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Exosomes Derived from IDO1-Overexpressing Rat Bone Marrow Mesenchymal Stem Cells Promote Immunotolerance of Cardiac Allografts

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Abstract

Background: The immunosuppressive activity of mesenchymal stem cells (MSCs) has been exploited to induce tolerance after organ transplantation. The indoleamine 2,3-dioxygenase (IDO) may have beneficial effects in the immunoregulatory properties of MSCs. It was recently revealed that exosomes derived from MSCs play important roles in mediating the biological functions of MSCs. This study aimed to explore the roles of exosomes derived from MSCs in the induction of immune tolerance. **Methods:** Dendritic cells (DCs) and T-cells were cultured with exosomes derived from rat bone marrow MSCs (BMSCs) overexpressing IDO1 or controls. For the in-vivo study, rats received heart transplants and were treated with exosomes from IDO-BMSCs and heart function was evaluated. Flow cytometry was used to detect expression of cell surface markers. Cytokine levels were detected in culture supernatants or serum samples. Protein and microRNA expressions in exosomes were investigated by chips. **Results:** Exosomes from IDO-BMSCs cultured with DCs and T-cells (1) downregulated CD40, CD86, CD80, MHC-II, CD45RA, CD45RA+CD45RB, OX62, and upregulated CD274 expression, (2) increased the number of regulatory T-cells (Tregs) and decreased the number of CD8+ T-cells, and (3) decreased the levels of pro-inflammatory cytokines, but increased the levels of anti-inflammatory cytokines compared with the other groups. Transplanted rats, which were injected with exosomes from IDO-BMSCs, had reduced allograft-targeting immune responses and improved cardiac allograft function. Exosomes secreted by IDO-BMSCs exhibited significant upregulations of the immunoregulatory protein FHL-1, miR-540-3p, and a downregulation of miR-338-5p. **Conclusion:** Exosomes derived from IDO-BMSCs can be used to promote immunotolerance and prolong the survival of cardiac allografts.

Keywords

bone marrow mesenchymal stem cells, indoleamine 2, 3-dioxygenase, exosomes, cardiac allograft, immunotolerance

Introduction

Heart failure is a major public health challenge, with a worldwide prevalence of more than 23 million[1]. Cardiac transplantation is the current accepted therapy for patients with end-stage heart failure. However, prolonged acceptance of the allograft requires long-term administration of strong immunosuppressive drugs, which have significant side effects[2]. Induction of transplantation tolerance without long-term immunosuppression remains an important goal in the field of transplantation biology[3].

Mesenchymal stem cells (MSCs) have been reported to exert anti-inflammatory and immunomodulatory effects[4–6], which are mediated via cell–cell interactions, as well as via secretion of factors modulating T-cell proliferation[7]. The immunomodulatory activity of MSCs is mediated by the transformation of pro-inflammatory M1 macrophages to anti-inflammatory M2 macrophages, as well as by inhibition of natural killer cells[8]. Additionally, MSCs have been shown to promote an anti-inflammatory response via secretion of

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cytokines, growth factors, interleukin (IL)-10, hepatocyte growth factor, transforming growth factor (TGF)β1, indoleamine 2,3-dioxygenase (IDO), prostaglandin E2 (PGE2), and human leukocyte antigen G (HLA-G)[9]. Their immunosuppressive properties make MSCs attractive candidates for cellular therapy of graft-versus-host disease and prevention of transplant rejection[10].

IDO, which is mainly expressed in lymphoid tissue and the placenta, catalyzes the rate-limiting cleavage of tryptophan via the kynurenine pathway[11] Treatment with an IDO1 inhibitor was previously shown to result in T-cell-dependent allograft rejection, and IDO has shown promise as an immunomodulator to suppress allograft rejection[12,13]. Activation of IDO-expressing DCs was shown to promote the survival of grafts[14]. IDO has been shown to mediate the immunoregulatory activity of CD4+ CD25+ FoxP3+ regulatory T-cells (Tregs)[15]. Interestingly, this interplay between IDO and Tregs has been shown to be important for CTLA4Ig-induced tolerance to murine cardiac allografts[16].

Exosomes are membrane-bound vesicles formed by the inward budding of multivesicular endosomes, which fuse with the plasma membrane and then undergo extracellular secretion[17–19]. Exosomes are secreted by several cells types including B-cells[20], dendritic cells (DCs)[21] and T-cells[22], and have been reported to contain proteins and RNA of the secretory cells. They are thought to represent the bioactive component of stem cells, and play an important role in intercellular communication[23,24]. Exosomes secreted by activated antigen-presenting cells (APCs) are more enriched in major histocompatibility (MHC) class I and II, CD86 and CD45 compared with exosomes secreted by quiescent APCs[25]. Exosomes secreted by DCs and B-cells were shown to play an important role in regulation of the adaptive immune response to pathogens and tumors[26]. Furthermore, graft-derived exosomes which transfer non-self MHC antigens and APC-activating mediators to recipient APCs are thought to mediate the rapid adaptive immune response leading to acute rejection of allografts[27]. There has been a recent focus on using MSC-derived exosomes as a cell-free therapy for cardiac regeneration following myocardial infarction[28].

In this study, we established a rat heterotopic heart transplant model. We used exosomes secreted by IDO-overexpressing BMSCs to investigate mechanisms underlying immune tolerance during allogeneic heart transplantation.

Materials and Methods

Animals

Healthy specific-pathogen-free (SPF) male Sprague–Dawley (SD) rats aged 4 weeks were purchased from Chengdu Dasuo Biological Technology Co., Ltd. (Chengdu, Sichuan, China). All animal studies were approved by the Animal Care and Use Committee of the First People's Hospital of Yunnan Province, China and were performed according to Good Laboratory Practice.

BMSCs were isolated from SPF rats as previously described[29]. Briefly, rats were sacrificed by cervical dislocation, the femur and tibia were collected, and immersed in 75% ethanol for 1–2 min and then in 0.9% normal saline. Both ends of the femur and tibia were removed to expose the bone marrow cavity, which was flushed. The femur and tibia were cut into blocks, rinsed repeatedly with saline, and the liquid was then transferred into a sterilized tube. After centrifugation at 1500×*g*/min for 10 min, the cell pellet was collected, and the cells were resuspended in C57BL/6 mouse BMSC medium (Cyagen Biosciences, Santa Clara, CA, USA) containing 10% fetal bovine serum (FBS). Cells were cultured at 37 in the presence of 5% CO_2, and the medium was refreshed after 72 h and every 3 days thereafter. Cells at passage 3 (P3) were purified with CD11b (Microglia) MicroBeads (Miltenyi, Auburn, CA, USA), and cultured until P7.

Transduction of BMSCs with Lentivirus Carrying IDO1

BMSCs were transduced with GV308 lentivirus carrying IDO1 as previously described[30]. Total RNA was extracted from transduced cells using Trizol reagent (Thermo Scientific, Waltham, MA, USA), according to the manufacturer's instructions. cDNA was prepared using the RevertAid™ First Strand cDNA Synthesis Kit (Thermo Scientific), and IDO1 was amplified using a template (10 ng/μl), with 10 μM each of IDO1 forward primer 5'-TTAAGACGCAATGAAGACT-3' and IDO1 reverse primer 5'-GAGGTGGAACATTCTGAG-3' (Shanghai Genechem Co., Ltd., Shanghai, China), dNTP mix (2.5 mM each), and PrimeSTAR HS DNA polymerase (0.5 μl, Takara Bio Inc., Otsu, Japan).

Extraction of Exosomes

At 16 h following lentivirus transduction, IDO1 expression was induced by treating the cells with 5 μg/ml of doxycycline (DOX) for 48 h, and exosomes were extracted using the Exosome Antibodies, Array & ELISA Kit (System Biosciences, Mountain View, CA, USA). Briefly, the cells were pelleted at 300×*g* for 15 min at 4°C, the supernatant was centrifuged at 15,000×*g* for 30 min at 4°C, and the resulting supernatant passed through a 0.2-μm filter. The filtrate was then centrifuged at 120,000×*g* for 70 min at 4°C, and the exosomes were harvested using the ExoQuick TC kit according to the manufacturer's instructions (System Biosciences, Mountain View, California, USA). Serum exosomes were removed by ultra-centrifugation at 120,000×*g* at 4°C overnight.

Separation and Culture of DCs from Peripheral Blood

Male SPF rats were anesthetized, the aorta was separated after laparotomy, and 10 ml of blood was collected from the aorta in a heparinized syringe. The blood was mixed with

erythrocyte lysis buffer and incubated on ice for 15 min with intermittent vortexing. Peripheral blood lymphocytes were collected using the Lymphocyte Separation Medium (RAT) (Catalog No: P8630; Solarbio CO., Beijing, China). The cells were resuspended in two volumes of erythrocyte lysis buffer and centrifuged at 450×*g* for 10 min at 4. The cell pellet was resuspended in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% FBS, 20 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) , 10 ng /ml IL-4 and 0.1mg/ml of penicillin and streptomycin and incubated for 10 days at 37 in the presence of 5% CO_2. The immature DCs and mature DCs were observed under a phase contrast microscope. After shaking, DCs were collected, fixed in 30 g/l glutaraldehyde and processed for electron microscopy as mentioned above. Then, cells were observed under an electron microscope (S-3000 N).

Separation of T-cells

Spleens were harvested from SD rats under aseptic conditions, minced, and single cell suspensions were prepared. Samples were lysed in erythrocyte lysis buffer (Solarbio Science & Technology Co.) and incubated on ice for 15 min with intermittent vortexing. The suspension was centrifuged at 450×*g* for 10 min at 4, and cells were harvested. For cell sorting, cell suspensions (10^7 cells) were centrifuged at 300×*g* for 10 min, and cells were resuspended in MACS buffer (10^7 cells per 80 μl of buffer). and incubated with anti-Rat DC (OX62) microBeads at 4 for 15 min. Cells were washed with 2 ml of buffer, centrifuged at 300×*g* for 10 min, resuspended in 500 μl of buffer, and passed through the column according to the manufacturer's instructions. The resultant T-cells were observed under a phase contrast microscope[7].

