ANNALS OF TRANSPLANTATION

ORIGINAL PAPER

e-ISSN 2329-0358
© Ann Transplant, 2020; 25: e921287
DOI: 10.12659/AOT.921287

Received: 2019.11.09
Accepted: 2020.02.18
Available online: 2020.04.24
Published: 2020.05.05

Toll-Like Receptor 3 Activator Preconditioning Enhances Modulatory Function of Adipose-Derived Mesenchymal Stem Cells in a Fully MHC-Mismatched Murine Model of Heterotopic Heart Transplantation

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Source of support: This work was supported by the National Natural Science Foundation of China (Grants No. 81373173)

Background: Donor-specific tolerance is the ultimate goal in organ transplantation. Diverse approaches, including the use of mesenchymal stem cells (MSCs), have been investigated to induce graft tolerance. Non-stimulated MSCs showed limited regulatory functions through interaction with multiple immune-regulatory cells, such as regulatory T cells (Tregs). To augment their functions, MSCs have been preconditioned with toll-like receptor (TLR3/4) agonist in autoimmune disease models, but results were conflicting.

Material/Methods: We evaluated the immunomodulatory effects of mouse adipose-derived mesenchymal stem cells (ADSCs) preconditioned with various combinations of TLR3/4 agonist and antagonists, including polyinosinic-polycytidylic acid poly(I:C)-TLR3 agonist, lipopolysaccharide (LPS) -TLR4 agonist, and TAK242-TLR4 antagonist. *In vitro* and *in vivo* experiments including mixed lymphocyte reaction, cytokines measurement, Tregs analysis, and a fully mismatched MHC heterotopic heart transplantation in mice (BALB/c to C57BL/6) were conducted.

Results: ADSCs preconditioned with poly(I:C) showed the highest efficiency in inhibiting lymphocyte proliferation, which was correlated with the upregulation of fibrinogen-like protein 2 (FGL2), an effector molecule of Tregs. The mean survival of cardiac allografts was extended from 8 to 12 days by intravenous injection of a single dose of ADSCs preconditioned with TLR3 agonist. The proportion of Tregs in the recipient's spleen was significantly increased by injecting the poly(I:C)-stimulated ADSCs.

Conclusions: These results show that short-term TLR3 agonist preconditioning enhances the immunomodulatory efficacy of ADSCs, which can induce the generation of Tregs and upregulate the expression of FGL2, thereby improving the outcome of patients receiving organ transplantation.

MeSH Keywords: Heart Transplantation • Mesenchymal Stromal Cells • Toll-Like Receptor 3 • Toll-Like Receptor 4

Full-text PDF: https://www.annalsoftransplantation.com/abstract/index/idArt/921287

Indexed in: [Science Citation Index Expanded] [Index Medicus/MEDLINE] [Chemical Abstracts] [Scopus]

Background

Graft rejection remains the main challenge in organ transplantation, and immune tolerance is considered as the ultimate goal [1]. Nonetheless, adoptive transfer of several regulatory cells were studied to induce tolerance [2,3], including regulatory T lymphocytes [4,5], regulatory B lymphocytes [6–8], tolerogenic dendritic cells [9], regulatory macrophages [10], myeloid-derived suppressor cells (MDSC) [11], and mesenchymal stem cells (MSCs) [12,13].

MSCs exert immunomodulation in 2 ways: directly by cell-to-cell contact, and indirectly through the secretion of cytokines, such as prostaglandin E_2 (PGE_2) [14], interleukin-10 (IL-10), nitric oxide (NO), indoleamine 2, 3-dioxygenase (IDO), and tumor necrosis factor α-stimulated gene 6 (*TSG-6*). MSCs can be divided into MSC1 and MSC2 according to the stimulation of toll-like receptor (TLR) on the surface of MSCs. The activation of TLR4 on the surface of MSCs modifies the MSCs into pro-inflammatory MSC1, while activation of TLR3 changes MSCs into anti-inflammatory MSC2 [15].

We aimed to investigate the effects of activation/inhibition of TLR3 and TLR4 on the immunomodulatory function of ADSCs in the context of mouse MHC fully mismatched heterotopic heart transplantation. We also sought to determine whether TLR3 stimulator plus TLR4 blocker can induce stronger effects on ADSCs than using TLR3 stimulator alone. In addition, we tried to identify the molecules exerting the major effect in these immunomodulatory procedures.

In this study, we isolated ADSCs from mice and confirmed their phenotypes and multilineage differentiating capabilities. Then, we assessed their immunomodulatory functions in both *ex vivo* and *in vivo* models. TLR3 stimulation alone induced the highest regulatory effects in these ADSCs, even better than the combination of TLR3 stimulator with TLR4 blocker. In addition, expression of *FGL2*, a reported effector molecule of Tregs, was significantly increased in ADSCs stimulated with TLR3 agonist. The results of our study clarified the roles of TLR3/4 stimulations in MSC-related immunomodulation, elucidating the effector molecule, and providing important insights for designing MSC-related therapeutics in the field of organ transplantation.

Material and Methods

Animals

Male C57BL/6 mice and BALB/c mice, 6–8 weeks old, were purchased from the Department of Laboratory Animal Science of China Medical University (Shenyang, China). The animals were maintained under standard conditions. All animal protocols were performed according to institutional guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC Issue No. 2018084) of China Medical University.

ADSC culture *in vitro*

First, ADSCs were isolated from C57BL/6 using the criteria of the International Society for Cellular Therapy (ISCT) for the isolation of MSCs from humans [16]. ADSCs were obtained from the abdominal subcutaneous lipid tissue isolated from 6–8-week-old C57BL/6 mice through mechanical and enzymatic digestion [17,18]. After tissue mincing, type I collagenase was added for 1 h at 37°C with agitation. The digested adipose tissue was centrifuged at 1000 rpm for 5 min. The resulting cell pellet was cultured in DMEM supplemented with 10% fetal bovine serum (FBS), antibiotic solution (100 U/mL penicillin, and 100 µg/mL streptomycin), and incubated at 37°C in a humidified environment containing 5% CO_2. Subsequently, the cells between passages 3 and 6 were used for further experiments.

