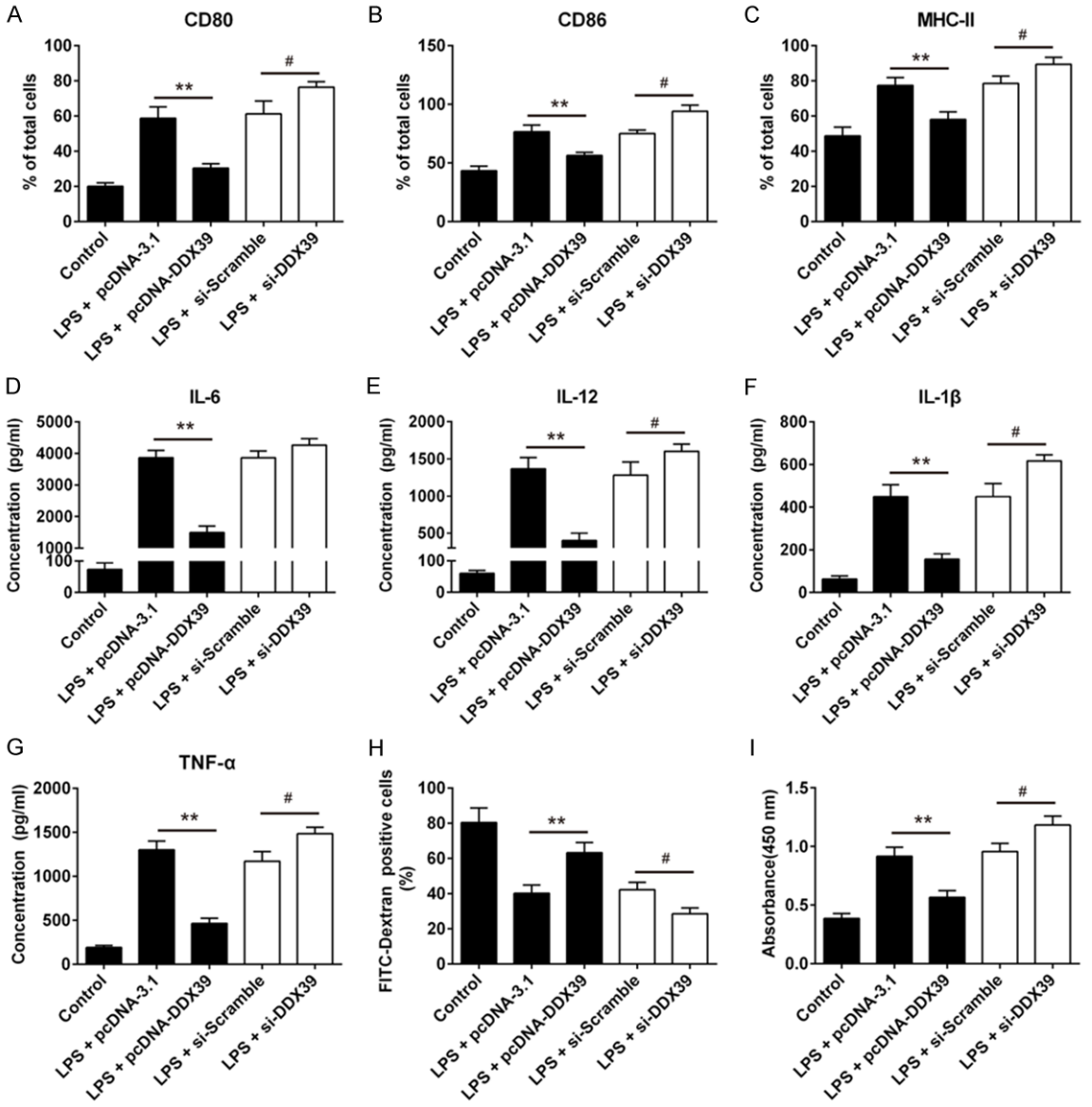

Figure 4. Interference of DDX39 promoted DCs maturation but overexpression of DDX39 inhibited DCs maturation. To evaluate the role of DDX39 in LPS-induced DCs maturation, recombinant plasmid pcDNA-DDX39 and si-DDX39 were respectively transfected into DCs in the presence of LPS, besides, pcDNA-3.1 and si-Scramble were also respectively transfected into DCs in the presence of LPS as negative control, and immature DCs were cultured as blank control. A-C: Detection of surface markers of mature DCs using flow cytometry, $N = 3$, $^{**}P < 0.01$, $^{\#}P < 0.05$; D-G: Detection of cytokines in cell culture supernatants using ELISA, $N = 3$, $^{**}P < 0.01$, $^{\#}P < 0.05$; H: The endocytic activity of the DCs was detected using flow cytometer and the comparisons were did between the proportion of FITC-dextran positive cells, $N = 3$, $^{**}P < 0.01$, $^{\#}P < 0.05$; I: Allogeneic mixed lymphocyte reaction was did at the ratio of DC/T cell 1:10, $N = 3$, $^{**}P < 0.01$, $^{\#}P < 0.05$.

Interference of DDX39 abolishes the inhibition of miR-15a on LPS-induced DCs maturation

Cell surface markers expressions and cytokines levels of LPS-induced DCs were declined considerably when transfected with miR-15a inhibitor than transfected with inhibitor NC ($P < 0.01$), LPS-induced DCs transfected with miR-15a inhibitor and si-DDX39 simultaneously would increase cell surface markers expressions and cytokines levels (**Figure 5A-G**). In the same time, compared with LPS-induced DCs transfected with miR-15a inhibitor, LPS-induced DCs transfected with miR-15a inhibitor and si-DDX39 simultaneously would decrease the endocytic activity and increase