Co-culture and Grouping

The different groups for the co-culture experiments included: (A) IDO1-BMSC-secreted exosomes co-cultured with DCs; (B) IDO1-BMSC-secreted exosomes co-cultured with T-cells; (C) IDO1-BMSC-secreted exosomes co-cultured with DCs + T-cells; (D) Empty vector-BMSC-secreted exosomes co-cultured with DCs; (E) Empty vector -BMSC-secreted exosomes co-cultured with T-cells; (F) Empty vector -BMSC- secreted exosomes co-cultured with DCs + T-cells; (G) BMSC-secreted exosomes co-cultured with DCs; (H) BMSC-secreted exosomes co-cultured with T-cells; (I) BMSC-secreted exosomes co-cultured with DCs + T-cells; (J) DCs only; (K) T-cells only; (L) DCs co-cultured with T-cells.

The concentration of exosomes from corresponding BMSCs was adjusted to 800 mg/ml to make the exosome concentrations consistent among groups. After cell counting, DCs were mixed with T-cells at a ratio of 1:1, followed by addition of 5 μg/ml lipopolysaccharide. The mixture was incubated for 24 h, 48 h or 72 h, and then processed for flow cytometry. The supernatant of co-cultured with DCs + T-cells was collected at the designated time points for reverse transcription polymerase chain reaction (RT-PCR) to detect the IDO1 expression, and for liquid-phase microarray assays (to detect cytokine levels).

The A, D, G and J groups were evaluated for CD40, CD86, CD80, MHC-II, CD274, CD45RA, CD45RA+CD45RB and OX62 expression. The B, E, H and K groups were evaluated for Treg, CD3/CD4 and CD3/CD8 expression. The C, F, I and L groups were evaluated for CD40, CD86, CD80, MHC-II, CD274, CD45RA, CD45RA+CD45RB, OX62, Treg, CD4 and CD8 expression.

Quantitative Analysis of Cytokine Levels

Cells were co-cultured for 48, 72 and 96 h, and the supernatant was collected. Cells from the different co-culture groups were harvested at the designated time points and processed for flow cytometry according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). Supernatant was assayed for in-vitro experiments, and serum samples were assayed for in-vivo experiments. Samples were assayed for IL-1α, IL-4, IL-1β, IL-2, IL-10, interferon (IFN)γ, IL-18, TGFβ1, TGFβ2 and TGFβ3 using the RECYTMAG-65K-07 kit (Merck, Millipore Corporation, Billerica, MA, USA) and TGFBMAG-64K-03 kit (Merck) according to the manufacturer's instructions.

Echocardiography Assessment of Ventricular Function

Rats that received heart transplants were injected in the tail vein with exosomes after 48 h as follows: IDO1-BMSC-exosomes (1 mL; 20 mg/ml), vector-BMSC-exosomes (exosomes from BMSCs with control vector transduction; 1 ml; 20 mg/ml), BMSCs exosome (1 ml; 20 mg/ml), and no exosomes (1 ml of saline). Cardiac function was evaluated by Doppler echocardiography with a Philips IE33 ultrasound machine at 48 h following heart transplantation[31,32], as well as at 2, 4, and 7 days after exosome injection. M-mode echocardiography was performed simultaneously. Left ventricular fractional shortening (FS) and ejection fraction (EF) were measured in three cardiac cycles.

Histological and Morphological Examination

At 48 h following heart transplantation, rats were sacrificed by injection of 10% KCl (2 ml) via the femoral vein, and the hearts were rapidly collected. The hearts were fixed in 4% paraformaldehyde for 24 h, embedded in paraffin, and cut into 5-μm sections, followed by hematoxylin and eosin (H&E) staining. The left ventricular myocardium was examined under a light microscope.

Table 1. Flow Cytometry to Detect Expression of Surface Markers at 48 Hours.

		IDO-BMSCs exosome + DC	Vector-BMSCs exosome + DC	BMSCs exosome + DC	DC cell	*p*-value	IDO-BMSCs exosome + T-cell	Vector-BMSCs exosome + T-cell	BMSCs exosome + T-cell	T-cell	*p*-value	IDO-BMSCs exosome + DC + T-cell	Vector-BMSCs exosome + DC + T-cell	BMSCs exosome + DC + T-cell	DC + T-cell	*p*-value
CD40	mean	8.6[c,d]	9.4 [c,d]	15.5[a,b,d]	27.0[a,b,c]	<0.0001						9.2 [b,d]	12.6[a,d]	11.6[d]	15.2[a,b,c]	0.003
	SD	0.60	0.12	1.36	2.12							1.26	0.81	1.86	0.85	
CD86	mean	41.6[b,c,d]	86.1[a,d]	85.0[a,d]	65.4[a,b,c]	<0.0001						32.7[b,c,d]	43.1[a,c,d]	49.0[a,b,d]	36.3[a,b,c]	<0.0001
	SD	11.50	1.63	1.86	5.45							1.01	1.89	0.72	2.91	
CD80	mean	43.8[b,c]	70.6[a,d]	72.3[a,d]	50.9[b,c]	0.009						18.1[b,c,d]	37.5[a]	34.4[a]	35.3[a]	0.0001
	SD	0.81	3.06	5.04	16.63							5.35	1.55	0.47	0.95	
MHC-II	mean	56.4[b,c]	88.1[a]	91.0[a]	71.7	0.02						35.8[b,c,d]	44.4[a,d]	45.2[a,d]	54.1[a,b,c]	0.0002
	SD	18.02	8.34	4.47	9.29							1.55	3.71	3.18	0.80	
CD274	mean	95.3 [d]	93.3[d]	89.1[d]	49.6[a,b,c]	<0.0001						66.9[b,c,d]	59.7[a,c]	56.1[a,b,d]	59.4[a,c]	<0.0001
	SD	0.55	0.85	2.40	7.23							0.32	1.26	1.99	1.43	
CD45RA	mean	76.6 [b]	89.7[a,c,d]	71.9[b]	73.9[b]	0.005						20.5[b,c,d]	25.8[a,c]	29.6[a,b]	27.8[a]	<0.0001
	SD	1.15	2.42	8.41	1.72							1.47	0.62	1.68	0.40	
CD45RA	mean	47.7[b,c,d]	83.6[a,c,d]	70.9[b]	60.7[a,b]	0.0005						35.6[b]	43.4[a,c,d]	38.9[b]	37.1[b]	0.009
+CD45RB	SD	9.71	1.64	2.36	6.19							1.85	2.45	2.35	1.58	
OX62	mean	93.2 [b,d]	98.3[a,c]	94.0[b,d]	99.0[a,c]	0.0003						70.0[b,c,d]	78.7[a,c,d]	73.3[a,b,d]	89.1[a,b,c]	<0.0001
	SD	1.33	1.60	0.44	0.15							0.95	0.82	2.76	0.81	
Treg	mean						3.1[b,c,d]	2.2[a]	2.2[a]	1.7[a]	0.005	1.3[b,c,d]	0.3[a]	0.6[a]	0.7[a]	0.006
	SD						0.25	0.47	0.20	0.26		0.36	0.15	0.21	0.15	
CD3+CD4	mean						23.8[d]	25.6[c,d]	23.0[b,d]	30.5[a,b,c]	0.0002	4.7[d]	3.2[c,d]	5.4[b,d]	12.8[a,b,c]	<0.0001
	SD						1.27	0.67	1.56	0.78		1.12	0.20	0.49	1.66	
CD3+CD8	mean						4.8[c,d]	5.0[c,d]	6.0[a,b,d]	15.4[a,b,c]	<0.0001	1.0[b,c,d]	4.0 [a,c,d]	6.5[a,b,d]	18.5[a,b,c]	<0.0001
	SD						0.21	0.06	0.15	0.36		0.49	0.51	0.81	0.46	

[a,b,c,d] ($p < 0.05$) Significantly different from:
[a] IDO-BMSC-exosome + cell.
[b] Vector-BMSCs exosome + cell,
[c] BMSC exosome + cell.
[d] cell only.
BMSC: bone marrow mesenchymal stem cell; DC: dendritic cell; MHC: major histocompatibility complex; SD: standard deviation; Treg: regulatory T-cell.