ADSC characterization

The ADSCs were differentiated towards adipogenic, chondrogenic, and osteogenic lineages. For adipogenic differentiation, ADSCs were cultured at 8×10^3 cells/cm^2 in 6-well plates. The cells were cultured to 80% confluency in adipogenesis differentiation medium (10% FBS, 1 µm/L dexamethasone, 200 µmol/Indomethacin, 0.5 µmol/L isobutyl methylxanthine, 10 µmol/L insulin, low-glucose DMEM, Solarbio). After 14 days, cells differentiating into adipocytes were confirmed with a working solution of oil red O stain (Sigma-Aldrich).

To induce chondrogenic differentiation, ADSCs were incubated at 8×10^3 cells/cm^2 in a 35-mm culture dish. The cells were grown to 80% confluency in chondrogenic differentiation medium (7 µm/L dexamethasone, 500 µg/LTGF-β, 50 µmol/L ascorbate, 10 µmol/L insulin, and high-glucose DMEM, Solarbio) for 21 days, and the chondrogenic differentiation was assessed using toluidine blue stain.

For osteogenic differentiation, ADSCs were incubated at 8×10^3 cells/cm^2 (2 mL/well) in a 6-well plate. The cells were cultured to 80% confluency in osteogenesis differentiation medium (10% FBS, 0.1 µm/L dexamethasone, 50 µmol/L ascorbate, 10 mmol/L glycerophosphate disodium salt, low-glucose DMEM, Solarbio). After 3 weeks, calcium deposition was detected by alizarin red stain.

ADSC phenotypes were analyzed by flow cytometry according to the Cell Surface Immunofluorescence Staining Protocol (BioLegend). The expression level of 4 surface markers, including CD29 (beta-1 integrin), stem cells antigen-1 (Sca-1), CD34, and CD45 were measured. CD29 is a mesenchymal cell marker

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that is expressed in both BMSCs and ADSCs. Sca-1 is commonly used for the identification of stem cells in mouse models. CD34 and CD45 are the surface markers of hematopoietic stem cells.

Antibodies and flow cytometric analysis

FITC anti-mouse CD29 (Catalog #102205), FITC anti-mouse Sca-1 (Catalog #108105), PE anti-mouse CD34 (Catalog #119307), PE anti-mouse CD45 (Catalog#103105), FITC anti-mouse CD4 (Catalog #100509), and Alexa Fluor 647anti-mouse FOXP3 (Catalog #126408) were purchased from BioLegend (San Diego, USA). FACS CantoII (BD Biosciences) was used for flow cytometry, and data were analyzed by BD FACSDiva software (BD Biosciences) and FlowJo (v X.0.7) software.

Treatment of ADSCs

MSCs were primed with LPS or poly(I:C) as previously described [15]. ADSCs were grown to sub-confluence in DMEM and incubated with agonists/antagonists for 1 h. Poly(I:C) (10 μg/mL) was used as an agonist for TLR3. LPS (500 ng/mL) was used as an agonist for TLR4. TAK242 [19,20] (1 μM, MCE) was used as an antagonist for TLR4. The cells were washed twice in growth medium before use in subsequent assays.

CD4$^+$ T cell sorting from C57BL/6 mice

Single splenic lymphocytes were prepared from C57BL/6 mice. CD4$^+$ T cells were isolated using the Mojosort™ mouse CD4$^+$T cell isolation kit (BioLegend), with >95% purity. Next, the cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) (1 μl/mL; Abcam) [21,22]. Freshly purified T cells were resuspended in 0.1% BSA-PBS at a density of 2×10^6 cells/mL and incubated with CFSE for 15 mins at 37°C. Subsequently, the cells were washed and resuspended in 1640 Medium for 10 min to stabilize the CFSE staining. Then, these CFSE labeled CD4$^+$T cells were seeded in 96-well plate at a density of 2×10^5 cells/well, as responding cells in the mixed lymphocyte reaction (MLR).

Allogeneic mixed lymphocyte and ADSC reactions

An allogeneic mixed lymphocyte reaction (MLR) assay was used to determine the effect of poly(I:C), LPS, or TAK242 preconditioning on the immunomodulatory properties of ADSCs. Splenocytes were prepared from BALB/c mice, followed by PBS washes and centrifugation at 1000 rpm for 5 min. Subsequently, the cells were cultured with mitomycin C (25 μg/mL, MCE) and used as donor stimulating cells at a 1:1 ratio (CD4$^+$T cell from C57BL/6 mice: splenocytes from BALB/c mice). Next, the splenocytes were seeded in a 96-well plate at a density of 2×10^5 cells/well. Then, 5 groups of ADSCs, including ADSCs (as control group), ADSC-poly(I:C), ADSC-LPS, ADSC-TAK242, and ADSC-poly(I:C) plus TAK242, were utilized in the MLR assay at ratios 1:0.1, 1:0.2, 1:1, 1:2 (CD4$^+$T cells: ADSC) in RPMI medium. After 5 days, the peak of CFSE on the flow cytometric histograms were evaluated, representing the division cycles for CD4$^+$T cells. CFSE was purchased from Abcam (Cambridge, UK).

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from each group of ADSCs using TRIzol reagent (Ambion), and 1 mg was reverse transcribed using PrimeScript RT reagent kit (Takara). The transcripts were quantified by qRT-PCR using 100 ng of cDNA, SYBRMix Ex Taq (Takara) and 10 mM forward and reverse primers. The primers were designed using Primer Express 2.0 software (Applied Biosystems) or ProbeFinder (Roche). *GAPDH* gene was used as a housekeeping gene to quantify and normalize the expression of the target genes. The reactions were carried out using the Thermal Cycler Dice Real-Time System (Takara). Subsequently, the dissociation curves were generated, and the specificity of the PCR reactions was confirmed. The comparative $\Delta\Delta$Ct method was used for data analysis. The data were normalized against that of the *GAPDH* gene to obtain the ΔCt and then calibrated with the geometric mean of the *GAPDH* ΔCt to generate the $\Delta\Delta$Ct. Then, fold-changes were calculated by the formula $2^{-\Delta\Delta CT}$. Using this method, expressions of 3 cytokines – Fgl2, Cox-2, and IL-10 – were analyzed. The primers are listed in Table 1.