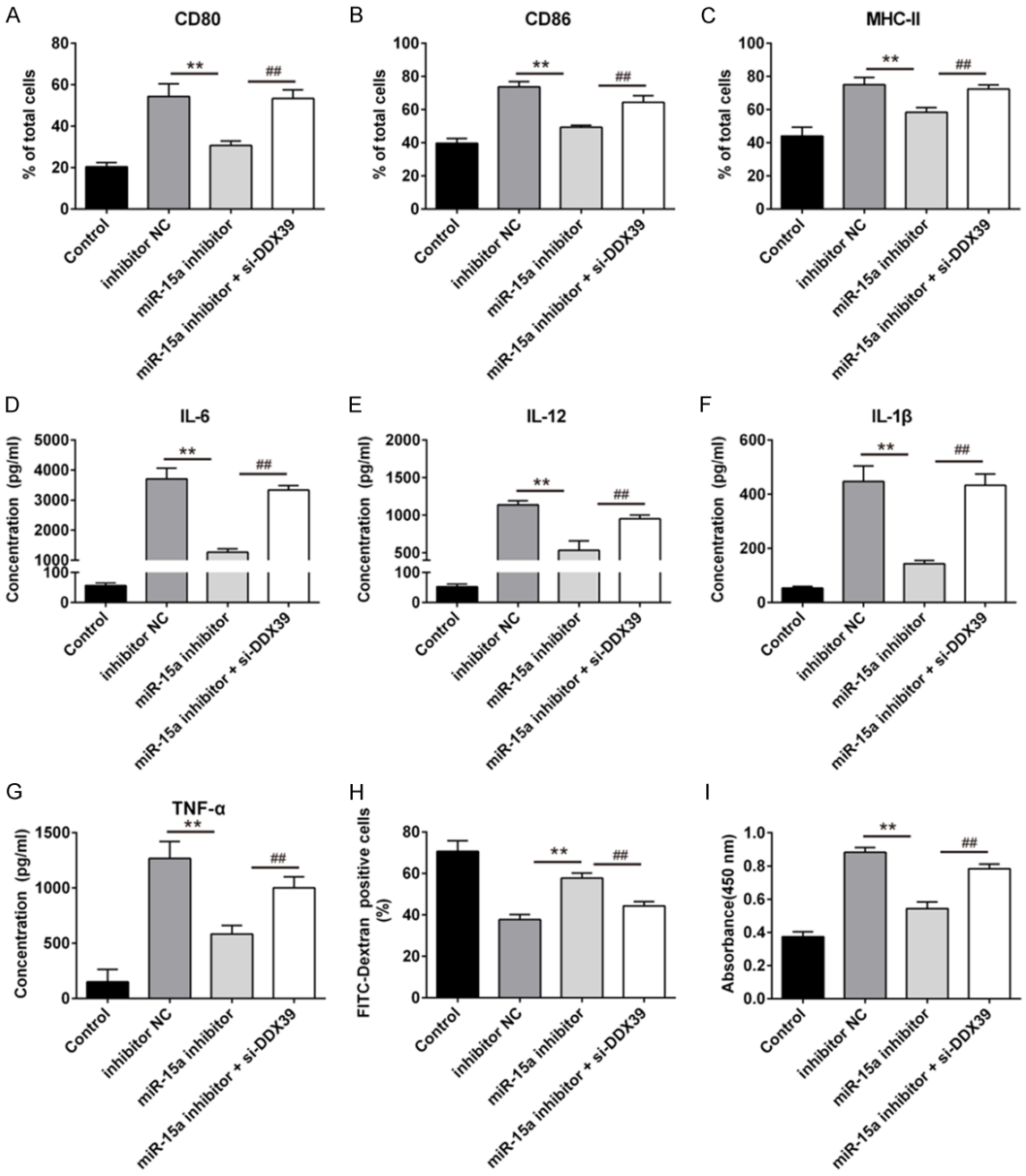

Figure 5. Interference of DDX39 removed the inhibition of miR-15a on DCs maturation. To evaluate the relationship between DDX39 and miR-15a in LPS-induced DCs maturation, miR-15a inhibitor and si-DDX39 were cotransfected into DCs in the presence of LPS, miR-15a inhibitor and inhibitor NC was respectively transfected into LPS-induced DCs as control, immature DCs were cultured as blank control. A-C: Detection of surface markers of mature DCs using flow cytometry, $N = 3$, $^{**}P < 0.01$, $^{\#\#}P < 0.01$; D-G: Detection of cytokines in cell culture supernatants using ELISA, $N = 3$, $^{**}P < 0.01$, $^{\#\#}P < 0.01$; H: The endocytic activity of the DCs was detected using flow cytometer and the comparisons were did between the proportion of FITC-dextran positive cells, $N = 3$, $^{**}P < 0.01$, $^{\#\#}P < 0.01$; I: Allogeneic mixed lymphocyte reaction was did at ratio of the DC/T cell 1:10, $N = 3$, $^{**}P < 0.01$, $^{\#\#}P < 0.01$.

the allostimulatory capacity ($P < 0.01$) (**Figure 5H**, **5I**). These indicate that DDX39 is the target gene of miR-15a involved in DCs maturation.

Discussion

Heart transplantation is the only effective means for treatment of end-stage heart dis-

ease [16]. Although the prognosis of heart transplantation is improving, but the long-term survival rate is still not satisfactory; and the incidences of chronic homograft rejection in the first year, the fifth year and the eighth year after the surgery for patients with heart transplantation were 8%, 32% and 