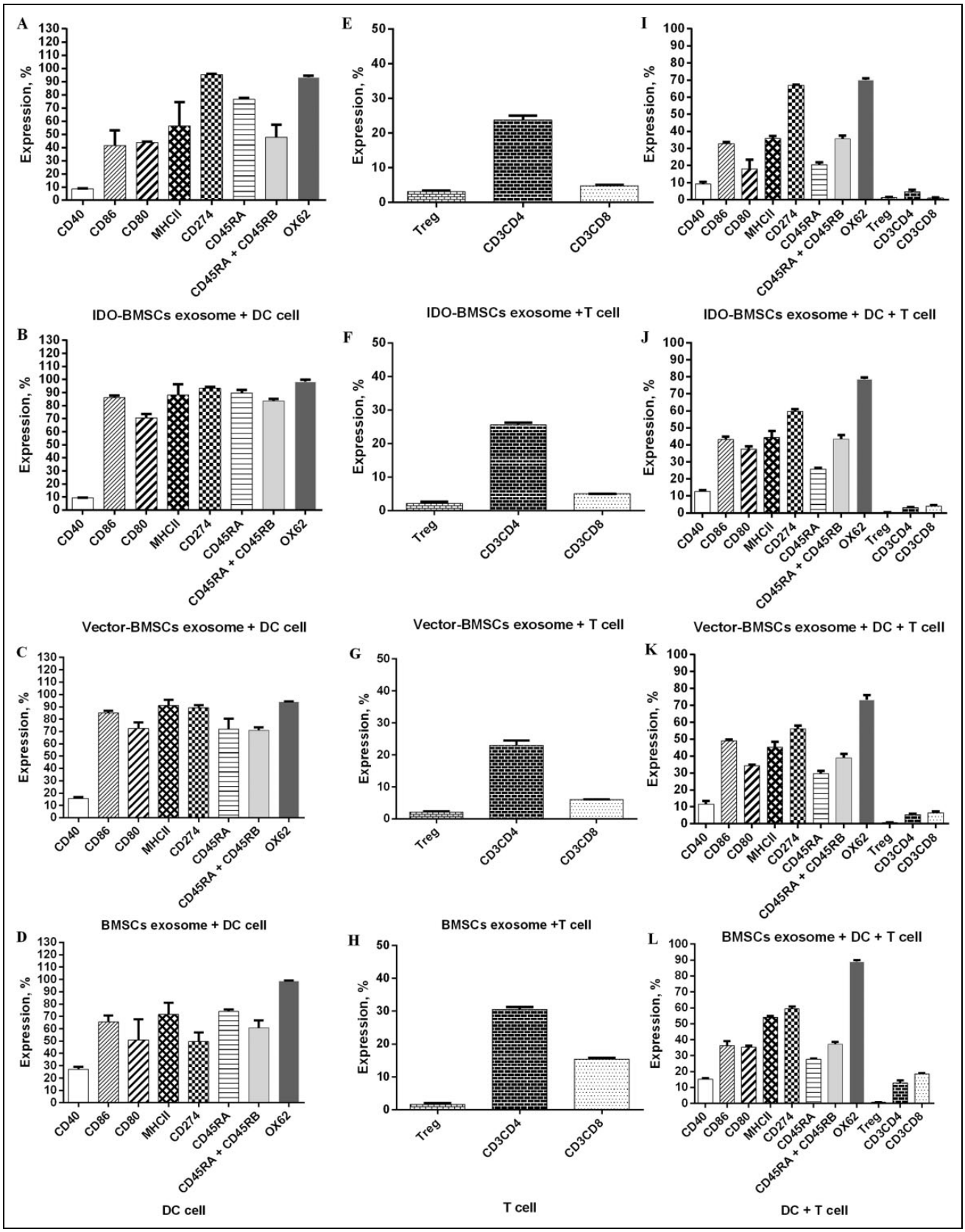

Figure 1. Surface marker expression at 48 hours (in-vitro flow cytometer experiments).

Table 2. Flow Cytometry to Detect Expression of Surface Markers at 72 Hours.

		IDO-BMSCs exosome + DC	Vector-BMSCs exosome + DC	BMSCs exosome + DC	DC cell	*p*-value	IDO-BMSCs exosome + T-cell	Vector-BMSCs exosome + T-cell	BMSCs exosome + T-cell	T-cell	*p*-value	IDO-BMSCs exosome + DC + T-cell	Vector-BMSCs exosome + DC + T-cell	BMSCs exosome + DC + T-cell	DC + T-cell	*p*-value
CD40	mean	6.8[c,d]	8.5[d]	10.9[a]	11.8[a,b]	0.02						9.0[b,c,d]	52.1[a]	53.5[a]	52.7[a]	<0.0001
	SD	1.99	1.95	0.40	1.40							0.44	0.26	1.30	0.64	
CD86	mean	12.1[b,c,d]	20.5[a,c,d]	17.5[a,b,d]	15.1[a,b,c]	<0.0001						6.1[b,c,d]	35.4[a, d]	35.3[a,d]	31.3[a,b,c]	<0.0001
	SD	0.78	0.56	1.21	0.10							0.45	1.22	1.04	0.29	
CD80	mean	4.1[b,c,d]	12.7[a]	16.3[a]	13.2[a]	0.0004						7.0[b,c,d]	14.1[a, c]	12.1[a,b,d]	13.1[a,c]	<0.0001
	SD	1.61	0.92	3.40	0.85							0.17	0.99	0.15	0.25	
MHC-II	mean	28.1[b,c]	51.7[a,c,d]	39.4[a,b,d]	31.9[b,c]	<0.0001						13.9[b,c,d]	34.7[a]	35.7[a]	31.5[a]	0.0002
	SD	2.88	1.68	4.20	2.19							0.52	2.19	6.38	1.33	
CD274	mean	75.6[d]	72.4[d]	72.2[d]	58.2[a,b,c]	<0.0001						31.0[b,c,d]	29.8[a, d]	28.8[a,d]	7.5[a,b,c]	<0.0001
	SD	2.53	1.62	2.33	2.57							0.12	1.12	0.32	0.15	
CD45RA	mean	21.8[c,d]	25.3[c,d]	41.8[a,b]	45.8[a, b]	<0.0001						40.0[b,c,d]	58.6[a, c]	51.1[a,b,d]	60.5[a, c]	<0.0001
	SD	1.40	1.33	4.27	0.46							1.33	0.61	0.72	1.21	
CD45RA	mean	10.6[b,c,d]	26.6[a,c,d]	21.0[a,b]	18.9[a,b]	0.0002						27.0[b,c,d]	54.8[a, d]	55.4[a,d]	47.9[a,b,c]	<0.0001
+CD45RB	SD	0.31	0.86	4.39	0.59							0.78	0.60	0.66	1.65	
OX62	mean	95.6[d]	97.5[d]	95.7[d]	92.5[a,b,c]	0.01						20.0[b,c,d]	54.7[a,c,d]	56.9[a,b,d]	64.7[a,b,c]	<0.0001
	SD	2.08	0.56	0.57	1.45							0.56	0.69	0.76	1.20	
Treg	mean						14.0[b,c,d]	0.5[a]	0.7[a]	0.7[a]	<0.0001	10.1[b,c,d]	0.4[a]	0.5[a]	0.4[a]	<0.0001
	SD						1.51	0.31	0.20	0.23		2.15	0.06	0.36	0.10	
CD3+CD4	mean						40.4[b,d]	36.0[a,c,d]	42.2[b,d]	51.4[a,b,c]	<0.0001	38.1[d]	36.3[c,d]	39.0[b,d]	62.6[a,b,c]	<0.0001
	SD						1.30	1.76	0.75	1.35		0.60	1.92	0.55	0.93	
CD3+CD8	mean						18.3[b]	13.4[a,c,d]	18.8[b]	19.0[b]	<0.0001	12.9[d]	13.3[d]	14.0[d]	18.7[a,b,c]	<0.0001
	SD						0.74	0.67	0.75	0.44		0.93	0.38	0.75	0.64	

[a,b,c,d] ($p < 0.05$) Significantly different from:
[a] IDO-BMSCs exosome + cell.
[b] Vector-BMSCs exosome + cell.
[c] BMSCs exosome + cell.
[d] Cell only.
BMSC: bone marrow mesenchymal stem cell; DC: dendritic cell; MHC: major histocompatibility complex; SD: standard deviation; Treg: regulatory T-cell.

Table 3. Flow Cytometry to Detect Expression of Surface Markers at 96 Hours.

		IDO-BMSCs exosome + DC	Vector-BMSCs exosome + DC	BMSCs exosome + DC	DC	*p*-value	IDO-BMSCs exosome + T-cell	Vector-BMSCs exosome + T-cell	BMSCs exosome + T-cell	T-cell	*p*-value	IDO-BMSCs exosome + DC + T-cell	Vector-BMSCs exosome + DC - + T-cell	BMSCs exosome + DC + T-cell	DC + T-cell	*p*-value
CD40	mean	$24.5^{b,c,d}$	$39.2^{a,c}$	$32.1^{a,b}$	34.2^{a}	0.006						$55.3^{b,c,d}$	$85.5^{a,b,d}$	$86.2^{a,d}$	$83.4^{a,b,c}$	<0.0001
	SD	0.82	5.87	3.23	2.07							0.40	0.75	0.42	0.31	
CD86	mean	$7.9^{b,d}$	$13.9^{a,c,d}$	$8.6^{b,d}$	$16.3^{a,b,c}$	<0.0001						10.0^{c}	11.3^{c}	$14.1^{a,b,d}$	10.8^{c}	0.0007
	SD	0.31	1.92	0.55	0.32							0.95	0.17	0.62	0.93	
CD80	mean	$12.1^{b,d}$	16.8^{a}	13.4^{d}	$19.8^{a,c}$	0.007						$2.4^{b,c,d}$	$3.9^{a,c,d}$	$5.7^{a,b,d}$	$6.9^{a,b,c}$	<0.0001
	SD	1.68	2.93	1.10	2.08							0.29	0.29	0.32	0.58	
MHC-II	mean	3.30^{d}	4.33^{d}	3.33^{d}	$48.6^{a,b,c}$	<0.0001						$34.2^{b,c,d}$	43.1^{a}	41.6^{a}	40.5^{a}	0.001
	SD	0.44	0.61	0.15	1.63							3.04	0.82	1.06	1.08	
CD274	mean	$34.2^{b,d}$	$13.6^{a,c,d}$	$32.8^{b,d}$	$8.2^{a,b,c}$	<0.0001						$54.0^{b,c}$	$47.8^{a,d}$	$46.3^{a,d}$	$52.6^{b,c}$	0.0007
	SD	2.77	1.67	0.25	0.38							1.14	2.19	1.01	1.53	
CD45RA	mean	50.8^{d}	52.1^{d}	54.5^{d}	$59.2^{a,b,c}$	0.004						$17.2^{b,c,d}$	$38.9^{a,c,d}$	$32.3^{a,b,d}$	$34.0^{a,b,c}$	<0.0001
	SD	0.45	0.90	3.13	2.30							0.66	0.55	0.80	0.91	
CD45RA	mean	$6.8^{b,d}$	$11.5^{a,c,d}$	$8.2^{b,d}$	$20.6^{a,b,c}$	<0.0001						$3.8^{b,c,d}$	$9.2^{a,c,d}$	$31.5^{a,b,d}$	$51.5^{a,b,c}$	<0.0001
+CD45RB	SD	0.62	1.46	0.26	1.72							0.50	0.40	1.78	4.61	
OX62	mean	$12.3^{b,c,d}$	$31.3^{a,d}$	$32.3^{a,d}$	$19.5^{a,b,c}$	<0.0001						$52.4^{b,c,d}$	$63.3^{a,c,d}$	$66.4^{a,b,d}$	$73.1^{a,b,c}$	<0.0001
	SD	3.00	4.10	1.10	2.25							1.57	0.55	0.72	0.44	
Treg	mean						4.9^{b}	3.1^{a}	3.8	4.5	0.12	1.9^{d}	1.1	1.2	0.6^{a}	0.10
	SD						0.87	1.31	0.58	0.32		0.87	0.21	0.15	0.55	
CD3+CD4	mean						$8.4^{c,d}$	$7.4^{c,d}$	$5.2^{a,b,d}$	$3.7^{a,b,c}$	0.0001	$6.7^{c,d}$	$8.0^{c,d}$	$11.0^{a,b,d}$	$16.3^{a,b,c}$	0.0003
	SD						1.07	0.71	0.21	0.50		1.55	2.11	1.07	1.23	
CD3+CD8	mean						$6.3^{b,c,d}$	11.9^{a}	11.4^{a}	13.3^{a}	0.02	$6.3^{c,d}$	6.9^{d}	$7.5^{a,d}$	$9.7^{a,b,c}$	0.0006
	SD						0.55	4.23	1.46	0.50		0.30	0.10	0.36	1.12	