ELISA for cytokines

The cell culture supernatants of ADSCS following the treatments with different combinations of TLR3/4 activators/blocker were collected. PGE_2, FGL2, and IL-10 levels were measured using an ELISA kit (Cusabio) according to the manufacturer's protocol.

Vascularized cardiac transplantation and treatment schedule of ADSCs

Heterotopic transplantations of intact allogeneic BALB/c hearts into C57BL/6 recipients were performed as described previously [23]. Briefly, the ascending aorta and pulmonary artery of the donor's heart were anastomosed end-to-side to the recipient's abdominal aorta and inferior vena cava, respectively. As a result of perfusion with the recipient's blood, the transplanted heart resumed contraction. The graft function was monitored by abdominal palpation, and the rejection was scored based on the cessation of heart contraction. BALB/c hearts were transplanted into BALB/c recipients as a technical control group. In the tolerant control group, BALB/c hearts were transplanted into C57BL/6 recipients, and rapamycin (0.4 mg/kg) was injected intraperitoneally on postoperative days (POD) 0, 1, 2, 4, 6, 8, 10, 12, 14, and 16. A total of 0.5×10^6 ADSCs were injected into the recipient via the caudal vein on POD1. A subset of the recipients was sacrificed on POD 4 to obtain spleens

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Table 1. Primer information.

Gene name	Forward primer (5'-3')	Reverse primer (5'-3')	Access number GenBank
FGL2	CGTGGACAACAAAGTGGCAAAT	CACGTAGTGGTCGGAACAATCT	NM_008013.4
COX2	GCTCTCCCCTCTCTACGCATTC	GCTTCAGTATCATTGGTGCCCTA	NM_005089
IL-10	TGGACAACATACTGCTAACCGACTC	GGGCATCACTTCTACCAGGTAAAAC	NM_010548.2
GAPDH	CCTCGTCCCGTAGACAAAATG	TGAGGTCAATGAAGGGGTCGT	NM_008084.2

and hearts, and the other recipients were investigated until allografts stopped beating (Figure 1).

Ex vivo analysis of CD4$^+$ Foxp3$^+$ Treg cell from the spleens of recipient mice

Splenocytes were freshly isolated from the spleens of recipient mice. Briefly, the spleen was mashed through a cell strainer and centrifuged at 1000 rpm for 5 min. Then, the cells were washed in cell staining buffer (BioLegend) and centrifuged at 1400 rpm for 5 min. The red blood cells were lysed by ammonium chloride solution (Stemcell) for 10 min, followed by washes and centrifugation. Finally, the cells were stained by FITC anti-mouse CD4 (Catalog #100509) according to the Cell Surface Immunofluorescence Staining Protocol (BioLegend), followed by staining with Alexa Fluor 647 anti-mouse FOXP3 (Catalog #126408) according to True-Nuclear™ Transcription Factor Staining Protocol (BioLegend). After that, the percentage of CD4$^+$ Foxp3$^+$Treg cells was evaluated by flow cytometry.

Histopathological analysis and damage score

The grafted hearts were harvested on POD7. The graft was formalin-fixated and embedded in paraffin. We made 3-mm sections at one-third of the distance from the base to the apex of the heart and stained them with hematoxylin and eosin (HE). According to the standardized grading system [24] for the pathologic diagnosis of rejection in cardiac biopsies of the International Society for Heart and Lung Transplantation (ISHLT), acute cellular rejection was divided into Grade 0 R (no rejection); Grade 1 R (mild: interstitial and/or perivascular infiltrate with up to 1 focus of myocyte damage); Grade 2 R (moderate: 2 or more foci of infiltrate with associated myocyte damage); and Grade 3 R (severe: diffuse infiltrate with multifocal myocyte damage±edema, hemorrhage±vasculitis). Two observers evaluated the histological slides individually, with 5 fields being checked in each slide. The average scores were calculated; final results are expressed as mean±standard deviation (SD).

Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the significance of differences between groups. Cardiac graft survival was reported in terms of median survival time, and comparative analysis was accomplished via the Kaplan-Meier cumulative survival method. The differences in the survival between the groups were determined using the log-rank (Mantel-Cox) test. Data of HE staining grading system were analyzed using rank test with a Bonferroni post hoc test. Statistical analyses were performed using GraphPadPrism7 software. Values of $P<0.05$ were considered as statistically significant.

Results

ADSCs have the full capabilities of MSCs

Mouse ADSCs were cultured in DMEM to a stable fibroblast-like morphology for subsequent experiments (Figure 2A). As shown in Figure 2B–2D, we confirmed the differentiation potential of ADSCs into adipocytes, chondrocytes, and osteoblasts by established methods. The phenotypes were analyzed by flow cytometry examinations. The cells were positive for CD29 and Sca-1 (90–99%) and negative for CD34 and CD45 (<5%) (Figure 2E).

Preconditioning of ADSCs with poly(I:C) had a maximal inhibitory effect on CD4$^+$T cells in MLR

Splenocytes were prepared from the spleens of C57BL/6 mice. Then, CD4$^+$T cells were selected with >95% purity. The addition of ADSCs significantly inhibited the proliferation of CD4$^+$T cells in a dose-dependent manner as compared to that in the control condition ($P<0.05$, Figure 3A). ADSCs preconditioned by poly(I:C) were more effective than untreated ADSCs in inhibiting the proliferation of CD4$^+$T cells at the different ratios ($P<0.05$, Figure 3A). In contrast, ADSCs preconditioned by LPS induced a significant increase in the proliferation of CD4$^+$T cell in a dose-dependent manner. When CD4$^+$T cell: ADSC ratio was 1:1, ADSCs pretreated with poly(I:C) significantly inhibited CD4$^+$T cells proliferation (mean inhibition rate 42.53±3.82%), which was better than the results of ADSCs preconditioned

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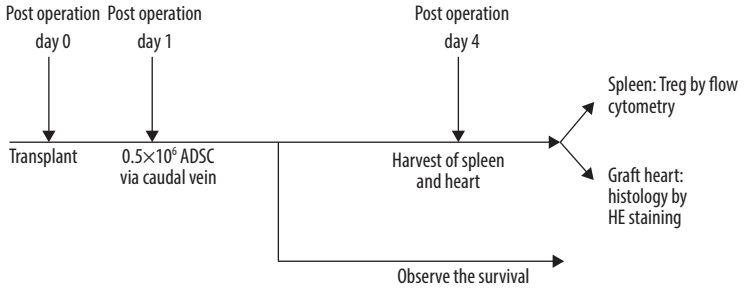

Figure 1. Treatment schedule in mouse heart transplantation and specimen harvest. ADSCs were injected into the recipient via the caudal vein on POD 1. A subset of the recipients was sacrificed on POD 4 to obtain spleens to measure the proportion of Tregs. The others were kept for observing the survival until graft hearts stop contracting by daily palpation.