43% respectively [17], which was the most important reason of death [18, 19]. A typical manifestation of chronic rejection is CAV, and its severity is far higher than that of other organs for transplant vasculopathy, but there is no effective treatment [20, 21]. At present, immunosuppressant therapy is the main treatment of CAV, but this method doesn't controls rejection occurring from the source and has wide side effects. So, exploring the mechanism of chronic rejection and its intervention strategy has an important theoretical and practical significance. DCs are in the central part of the adaptive immunity and play a key role in chronic rejection [12, 22]. In the indirect identification process of allogeneic antigens, DCs activate the T cell response by uptaking, processing and presenting the allogeneic antigens to T cells [23-25]. Activation and mature state of DCs decide the type and degree of subsequent immune response, and immature DCs will mediate the immune tolerance if DCs secrete regulatory cytokines (like IL-10) too much and induce the activation of regulatory T cells; but mature DCs can secrete a lot of proinflammatory cytokines and stimulate effect T cell response [26, 27]. In this study, we indicated the effects of overexpressed miR-15a and lower expressed DDX39 on the phenotypic and functional maturation of DCs induced by LPS through analyzing the expression of cell surface markers (CD80, CD86 and MHC-II) and cytokines (IL-1β, IL-6, IL-12 and TNF-α), and endocytosis, stimulatory capacity of DCs for T cell proliferation.