[a,b,c,d] ($p < 0.05$) Significantly different from:
[a] IDO-BMSCs exosome + cell.
[b] Vector-BMSCs exosome + cell.
[c] BMSCs exosome + cell.
[d] Cell only.
BMSC: bone marrow mesenchymal stem cell; DC: dendritic cell; MHC: major histocompatibility complex; SD: standard deviation; Treg: regulatory T-cell.

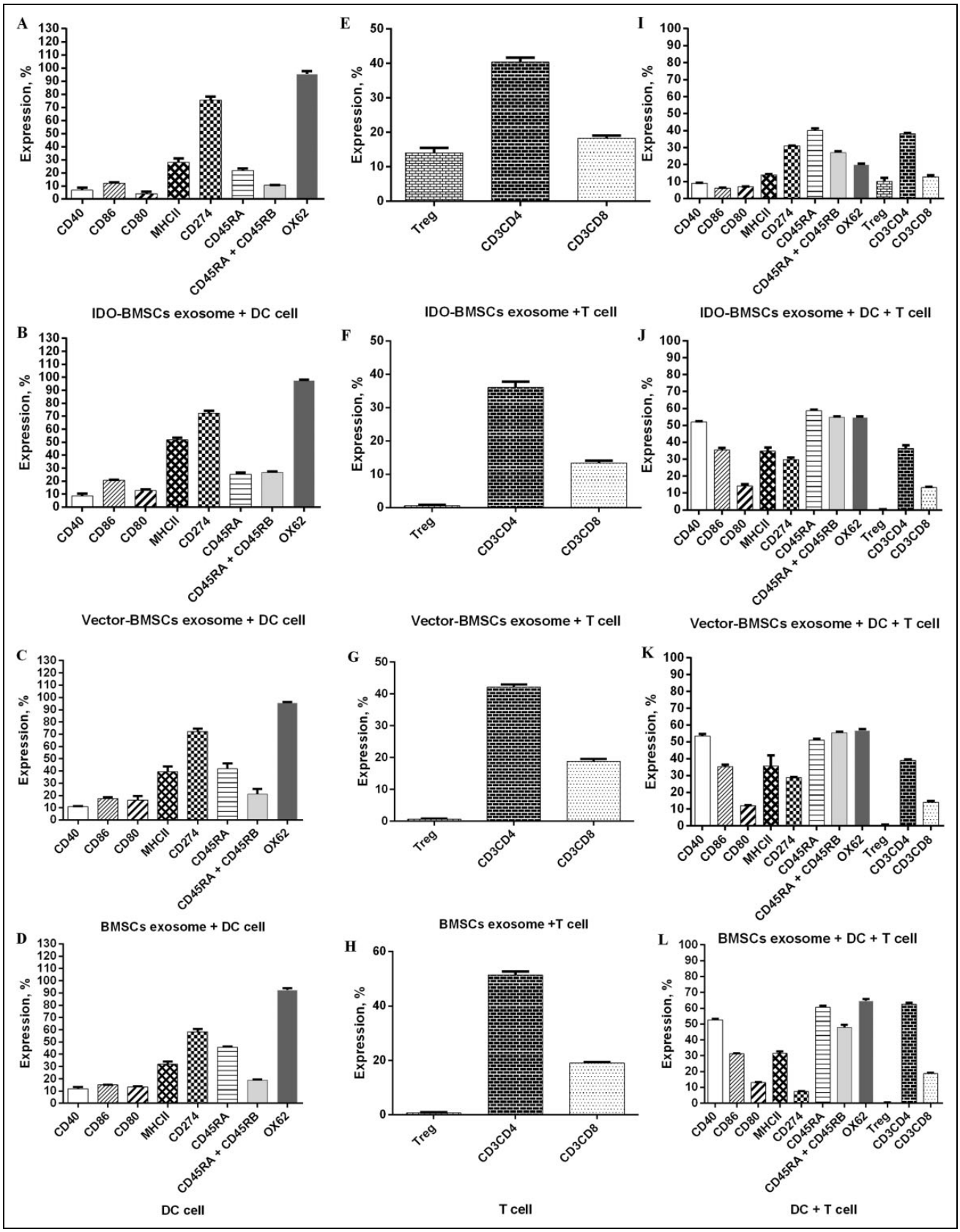

Figure 2. Surface marker expression at 72 hours (in-vitro flow cytometer experiments).

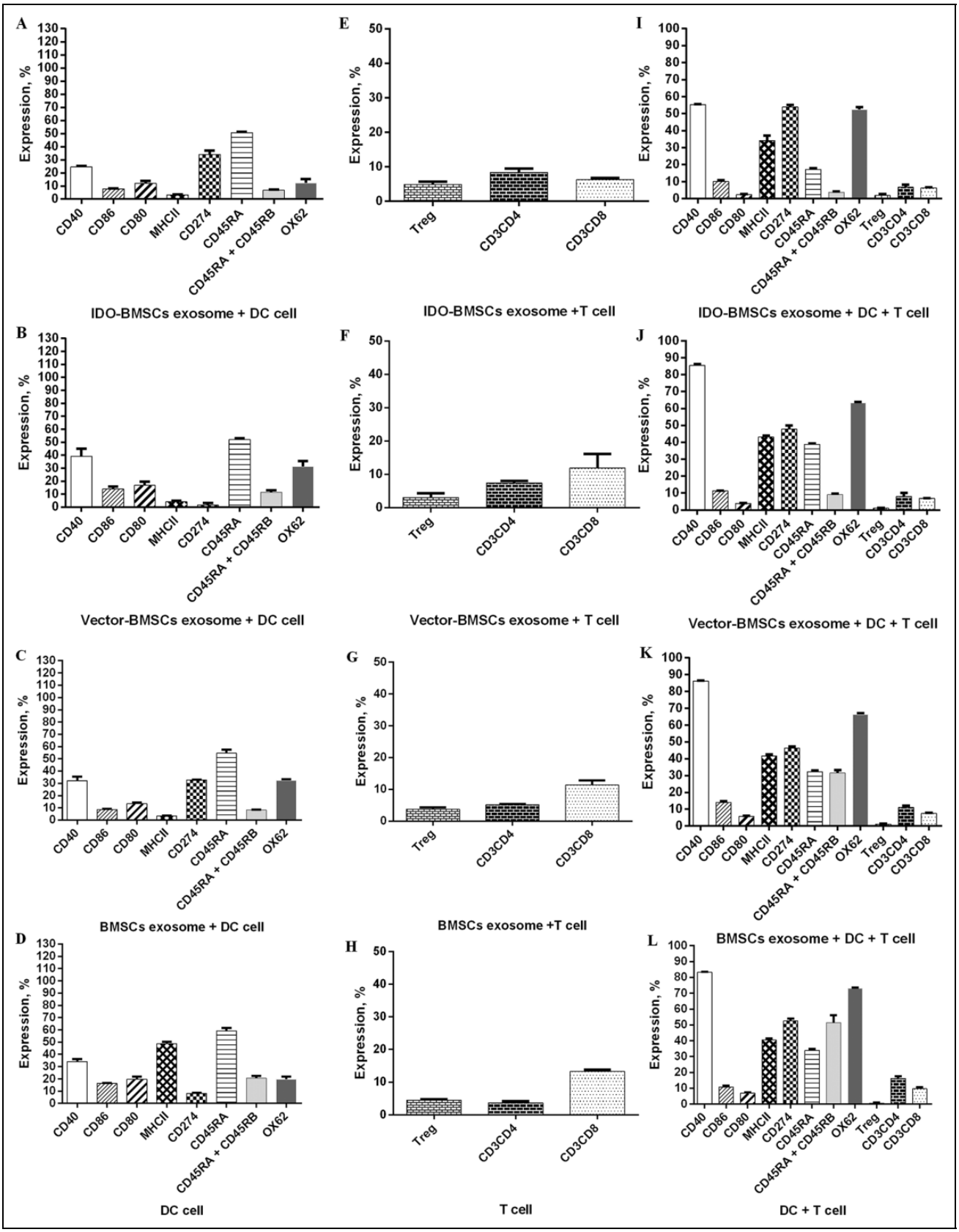

Figure 3. Surface marker expression at 96 hours (in-vitro flow cytometer experiments).