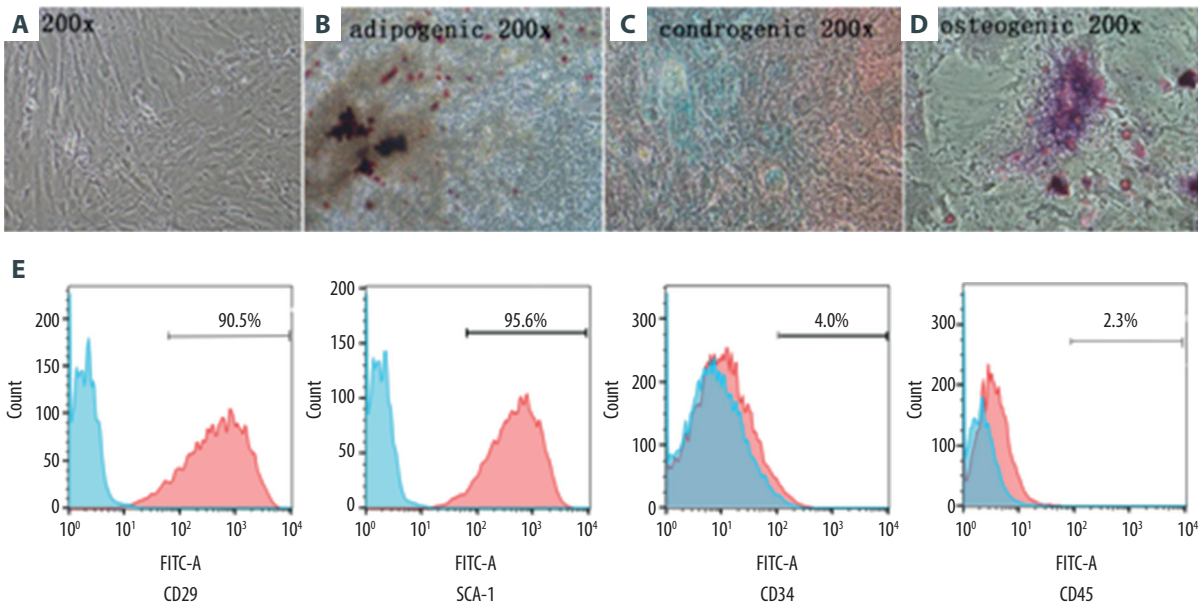

Figure 2. Morphology, tri-lineage differentiation capacity, and surface marker expression of ADSCs. (**A**) Morphology of ADSCs. (**B–D**) *In vitro* adipogenic, chondrogenic, osteogenic differentiation of ADSCs were evaluated at passage 5, as shown by oil red O, toluidine blue, and alizarin red staining. (**E**) Histograms represent the immunophenotypic profile of ADSCs in passage 5. Representative data of 3 separate experiments are shown.

with poly(I:C) plus TAK242 (mean inhibition rate 28.70±3.74%) ($P<0.05$, Figure 3B, 3C).

FGL2, but not PGE_2 or IL-10, is the main effector molecule in ADSCs preconditioned with TLR3 activator

Next, we explored the effect of poly(I:C), LPS, or TAK242 preconditioning on the expression of immune modulators (IL-10, PGE_2, and sFGL2). The expressions of FGL2, Cox-2, and IL-10 were evaluated quantitatively by RT-PCR. ADSCs preconditioned by poly(I:C) exerted a significant increase in the relative expression of *FGL2* mRNA as compared to the untreated ADSCs or the other 3 groups of pretreated ADSCs ($P<0.05$, Figure 4A). In addition, the secretion of FGL2 in the supernatants of the ADSC-poly(I:C) was significantly higher than that in the other 4 groups ($P<0.05$, Figure 4B).

We observed no significant differences in the relative expression of *Cox-2* mRNA or *IL-10* mRNA among untreated ADSCs, ADSCs preconditioned by poly(I:C), and other groups as assessed by RT-PCR. Furthermore, there were no significant differences in PGE_2 or IL-10 secretion between untreated ADSCs and those preconditioned by different drugs by the ELISA assay (data not shown).

These results suggested that ADSCs preconditioned by poly(I:C) exert a higher immunosuppressive effect through expression of FGL2, but not PGE_2 or IL-10, *in vitro* as compared to the untreated ADSCs or ADSCs pretreated with LPS or TAK242.

Intravenous injection of ADSCs pretreated with poly(I:C) prolonged the survival of cardiac allograft

The median survival of cardiac allografts in the recipients without any intervention (untreated control) was 7 days. In the