LPS can stimulate the maturation of DCs and trigger the production of proinflamatory cytokines (IL-1β, IL-6, IL-12, and TNF-α) in vitro [28], furthermore, mature DCs highly express MHC-II and co-stimulators (CD80 and CD86) [29], which is essential for an antigen-specific T cell response [30]. In addition, CD11c is a marker of DCs and involves in phagocytosis, cell migration, cytokine production and inflammation [31]. So we detected these cytokines and surface markers to evaluate LPS induced DCs maturation. The results showed that expression rate of CD11c in all cells was above 90% and indicated DCs culturing was successful. High expression of cytokines and surface markers detected in LPS-induced DCs indicated that DCs were fully mature with treatment of LPS.

MicroRNA possesses a wide range of expression profile and regulatory function, there have reports about expression and function of microRNA almost in every system [32, 33], including immune system [34, 35], but its functions in immune system are still poorly understood. And more studies about microRNA functions in DCs maturation and activation should be done to further understand the mechanism of DCs maturation and activation. The results of microRNA array showed difference about microRNAs expression in immature DCs and LPS-induced DCs and high level of miR-15a in LPS-induced DCs. Furthermore, miR-15a inhibitor depressed the maturation of LPS-induced DCs which concluded from downregulation of the expressions of cell surface markers and cytokines, increased endocytic activity, and decreased stimulatory capacity for T cell proliferation. These turned out that miR-15a participates in the process of LPS-induced DCs maturation.

DExD/H-box helicases participate in the metabolism process of RNA from transcription to degradation [36], DDX58 (RIG-I) and DDX60 are sensors of viral RNA molecules and several viruses and elicit antiviral interferon responses [37]. In this study, DDX39 was predicted as the target gene of miR-15a and was low expressed in LPS-induced DCs. Overexpression or lower expression of DDX39 all influenced the expressions of cell surface markers and cytokines, endocytic activity and stimulatory capacity on T cell activation of LPS-induced DCs, suggested that expression of DDX39 can make a significant impact on LPS-induced DCs maturation. To investigated the relationship between miR-15a and DDX39 in LPS-induced DCs maturation, we cotransfected DCs with miR-15a inhibitor and si-DDX39 in the presence of LPS, and the results showed that the inhibition of surface markers and cytokines expression by miR-15a inhibitor was suppressed by si-DDX39. Si-DDX39 depressed the endocytic activity which had been risen by miR-15a inhibitor and elevated the stimulatory capacity for T cell proliferation declined by miR-15a inhibitor. These

results indicated that overexpression of miR-15a promote the LPS-induced DCs maturation may directly via blocking DDX39 expression.

In summary, miR-15a is highly expressed in LPS-induced DCs, and miR-15a inhibitor can suppress the phenotype and functional maturation of LPS-induced DCs. Furthermore, DDX39 is the downstream target gene of miR-15a and the role of DDX39 in the phenotype and functional maturation of LPS-induced DCs is opposite to that of miR-15a. These results help understanding the mechanism of DCs maturation, which is important for T cell response in innate and adaptive immunity, including chronic rejection after heart transplantation. So miR-15a and DDX39 may be potential intervention targets for chronic rejection after heart transplantation.

Disclosure of conflict of interest

None.

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