Table 4. Mean RT-PCR threshold (Ct) values of IDO-1 at 48, 72, 96 h (In-Vitro Experiments).

	48h		72h		96h		
	mean (SD)	*p*-value	mean (SD)	*p*-value	mean (SD)	*p*-value	*p*-value for trend
IDO-BMSC-exosome + DC	2.8 (0.34)[b,c,d]	<0.0001	3.7 (0.20)[b,c,d]	<0.0001	4.4 (0.13)[b,c,d]	<0.0001	<0.0001
Vector-BMSC-exosome + DC	0.02 (0.01)[a,d]		0.3 (0.12)[a,d]		0.2 (0.01)[a,d]		0.30
BMSC-exosome + DC	0.03 (0.01)[a,d]		0.3 (0.04)[a,d]		0.1 (0.01)[a,d]		0.61
DC	1.00 (0.00)[a,b,c]		1.0 (0.00)[a,b,c]		1.0 (0.00)[a,b,c]		–
IDO-BMSC-exosome + T-cell	1.3 (0.03)[d]	0.0009	1.4 (0.19)[d]	0.0101	1.6 (0.10)[d]	0.0081	0.03
Vector-BMSC-exosome + T-cell	1.4 (0.13)[d]		1.6 (0.16)[d]		1.5 (0.21)[d]		0.49
BMSC-exosome + T-cell	1.5 (0.12)[d]		1.6 (0.27)[d]		1.4 (0.22)[d]		0.67
T-cell	1.0 (0.00)[a,b,c]		1.0 (0.00)[a,b,c]		1.0 (0.22)[a,b,c]		–
IDO-BMSC-exosome + DC + T-cell	5.0 (0.54)[b,c,d]	<0.0001	6.4 (0.02)[b,c,d]	<0.0001	7.8 (0.14)[b,c,d]	<0.0001	<0.0001
Vector-BMSC-exosome + DC + T-cell	1.3 (0.23)[a]		1.6 (0.53)[a,c,d]		1.6 (0.06)[a,d]		0.30
BMSC-exosome + DC + T-cell	1.0 (0.16)[a]		1.0 (0.01)[a,b]		1.4 (0.22)[a,d]		0.05
DC + T-cell	1.0 (0.00)[a]		1.0 (0.00)[a,b]		1.0 (0.00)[a,b,c]		–

[a,b,c,d]($p < 0.05$) Significantly different from.
[a]IDO-BMSCs exosome+ cell.
[b]Vector-BMSCs exosome + cell.
[c]BMSCs exosome + cell.
[d]cell only.
BMSC: bone marrow mesenchymal stem cell; DC: dendritic cell; SD: standard deviation.

Agilent miRNA Chip Analysis

IDO1-BMSCs and control BMSCs were maintained in serum-containing medium containing DOX without exosomes for at least 48 h. Total RNAs that included small RNA fraction from the exosome pellet were isolated using SeraMir™ Exosome RNA Amplification Kit (System Biosciences) according to the manufacturer's instructions. miRNA was purified with the mirVana™ miRNA Isolation Kit (AM1561) following the manufacturer's instructions. Total RNA (200 ng) was labeled using the Agilent miRNA Complete Labeling and Hyb Kit (Richardson, Texas, USA). Agilent Feature Extraction (version 10.7) was used to analyze the images after hybridization, followed by data extraction. miRNAs were considered to be upregulated at a ratio of >1.2 and downregulated at a ratio of <0.83. Agilent GeneSpring software was used for the data normalization. GeneSpring was used for the analysis of intergroup difference.

Quantitative Proteomic Analysis

Exosomes from BMSCs were incubated with lysis buffer (8 M urea,1% Triton X-100, 65 mM dithiothreitol, 1% protease inhibitor, 3 μM trichostatin A, 50 mM nicotinamide, and 2 mM ethylenediaminetetraacetic acid), followed by sonication on ice. Samples were centrifuged at 4°C for 10 min at 12,000×*g*, the supernatant was incubated with 15% trichloroacetic acid (TCA) at 4°C for 2 h, and processed as previously described[33] for reverse-phase high-performance liquid chromatography (HPLC) with high pH (Agilent 300 Extend C18 column; 5 μm particles, 4.6 mm ID, 250 mm length). The peptides were further validated by liquid chromatography (LC)-mass spectroscopy (MS)/MS analysis using standard protocols[34].

Quantitative Analysis of Global Proteome in Red Tree

Quantitative global proteome analysis was performed after tandem mass tag (TMT)-labeled peptides were subjected to HPLC fractionation followed by high-resolution LC-MS/MS analysis. After identification of upregulated and downregulated proteins in each exosome group, intensive bioinformatic analysis (protein annotation, functional classification, functional enrichment, and functional enrichment-based cluster analysis) was carried out to annotate quantifiable targets.

Small RNA Library Preparation

Total exosomal RNA (3 μg) was used as input material for the small RNA library. Sequencing libraries were generated using NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, Ipswich, MA, USA) following the manufacturer's recommendations and index codes were added to attribute sequences to each sample. The library quality was assessed on the Agilent Bioanalyzer 2100 system using DNA high sensitivity chips.

Clustering of the index-coded samples was performed on a cBot Cluster Generation System using TruSeq SR Cluster Kit v3-cBot-HS (Illumina, San Diego, CA, USA) according to the manufacturer's instructions. After cluster generation, the library preparations were sequenced on an Illumina Hiseq 2500 platform and 50 bp single-end reads were generated.

Statistical Analysis

Mean and standardized deviation were summarized for all numerical variables. A one-way analysis of variance was used

Table 5. Expression Levels of IL-1α, IL-4, IL-1β and IL-2 at 48, 72, and 96 h (In-Vitro Experiments).

	IL-1α	IL-4	IL-1β	IL-2
48 h				
IDO-BMSC-exosome + DC + T-cell	57.3 (2.56)[b,c,d]	2.7 (0.50)[b,c,d]	57.9 (1.91)[b,c,d]	53.7 (1.32)[b,c,d]
Vector-BMSC-exosome + DC+T-cell	93.0 (1.72)[a,d]	10.4 (0.24)[a,c,d]	78.5 (1.87)[a,d]	63.2 (2.81)[a,d]
BMSC-exosome + DC + T-cell	96.3 (3.45)[a,d]	11.6 (0.42)[a,b]	76.8 (2.50)[a,d]	64.5 (3.91)[a,d]
DC + T-cell	225.5 (0.70)[a,b,c]	12.2 (0.20)[a,b]	84.7 (1.30)[a,b,c]	87.6 (3.67)[a,b,c]
p-value	<0.0001	<0.0001	<0.0001	<0.0001
72 h				
IDO-BMSC-exosome + DC + T-cell	55.0 (1.65)[b,c,d]	2.3 (0.22)[b,c,d]	55.9 (1.39)[b,c,d]	52.4 (1.76)[b,c,d]
Vector-BMSC-exosome + DC + T-cell	91.7 (1.90)[a,d]	9.4 (0.44)[a,d]	77.6 (2.21)[a,d]	62.0 (2.91)[a,d]
BMSC-exosome + DC + T-cell	95.3 (3.55)[a,d]	10.3 (0.33)[a,d]	75.0 (2.81)[a,d]	63.2 (3.92)[a,d]
DC + T-cell	252.6 (29.36)[a,b,c]	17.1 (2.44)[a,b,c]	88.3 (1.17)[a,b,c]	89.2 (2.75)[a,b,c]
p-value	<0.0001	<0.0001	<0.0001	<0.0001
96 h				
IDO-BMSC-exosome + DC + T-cell	53.9 (1.42)[b,c,d]	2.2 (0.21)[b,c,d]	55.1 (1.58)[b,c,d]	51.2 (1.72)[b,c,d]
Vector-BMSC-exosome + DC + T-cell	130.9 (2.42)[a,d]	8.5 (0.37)[a,d]	77.1 (2.07)[a,c,d]	61.2 (2.64)[a,d]
BMSC-exosome + DC + T-cell	132.1 (9.48)[a,d]	9.5 (0.39)[a,d]	73.8 (1.88)[a,b,d]	62.2 (4.03)[a,d]
DC + T-cell	291.4 (0.96)[a,b,c]	18.2 (2.65)[a,b,c]	90.7 (0.18)[a,b,c]	90.2 (1.00)[a,b,c]
p-value	<0.0001	<0.0001	<0.0001	<0.0001

BMSC: bone marrow mesenchymal stem cell; DC: dendritic cell; IL: interleukin; SD: standard deviation.

Table 6. Expression Levels of IL-10, IFNγ and IL-18 at 48, 72, 96 h (In-Vitro Experiments).