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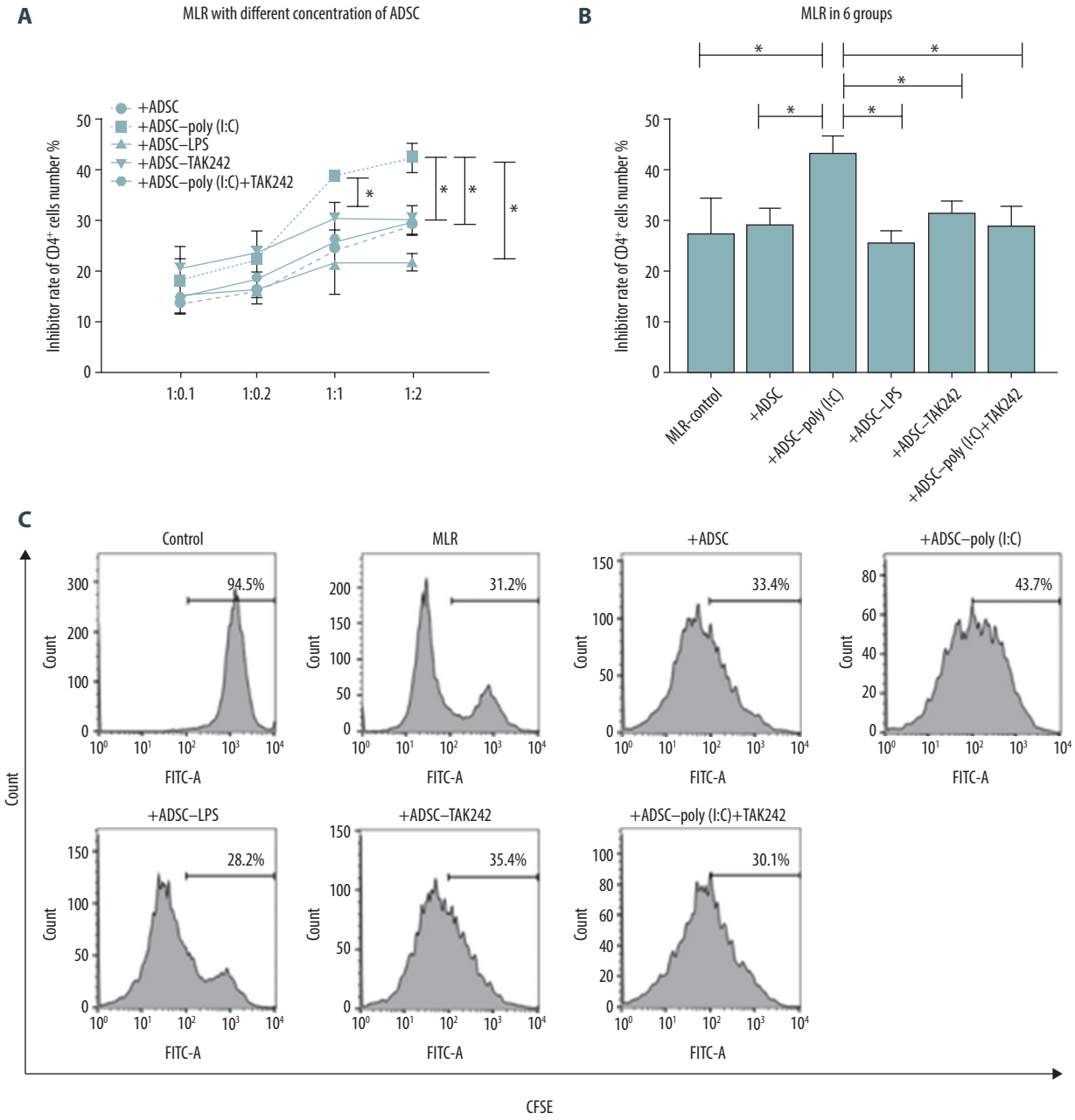

Figure 3. ADSCs preconditioned by poly(I:C) significantly inhibited the proliferation of CD4+T cells in MLR assay. CD4+T cells from C57BL/6, which were previously stained by CFSE, were cocultured with splenocytes at a ratio of 1:1 in RPMI medium in the presence or absence of ADSCs or different groups of preconditioned ADSCs at ratios 1:0.1, 1:0.2, 1:1, or 1:2 for 5 days. Proliferation of responding CD4+T cells were analyzed by flow cytometry. (**A**) The addition of ADSCs decreased the CD4+T cells proliferation in a dose-dependent manner. (**B, C**) When the CD4+T cell: ADSC ratio was 1:1, ADSCs preconditioned by poly(I:C) significantly inhibited the proliferation of CD4+T cell (inhibition rate 42.53±3.82%), which was better than that in the other groups. Representative data of 3 separate experiments are shown.

technical control group, the median survival of cardiac allografts was longer than 100 days. In the tolerant control group (n=10), 9 cases of cardiac allografts were still alive after stop injection of rapamycin, while 1 case of cardiac allograft stopped contracting on POD 20. The monotherapy of unstimulated ADSCs prolonged the median survival after cardiac allograft by 10.2 days. ADSC-poly(I:C) significantly prolonged the allograft survival to 12.3 days ($P<0.05$, n=10, Figure 5). In contrast, ADSCs treated with

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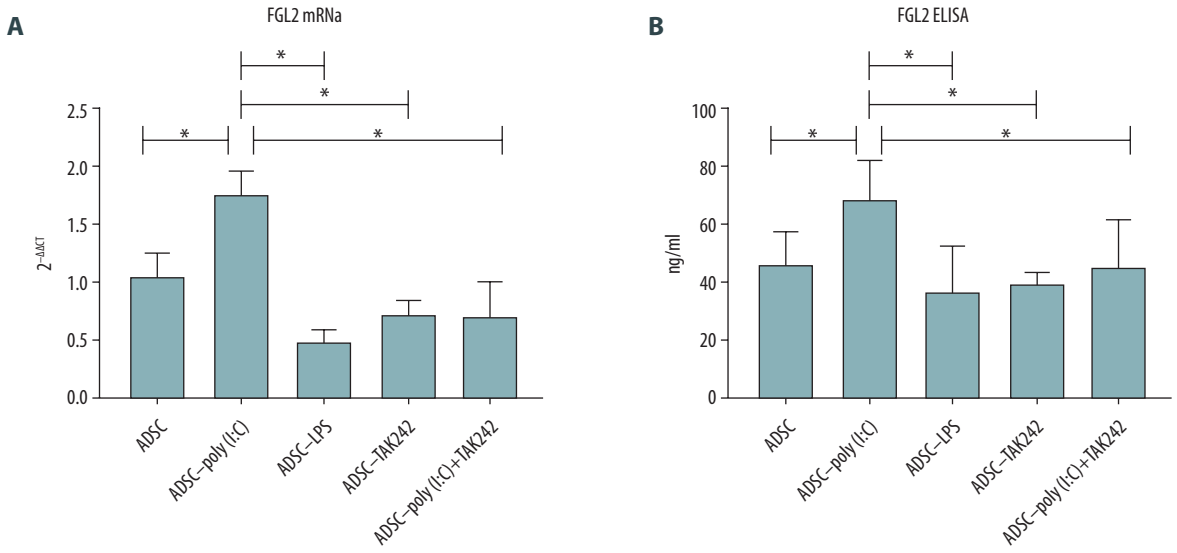

Figure 4. (**A, B**) The expression of FGL2 measured by RT-PCR or ELISA. ADSCs preconditioned by poly(I:C) caused a significant increase in the *FGL2* mRNA expression and secretion of FGL2 as compared to the untreated ADSCs or the other 3 groups. Data represent 3 independent experiments (mean±SD, * P<0.05).

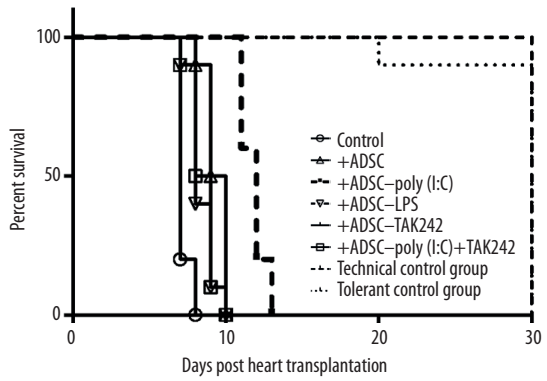

Figure 5. Survival curve of cardiac allografts (heart transplantation from BALB/c to C57BL/6). Survival in the recipients without any intervention (control group) was 7 days. In the technical control group, the median survival of cardiac allografts was longer than 100 days. In the tolerant control group (n=10), 9 cases of cardiac allografts were still alive after stopping injection of rapamycin and 1 case of cardiac allograft stopped contracting on POD 20. ADSC monotherapy prolonged the cardiac allograft median survival up to 10.2 days. ADSC-poly(I:C) significantly prolonged the allograft survival to up 12.3 days (P<0.05, n=10). ADSCs treated with LPS, TAK242, or poly(I:C) plus TAK242 prolonged the survival to 8, 9.5, and 10.5 days, respectively.

LPS, TAK242, or poly(I:C) plus TAK242 prolonged the survival to 8, 9.5, and 10.5 days, respectively.

Preconditioning of ADSCs with poly(I:C) increased the proportion of CD4$^+$ Foxp3$^+$ Treg cells in the mouse spleen after heart transplantation

Next, we explored the effect on the Treg cells in the model of heart transplantation (Figure 6B, 6H). Treatment with untreated ADSCs increased the population of Treg subsets to 14.80±1.06% in the spleen of heart-transplanted mice (Figure 6C) as compared to 8.83±1.24% in the normal C57BL/6 mice (Figure 6A, P<0.05). In addition, the preconditioning of ADSCs with poly(I:C) significantly increased the Tregs subset to 20.33±1.91% (P<0.05, Figure 6D). However, the number of Treg cells in the ADSC group pretreated with LPS did not increase (13.53±1.37%, Figure 6E) when compared with untreated ADSCs. Also, no significant increase was detected in the ADSC-TAK242 (15.60±2.30%, Figure 6F) or ADSC-poly(I:C) plus TAK242 (15.9±1.78%, Figure 6G) groups.

ADSCs preconditioned with TLR3 stimulator show protective effect in graft histological findings

Histological assessments were carried out in grafted hearts 4 days after transplantation. There was a significant difference between the normal group and the other 6 groups (Figure 7A, 7H). The histological findings of heart grafts were consistent with the survival data. Grafts receiving injections with cell culture medium only showed mild rejection, shown by swollen cardiomyocytes with apparent infiltration of monocytes (Figure 7B).

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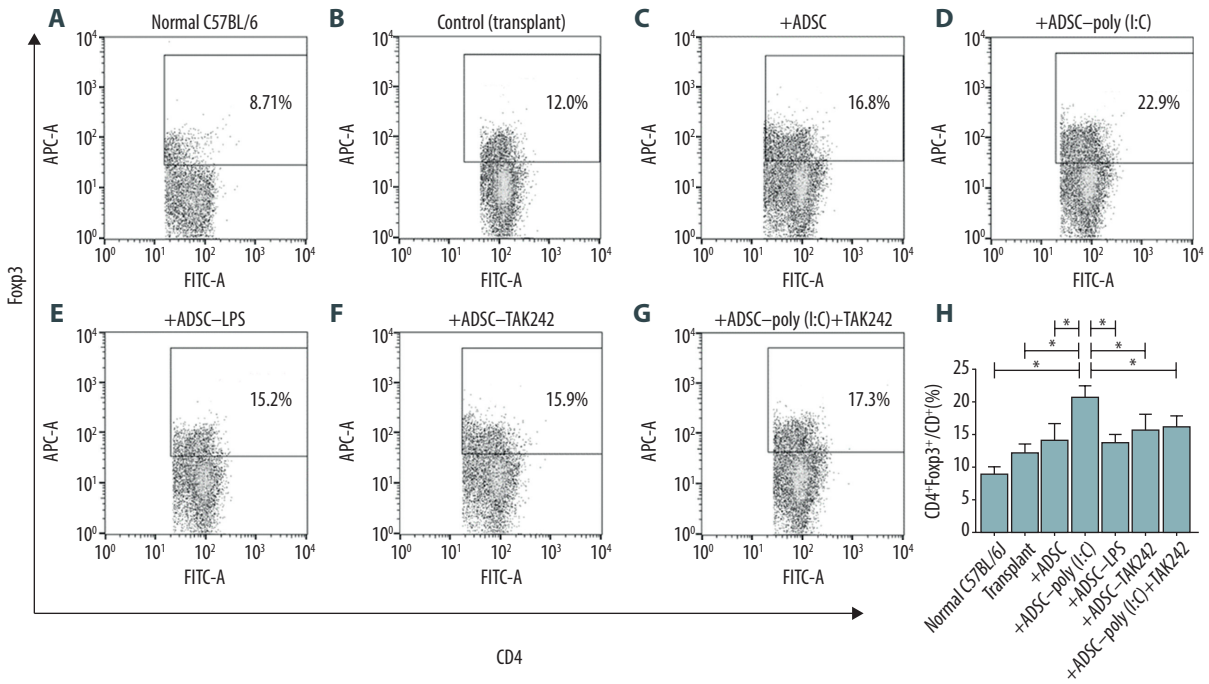

Figure 6. (**A–H**) Preconditioning of ADSCs with poly(I:C) increased the splenic proportion of $CD4^+$ $Foxp3^+$ Tregs in the heart transplantation model. Spleens were removed from different groups of treatments on POD 4, labeled by FITC anti-mouse CD4 and Alexa Fluor 647 anti-mouse Foxp3, and analyzed by flow cytometry. Data represent 3 independent experiments (* P<0.05).