	IL-10	IFNγ	IL-18
48 h			
IDO-BMSC-exosome + DC + T-cell	1149.0 (33.2)[b,c,d]	10.6 (0.37)[b,c,d]	62.5 (2.13)[b,c,d]
Vector-BMSC-exosome + DC + T-cell	820.3 (4.25)[a,c,d]	15.5 (0.40)[a,d]	75.9 (2.59)[a,d]
BMSC-exosome + DC + T-cell	881.6 (14.35)[a,b,d]	15.5 (0.30)[a,d]	77.3 (1.23)[a,d]
DC + T-cell	348.4 (2.99)[a,b,c]	18.2 (1.44)[a,b,c]	90.0 (0.44)[a,b,c]
p-value	<0.0001	<0.0001	<0.0001
72 h			
IDO-BMSC-exosome + DC + T-cell	1254.7 (35.70)[b,c,d]	9.7 (0.19)[b,c,d]	60.3 (1.79)[b,c,d]
Vector-BMSC-exosome + DC + T-cell	864.0 (26.51)[a,d]	14.4 (0.22)[a,d]	74.2 (1.28)[a,d]
BMSC-exosome + DC + T-cell	896.3 (15.31)[a,d]	14.5 (0.27)[a,d]	76.5 (1.98)[a,d]
DC + T-cell	313.8 (3.02)[a,b,c]	21.7 (1.05)[a,b,c]	93.0 (0.70)[a,b,c]
p-value	<0.0001	<0.0001	<0.0001
96 h			
IDO-BMSC-exosome + DC + T-cell	1266.2 (47.20)[b,c,d]	9.3 (0.15)[b,c,d]	59.5 (1.86)[b,c,d]
Vector-BMSC-exosome + DC + T-cell	870.4 (22.34)[a,d]	11.1 (0.29)[a,c,d]	73.2 (1.58)[a,d]
BMSC-exosome + DC + T-cell	902.5 (18.90)[a,d]	13.7 (0.23)[a,b,d]	75.4 (2.02)[a,d]
DC + T-cell	304.3 (2.42)[a,b,c]	28.1 (0.72)[a,b,c]	95.3 (0.27)[a,b,c]
p-value	<0.0001	<0.0001	<0.0001

§ Value were summarized as mean (SD); Unit: pg/ml.
[a,b,c,d]Significantly different from.
[a]IDO-BMSCs exosome + cell.
[b]Vector-BMSCs exosome + cell.
[c]BMSCs exosome + cell.
[d]cell only.
BMSC: bone marrow mesenchymal stem cell; DC: dendritic cell; IDO: indoleamine 2,3-dioxygenase; IFN: interferon; IL: interleukin; SD: standard deviation.

to test the differences in means between groups. Multiple comparisons were performed by a post-hoc test with Fisher's least significant difference. All statistical significance including pair-wise comparison tests were defined by the two-tailed test and $p < 0.05$. All analyses were conducted using SAS version 9.4 (SAS Institute Inc., Cary, NC, USA).

Results

Flow Cytometry to Detect Expression of Cell Surface Markers in the In-Vitro Model

Flow cytometry was used to determine the expression of cell surface markers in different groups of co-cultured BMSC-

Table 7. Expression Levels of TGFβ1, TGFβ2, TGFβ3 at 48, 72, and 96 h (In-Vitro Experiments).

	TGFβ1	TGFβ2	TGFβ3
48 h			
IDO-BMSC-exosome + DC + T cell	1244.0 (25.71)[b,c,d]	240.9 (5.67)[b,c,d]	6.6 (0.28)[b,c,d]
Vector-BMSC-exosome + DC + T cell	1169.0 (30.45)[a,d]	208.2 (0.80)[a,d]	5.4 (0.28)[a,d]
BMSC-exosome + DC + T cell	1165.1 (31.81)[a,d]	200.2 (5.90)[a,d]	5.5 (0.30)[a,d]
DC + T cell	1044.0 (15.87)[a,b,c]	138.3 (2.71)[a,b,c]	4.4 (0.30)[a,b,c]
p-value	0.0001	<0.0001	0.0001
72 h			
IDO-BMSC-exosome + DC + T cell	1313.4 (4.28)[b,c,d]	244.7 (1.86)[b,c,d]	6.8 (0.05)[b,c,d]
Vector-BMSC-exosome + DC + T cell	1178.7 (30.17)[a,d]	209.8 (1.07)[a,d]	5.8 (0.06)[a,d]
BMSC-exosome + DC + T cell	1172.4 (32.70)[a,d]	202.7 (7.25)[a,d]	5.7 (0.19)[a,d]
DC + T cell	837.4 (36.93)[a,b,c]	127.9 (4.27)[a,b,c]	4.3 (0.29)[a,b,c]
p-value	<0.0001	<0.0001	<0.0001
96 h			
IDO-BMSC-exosome + DC + T cell	1325.4 (6.68)[b,c,d]	247.4 (1.62)[b,c,d]	6.9 (0.06)[b,c,d]
Vector-BMSC-exosome + DC + T cell	1243.0 (3.21)[a,c,d]	210.8 (0.32)[a,d]	6.0 (0.02)[a,d]
BMSC-exosome + DC + T cell	1179.3 (28.85)[a,b,d]	209.7 (12.32)[a,d]	5.9 (0.14)[a,d]
DC + T cell	749.9 (34.53)[a,b,c]	118.2 (2.08)[a,b,c]	4.2 (0.25)[a,b,c]
p-value	<0.0001	<0.0001	<0.0001

§ Value were summarized as mean (std); Unit: pg/ml.
[a,b,c,d]Significantly different from.
[a]IDO-BMSCs exosome + cell.
[b]Vector-BMSCs exosome + cell.
[c]BMSCs exosome + cell.
[d]cell only.
BMSC: bone marrow mesenchymal stem cell; DC: dendritic cell; IDO: indoleamine 2,3-dioxygenase; IFN: interferon; IL: interleukin; SD: standard deviation.

exosomes. After 48 h, and 72 h of co-culture, IDO-BMSC-exosomes co-cultured with DCs, and IDO-BMSC-exosomes co-cultured with DCs + T-cells had significantly lower expression of CD40, CD86, CD80, MHC-II, and CD45RA + CD45RB compared with (1) vector-BMSC-exosomes or (2) BMSC-exosomes co-cultured with DCs or DCs + T-cells (all $p<0.05$). In contrast, IDO-BMSC-exosomes co-cultured with DCs, and IDO-BMSC-exosomes co-cultured with DCs + T-cells had the highest expression of CD274 (all $p < 0.0001$) compared with the other groups (Table 1, Fig. 1). IDO-BMSC-exosomes co-cultured with T-cells and IDO-BMSC-exosomes co-cultured with DCs + T-cells for 48 h and 72 h had a significantly higher proportion of Tregs (both $p < 0.05$), and a significantly lower expression of CD3 + CD8 compared with the other groups after 48 h, 72 h, and 96 h of co-culture (all $p < 0.0001$; Tables 1–3, Figs. 1–3).

RT-PCR to Detect IDO1 Expression

The expression of IDO1 was determined in the different co-cultured groups at 48, 72 and 96 h. IDO1 expression was significantly higher in IDO-BMSC-exosomes co-cultured with DCs + T-cells compared with the other groups ($p < 0.0001$) and showed a time-dependent increase. The mean RT-PCR threshold (Ct) values showed an increasing pattern with time in the IDO-BMSC-exosomes co-cultured with DCs, as well as in IDO-BMSC-exosomes co-cultured with DCs + T-cells (p-value for trend <0.0001; Table 4).

Quantitation of Cytokine Levels

The supernatant was collected from the different co-culture groups at 48, 72 and 96 h and processed for liquid microarray analysis to determine cytokine levels. IDO-BMSC-exosomes co-cultured with DCs + T-cells for 48 h, 72 h, and 96 h had significantly lower mean levels of IL-1α, IL-4, IL-1β, IL-2, IFNγ, and IL-18 compared with the other groups ($p < 0.05$). In contrast, IDO-BMSC-exosomes co-cultured with DCs + T-cells for 48 h, 72 h, and 96 h had significantly higher levels of IL-10, TGFβ1, TGFβ2, and TGFβ3 compared with the other groups (Tables 5–7; all $p < 0.05$).

Evaluation of Heart Function in Heart Transplant Model

Heart function was evaluated in the rat abdominal heterotopic heart transplantation model. Transplanted rats were injected with Dir-stained IDO-BMSC-secreted exosomes, empty vector-BMSC-secreted exosomes, or BMSC-secreted exosomes 48 h after transplantation. Animal in-vivo imaging was used to detect the cardiac local fluorescence intensity after intervention 2, 4 and 7 days. EF and FS were determined by color Doppler examination after 2, 4 or 7 days of exosome treatment. Untreated animals, and animals treated with mycophenolate mofetil were used as controls. Animals treated

Table 8. Evaluation of Heart Function in Rat Heart Transplantation Model.

	Difference between pre- and post-test	
	EF, %	FS, %
2 days		
IDO-exosome	16.8 (1.51)[b,c,d,e]	14.8 (1.49)[b,c,d,e]
Vector-exosome	3.8 (1.43)[a,e]	2.4 (0.31)[a,e]
BMSCs exosome	4.5 (1.08)[a,e]	4.3 (0.25)[a,e]
Mycophenolate mofetil	4.4 (0.35)[a,e]	5.0 (0.26)[a,e]
Untreated	−33.0 (11.13)[a,b,c,d]	−16.7 (5.43)[a,b,c,d]
p-value	<0.0001	<0.0001
4 days		
IDO-exosome	19.6 (7.10)[b,c,d,e]	11.6 (3.77)[b,c,d,e]
Vector-exosome	7.8 (7.24)[a,e]	5.0 (4.39)[a,e]
BMSCs exosome	7.7 (2.62)[a,e]	5.3 (1.67)[a,e]
Mycophenolate mofetil	4.3 (2.22)[a,e]	3.3 (0.88)[a,e]
Untreated	−8.3 (4.75)[a,b,c,d]	−5.6 (3.13)[a,b,c,d]
p-value	0.001	0.0007
7 days		
IDO-exosome	14.3 (1.98)[b,c,d,e]	12.3 (1.82)[b,c,d,e]
Vector-exosome	4.5 (1.65)[a,d,e]	2.2 (1.17)[a,d,e]
BMSCs exosome	4.4 (1.98)[a,d,e]	1.9 (1.31)[a,d,e]
Mycophenolate mofetil	−16.9 (10.23)[a,b,c,e]	−10.6 (6.47)[a,b,c,e]
Untreated	−47.1 (3.23)[a,b,c,d]	−27.4 (2.87)[a,b,c,d]
p-value	<0.0001	<0.0001

§ Value were summarized as mean (std).
[a,b,c,d,e] Significantly different from.
[a]IDO-exosome.
[b]Vector-exosome.
[c]BMSC-exosomes.
[d]mycophenolate mofetil.
[e] untreated.
BMSC: bone marrow mesenchymal stem cell; EF: ejection fraction; FS: fractional shortening; IDO: indoleamine 2,3-dioxygenase.

with IDO-BMSC-secreted exosomes had a significantly higher EF and FS on Days 2, 4 and 7 compared with the other groups (all $p < 0.05$; Table 8). Animal in-vivo imaging was used to detect the cardiac local fluorescence intensity after 2, 4 or 7 days of treatment. Animals treated with IDO-BMSC-secreted exosomes showed the highest average fluorescence intensity at each time point compared with the other groups (all $p < 0.0001$; Table 9, Fig. 4).