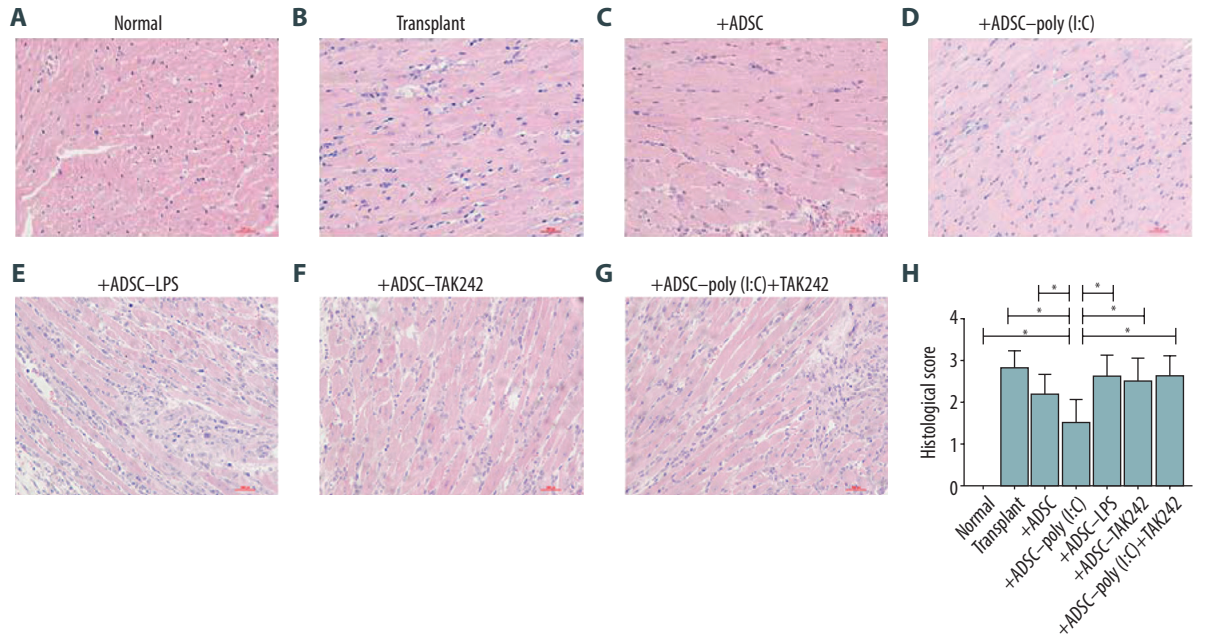

Figure 7. (**A–H**) Histology findings of heart grafts with the treatment of ADSC preconditioned with different combinations of TLR3/TLR4 activator and inhibitors, harvested at day 7 after transplantation (**B–G**). Heart from a BALB/c mouse without transplantation was used as a normal control (**A**). Sections were stained with hematoxylin and eosin (H&E) and zoomed fields (×200) are shown. In H&E staining, cell nucleus is stained purple blue, cytoplasm in pink, while red blood cells and fibrin are stained intensely red. Representative views of each group are shown. Histological scores of grafted hearts POD4 after transplantation were then evaluated and compared. * P<0.05 *vs*. the other groups.

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Treatment with ADSC alleviated the histological damage to some degree (Figure 7C), while ADSCs preconditioned with TLR3 activator had the best protective effects, in which grafts remained near to normal except for mild cell infiltration (Figure 7D). In contrast, grafts treated with ADSCs preconditioned with TLR3 activator plus TLR4 blocker did not show further histological improvement (Figure 7F, 7G), while grafts treated with ADSCs preconditioned with TLR4 activator had the most severe cellular destruction and perivascular infiltration (Figure 7E). In the histological score analysis, there were significant differences between the ADSCs preconditioned with TLR3 activator and other 6 groups ($P<0.05$).

Discussion

MSCs were first discovered in bone marrow in 1974 [25] and were termed bone marrow stem cells (BMSCs). These cells were found to have several functions, including supporting hematopoiesis, inducing tissue regeneration, and immunomodulation. The MSCs that were discovered in fat tissue were termed adipose-derived mesenchymal stem cells (ADSCs) in 2001 [26]. These cells have been demonstrated to have similar functions as BMSCs. By 15 February 2020, MSCs had been used in 979 clinical trials registered on ClinicalTrials.gov. A total of 30 trials are related to organ transplantation. In the field of organ transplantation, MSCs are primarily used in living kidney transplantation [27]. Considered as the father of mesenchymal stem cells, Arnold I. Caplan suggested changing the name of MSCs from mesenchymal stem cells to medicinal signaling cells in 2017 [28]. Despite the controversy in the field of regenerative medicine, MSCs were confirmed to have an immunomodulatory function in the year 2000, when human MSCs were transplanted into fetal sheep early in gestation and had unique immunological characteristics for persistence in a xenogeneic environment [29]. Furthermore, gene therapy has been used to improve the immunomodulatory function of MSCs, such as the transduction of genes *Foxp3* [30], *IL-10* [31], *HGF* [32], *IDO* [33], and *IL-7* [34]. The preconditioning of MSCs also strengthened their immunomodulatory function, which was easier and more cost-effective than gene therapy. Some studies used IL-1β [35] or IFN-γ [36] to precondition MSCs, while other studies used TLR agonist to strengthen the immunomodulatory function of MSCs.

TLR was first discovered in innate immune cells, especially macrophages [37]. Subsequently, some investigators demonstrated that mouse MSCs express TLRs on their surface [38]. In nature, during a virus attack, TLR3 on the surface of macrophages or dendritic cells is activated, whereas during bacterial infection, TLR4 is stimulated and the myeloid differentiation factor 88 (MyD88)-dependent pathway is activated. LPS is the TLR4 ligand, and TAK 242 is the antagonist of TLR4. After activation of TLR3 by dsRNA or poly(I:C), the TLR3-TIR-domain containing the adapter-inducing interferon-β (TRIF) -interferon regulatory factor 3 (IRF3)-IFN-β pathway is activated.

A short-term TLR3 agonist, poly(I:C), is the analog of double-stranded RNA (dsRNA) and has been found to strengthen the immunomodulatory function in a mouse model of dextran sulfate sodium (DSS)-induced acute colitis [39] and in an experimental autoimmune encephalomyelitis (EAE) model [40]. Conversely, infusion of MSCs preconditioned by LPS significantly increased clinical signs of disease, colon shortening, and histological disease index in the model of DSS-induced colitis. In our study, we attempted to explore whether TLR4 antagonist (TAK242) alone or the combination of poly(I:C) and TAK242 have a stronger immunomodulatory function than the monotherapy of poly(I:C).

MLR is an *in vitro* assay mimicking organ transplantation, which is primarily attributed to the helper T (Th1) cell reaction that induces the rejection [41]. Thus, we assessed the functions of preconditioned ADSCs with respect to MLR. When the $CD4^+$T cell: ADSC ratio was 1:1, ADSCs preconditioned by poly(I:C) significantly inhibited $CD4^+$T cell proliferation (inhibition rate 42.53±3.82%), which was better than the ADSCs pretreated with poly(I:C) plus TAK242 (inhibition rate 28.70±3.74%). To the best of our knowledge, the present study, for the first time, indicates that the modulatory function of ADSCs preconditioned by TLR3 activator plus TLR4 blocker is not better than that of TLR3 activator alone. Most probably, interactive compensations in signals transduction occur and the immunomodulatory function of ADSCs might be modulated to balance the pro- and anti- inflammation. The combination of 2 most effective factors unexpectedly decreased the ability of MSCs to regulate the immune response. These conflicts in the transduction of regulatory signals have also been suggested by others [42].

To investigate the mechanism underlying augmented immunoregulatory functions of ADSCs preconditioned with TLRs stimulators, we assessed the expression of the 3 molecules. Our results showed that significant over expression of FGL2 might be the main reason. RT-PCR and ELISA results demonstrated that *FGL2* is upregulated in mouse ADSCs preconditioned with poly(I:C). The *Fgl2* gene is also known as pT49 and fibroleukin, and was first cloned successfully in mouse cytotoxic T cells [43]. It was classified as membrane form FGL2 (mFGL2) and soluble FGL2 (sFGL2). FGL2 is primarily bound to FcgR, including FcgRIIB and FcgRIII [44]. By a previous report, *FGL2* mRNA expression was upregulated in human ADSCs with a cocktail of pro-inflammatory cytokines, specifically IL-1β, TNF-α, IFN-α, and IFN-γ [45]. FGL2was reported as a novel effector molecule in the immunoregulation of Treg cells [46]. Hence, our results suggested a correlation between the IFN-β expression induced by TLR3 activation and the overexpression of FGL2.

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Increased numbers of regulatory T cells were reported to be closely related with immune tolerance. Tregs represent<10% of $CD4^+$T cells in the periphery [47,48]. In the current study, we found that ADSCs preconditioned with poly(I:C) significantly increased the Treg subsets to 20.33±1.91% in the mouse model of heart transplantation. Thus, ADSCs may not only have an immunomodulatory function, but could also induce an increase in the number of regulatory T cells to strengthen this function. It was reported that after activation of TLR on the surface of MSCs, the expression of Notch ligand and Delta-like 1 (DL1) increased. The Notch pathway in the $CD4^+$T cell is stimulated through the cell contact mechanism, and the proportion of Tregs is therefore increased [49].

The present study, for the first time, used TLR agonist/antagonist-treated MSCs in the mouse model of fully MHC-mismatched heart transplantation. Although the goal of graft tolerance was not reached, using a single dose of TLR3 activated ADSCs prolonged the graft survival from 7 days up to 12.3 days. These imperfect improvements in the transplant results may be caused by several reasons. First of all, the dosage and timing of ADSC treatment. Single-dose treatment of ADSCs on POD1 may be inadequate to reverse the rejecting procedures, compared with the 10 dosages administration on different PODs with rapamycin. The second reason is the route of administration. Since the grafts were placed in the abdominal cavity, and tail vein-injected ADSCs are primarily engulfed by recipient macrophages in the lung and thus cannot reach the most appropriate sites for their functions. In the future, multiple injections with different dosages through varied routes of administration could be attempted. Also, a fully MHC-mismatched graft pairing may be too stringent for this immunomodulatory model. Therefore, haplotype-mismatched grouping, such as the first filial generation (F1) mice, could be used as the recipients of the heart transplantation, which will cause milder graft rejections and better simulate the clinical scenario. In addition, combination treatment of ADSCs with reduced dosage of immunosuppressors have been shown to significantly prolong graft survival [50,51]. This approach could also be used to show the regulatory potential of TLR-activated ADSCs.

Hearts in our heterotopic heart transplantation (HHT) model cannot provide the same pumping functions as grafts in clinical orthotopic heart transplantation (OHT), because the circulation route in these HHT grafts is totally different from that of OHT hearts [52]. This animal model is well accepted as the immune rejection-tolerance model because of its several advantages. First, mice used in these experiments are all inbred SPF animals, thus providing a consistent genetic background. Second, graft rejection can be easily monitored by simple palpation of the heart beating at the recipient abdominal wall. Also, lower dosages of reagents are required in these mice, as their body weights are lower than in larger animals, therefore reducing the total expenses in the overall study.

Conclusions

We proved that ADSCs preconditioned by TLR3 stimulator has the strongest immune regulatory function, both *in vitro* and *in vivo*, and is better than that of ADSCs preconditioned by the combination of TLR3 agonist with TLR4 antagonist. This function is probably mediated via ADSCs– Tregs interactions and secretion of FGL2. In future studies, new strategies to induce solid graft tolerance with function-augmented ADSCs will be investigated.

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