Flow Cytometry to Detect Expression of Cell Surface Markers in the In-Vivo Model

Spleens from transplanted rats injected with the different BMSC-exosome groups were processed for flow cytometry to evaluate expression of surface markers. The IDO-BMSC-exosome group had significantly lower expression of the CD40, CD86, CD80, MHC-II, CD45RA and CD45RA+CD45RB, but a significantly higher expression of CD274 and a higher proportion of Tregs compared with the other groups after 48 h, 72 h, and 96 h of treatment (all $p < 0.001$; Tables 10–12, Figs. 5–7).

Quantitation of Cytokine Levels for the In-Vivo Model

Liquid microarray analysis to evaluate serum cytokine levels showed that the IDO-BMSC-exosome group had significantly lower levels of IL-1α, IL-4, IL-1β, IL-2, IFNγ, and IL-18 on days 2, 4, and 7 after treatment compared with the other groups, and the levels of these cytokines tended to decrease over time. However, the IDO-BMSC-exosome group had significantly higher levels of IL-10, TGFβ1, TGFβ2, and TGFβ3 on days 2, 4, and 7 after treatment compared with the other groups, and the levels of these cytokines tended to increase over time (all $p < 0.0001$; Tables 13–15.

Histopathology

After 3 days from the establishment of the rat heart transplantation model, the animals were treated with the different groups of exosomes. The hearts were harvested after 2, 4, and 7 days of treatment for the preparation of paraffin sections and H&E staining. Animals injected with IDO-BMSCs exosomes exhibited a significantly lower number of infiltrated inflammatory cells compared with the other groups at all time points of examination (Figs. 8–10).

Bioinformatics

We analyzed exosome proteins from IDO-BMSC-exosomes, empty vector-BMSC-exosomes (NC), and BMSC-exosomes. We identified a total of 1392 proteins, of which 1158 proteins were quantified. When the threshold of fold change was defined as 1.2 and p-value <0.05 in a Student's t test was used as a criterion, we found that 288 proteins were upregulated, and 90 proteins were downregulated in IDO1-BMSC-exosomes compared with NC-BMSC-exosomes. On the basis of these findings, the quantified proteins were further subjected to systemic bioinformatics analyses including: (1) protein annotation, (2) functional classification, (3) functional enrichment, and (4) clustering analysis based on functional enrichment.

The IDO-BMSC/NC comparison represented the proteins meeting condition 1 and condition 2. For condition 1, differentially expressed proteins in NC/BMSC served as the background, and proteins in which the changes in the IDO-BMSC/BMSC and IDO-BMSC/NC were similar to those observed in the NC/BMSC comparison were excluded. For condition 2, proteins in which the changes in the IDO-BMSC/BMSC comparison were different from those in the IDO-BMSC/NC comparison were excluded. The ratio referred to the ratio of the value in the IDO-BMSC/BMSC comparison to that of the IDO-BMSC/NC comparison. The p-value referred to the p-value in the IDO-BMSC/NC comparison.

Proteins meeting condition 1 and condition 2 were further analyzed with the threshold of fold change set to1.2. The top 20 proteins which were upregulated or downregulated (Supplemental Table S5) were subjected to Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis, and immune-related proteins (Supplemental

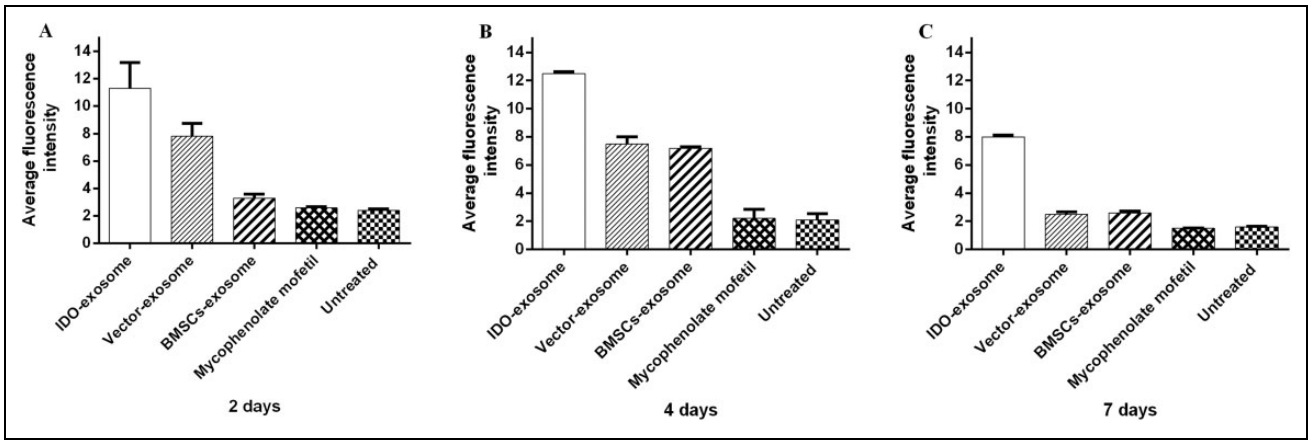

Figure 4. Average fluorescence intensity of transplanted heart in rat model, unit: (p/s/cm^2) × 107.

Table 9. In-Vivo Imaging of Transplanted Heart in Rat Model.

	Average fluorescence intensity (p/s/cm^2) × 10^7		
	2 days	4 days	7 days
Abdominal			
IDO-exosome	11.3 (1.88)[b,c,d,e]	12.5 (0.15)[b,c,d,e]	8.0 (0.13)[b,c,d,e]
Vector-exosome	7.8 (0.96)[a,c,d,e]	7.5 (0.50)[a,d,e]	2.5 (0.17)[a,d,e]
BMSCs exosome	3.3 (0.30)[a,b]	7.2 (0.09)[a,d,e]	2.6 (0.12)[a,d,e]
Mycophenolate mofetil	2.6 (0.07)[a,b]	2.2 (0.65)[a,b,c]	1.5 (0.03)[a,b,c]
Untreated	2.4 (0.11)[a,b]	2.1 (0.44)[a,b,c]	1.6 (0.04)[a,b,c]
p-value	<0.0001	<0.0001	<0.0001

§ Value were summarized as mean (std).
[a,b,c,d,e]Significantly different from.
[a]IDO-exosome.
[b]Vector-exosome.
[c]BMSC-exosomes.
[d]mycophenolate mofetil.
[e]untreated.
BMSC: bone marrow mesenchymal stem cell; IDO: indoleamine 2,3-dioxygenase.

Table S6) were selected for further analysis. Our results showed that FHL-1 was a key protein related to immunity in IDO-BMSC-exosomes.

Functional Classification of Differentially Quantified Proteins

According to the Gene Ontology (GO) annotation information of identified proteins (Supplemental Fig. S1, Table S3 and S4), we calculated the number of differentially expressed proteins in each GO term of level 2 (IDO-BMSC/NC; Supplemental Table S1 and S2). The GO-based enrichment analysis of upregulated and downregulated proteins is shown in Supplemental Fig. S2. The pathway obtained from KEGG pathway enrichment, and the KEGG-based enrichment analysis of upregulated and downregulated proteins are shown in Supplemental Figs S3 and S4.

Functional Enrichment-Based Clustering for Protein Groups

Hot plots were delineated according to the Pearson's correlation coefficient, which was used to evaluate the relationship between two groups. A coefficient close to −1 was defined as a negative correlation; a coefficient close to 1 was defined as a positive correlation; when the coefficient was close to 0, no relationship was shown. In Supplemental Fig. S5, red represents a coefficient of 1; green represents a coefficient of −1; white represents a coefficient of 0 (Supplemental Fig. S5).

Identification of Immune-Related microRNAs in IDO-BMSC-Exosomes

Our study employed small RNA sequencing to detect the immune-related microRNAs in IDO-BMSC-exosomes.

Table 10. Surface Marker Expression at 48 Hours (In-Vivo Experiments).

		CD40	CD86	CD80	MHC-II	CD274	CD45RA	CD45RA+CD45RB	Treg
IDO-BMSCs exosome	mean	2.1[b,c,d,e]	6.4[b,c,d,e]	5.2[b,c,e]	18.9[b,c,d,e,f]	11.6[b,c,d,e,f]	37.2[b,c,d,e,f]	30.1[b,c,d,e,f]	5.6[c,d,e,f]
	SD	0.12	0.61	0.40	0.23	0.76	0.95	0.31	0.31
Vector-BMSCs exosome	mean	4.0[a,e]	9.0[a,f]	9.2[a,f]	47.5[a,c,d,e,f]	6.9[a,d]	55.8[a,d,e,f]	35.3[a,d,e,f]	4.9[d,e]
	SD	1.18	0.25	0.74	0.42	0.75	0.25	0.95	0.32
BMSCs exosome	mean	4.2[a,e,f]	8.7[a,f]	8.9[a,e,f]	42.5[a,b,d,e,f]	6.8[a,d]	55.4[a,d,e,f]	34.5[a,e,f]	4.8[a,d,e]
	SD	0.98	0.44	0.81	1.76	0.45	0.55	1.10	0.38
Mycophenolate mofetil	mean	4.4[a,e,f]	8.4[a,f]	7.5[e]	30.9[a,b,c,e,f]	8.2[a,b,c,e]	43.2[a,b,c,e,f]	32.9[a,b,e,f]	3.6[a,b,c,f]
	SD	0.15	1.11	2.19	1.33	0.81	0.29	1.41	0.35
Untreated	mean	8.5[a,b,c,d,f]	9.0[a,f]	11.7[a,c,d,f]	33.5[a,b,c,d,f]	6.3[a,d,f]	54.1[a,b,c,d,f]	40.8[a,b,c,d,f]	2.9[a,b,c,f]
	SD	1.72	0.21	2.40	0.74	0.46	0.15	0.91	0.55
Normal	mean	2.5[d,e]	7.4[b,c,d,e]	5.9[b,c,e]	1.7[a,b,c,d,e]	7.5[a,e]	49.0[a,b,c,d,e]	26.4[a,b,c,d,e]	4.4[a,d,e]
	SD	0.26	0.21	0.35	0.47	0.17	0.81	0.61	0.46
	p-value	<0.0001	0.0006	0.001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[a,b,c,d,e,f] Significantly different from
[a]IDO-BMSCs exosome,
[b]Vector-BMSCs exosome,
[c]BMSCs exosome,
[d]mycophenolate mofetil,
[e]untreated,
[f]normal.
BMSC: bone marrow mesenchymal stem cell; IDO: indoleamine 2,3-dioxygenase; MHC: major histocompatibility complex; SD: standard deviation; Treg: regulatory T-cell.

Table 11. Surface Marker Expression at 72 Hours (In-Vivo Experiments).

		CD40	CD86	CD80	MHC-II	CD274	CD45RA	CD45RA + CD45RB	Treg
IDO-BMSCs exosome	mean	2.9[b,c,e,f]	6.4[b,c,d,e,f]	5.2[d,e,f]	25.3[b,c,d,e,f]	11.6[b,c,d,e,f]	35.7[b,e,f]	31.8[b,d,e,f]	6.4[b,c,d,e,f]
	SD	0.10	0.25	0.25	0.31	0.55	5.24	0.99	0.76
Vector-BMSCs exosome	mean	4.2[a,d,e,f]	10.4[a,d,e,f]	5.3[d,e,f]	42.8[a,d,e,f]	7.3[a,d,e,f]	40.0[a,e,f]	33.7[a,c,d,e,f]	4.3[a]
	SD	0.49	0.26	0.10	1.21	0.38	0.84	1.21	0.67
BMSCs exosome	mean	4.3[a,d,e,f]	10.9[a,d,e,f]	5.5[d,e,f]	43.9[a,d,e,f]	7.7[a,e,f]	38.2[e,f]	31.5[b,d,e,f]	4.1[a]
	SD	0.51	0.44	0.40	0.78	0.29	0.25	0.58	0.60
Mycophenolate mofetil	mean	3.1[b,c,e,f]	7.3[a,b,c,e]	7.6[a,b,c,e,f]	27.3[a,b,c,e,f]	8.5[a,b,e,f]	38.5[e,f]	38.4[a,b,c,e,f]	4.3[a]
	SD	0.06	0.10	0.35	1.30	0.21	0.76	0.35	0.47
Untreated	mean	5.9[a,b,c,d,f]	21.2[a,b,c,d,f]	8.2[a,b,c,d,f]	35.6[a,b,c,d,f]	5.8[a,b,c,d]	49.7[a,b,c,d]	48.0[a,b,c,d,f]	4.1[a]
	SD	0.26	1.01	0.10	0.70	0.65	0.72	1.26	0.21
Normal	mean	2.1[a,b,c,d,e]	7.9[a,b,c,e]	2.5[a,b,c,d,e]	30.2[a,b,c,d,e]	5.9[a,b,c,d]	48.0[a,b,c,d]	43.5[a,b,c,d,e]	3.5[a]
	SD	0.21	0.35	0.31	0.36	0.21	0.71	0.81	0.21
	p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0005

[a,b,c,d,e,f] Significantly different from
[a]IDO-BMSCs exosome,
[b]Vector-BMSCs exosome,
[c]BMSCs exosome,
[d]mycophenolate mofetil,
[e]untreated,
[f]normal.
BMSC: bone marrow mesenchymal stem cell; IDO: indoleamine 2,3-dioxygenase; MHC: major histocompatibility complex; SD: standard deviation; Treg: regulatory T-cell.

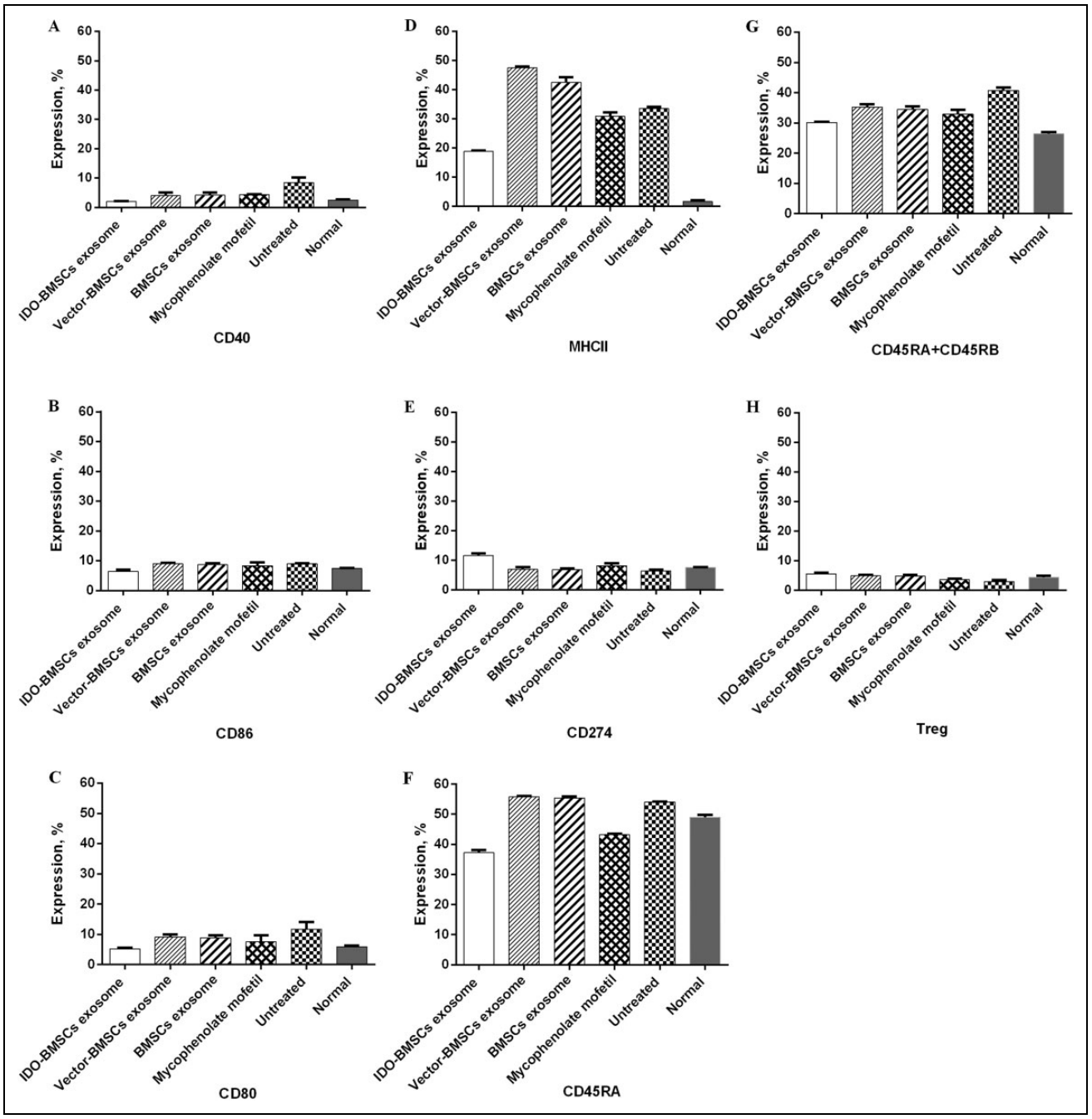

Figure 5. Surface marker expression at 48 hours (in-vivo experiments).

Differences in microRNAs meeting condition 1 and condition 2 as above described between IDO/BMSCs, between IDO/NC and between BMSCs/NC were determined according to the following criteria: a significant difference was defined if FC $\geq$ 1.5 or $\leq$0.67, and $p \leq 0.05$. The top 20 key microRNAs which were upregulated or downregulated in IDO/BMSCs were subjected to KEGG and GO enrichment (Supplemental Table S7), and the microRNAs related to immunity were further analyzed. Results showed miR-540-3p was a key microRNA which was upregulated, and miR-338-5p was a key microRNA which was downregulated (Supplemental Table S8).

Discussion

In this study, we investigated the molecular mechanisms of immunosuppression mediated by exosomes derived from IDO1-overexpressing BMSCs. We established a rat heart transplantation model to investigate immune and functional changes in transplanted animals treated with exosomes

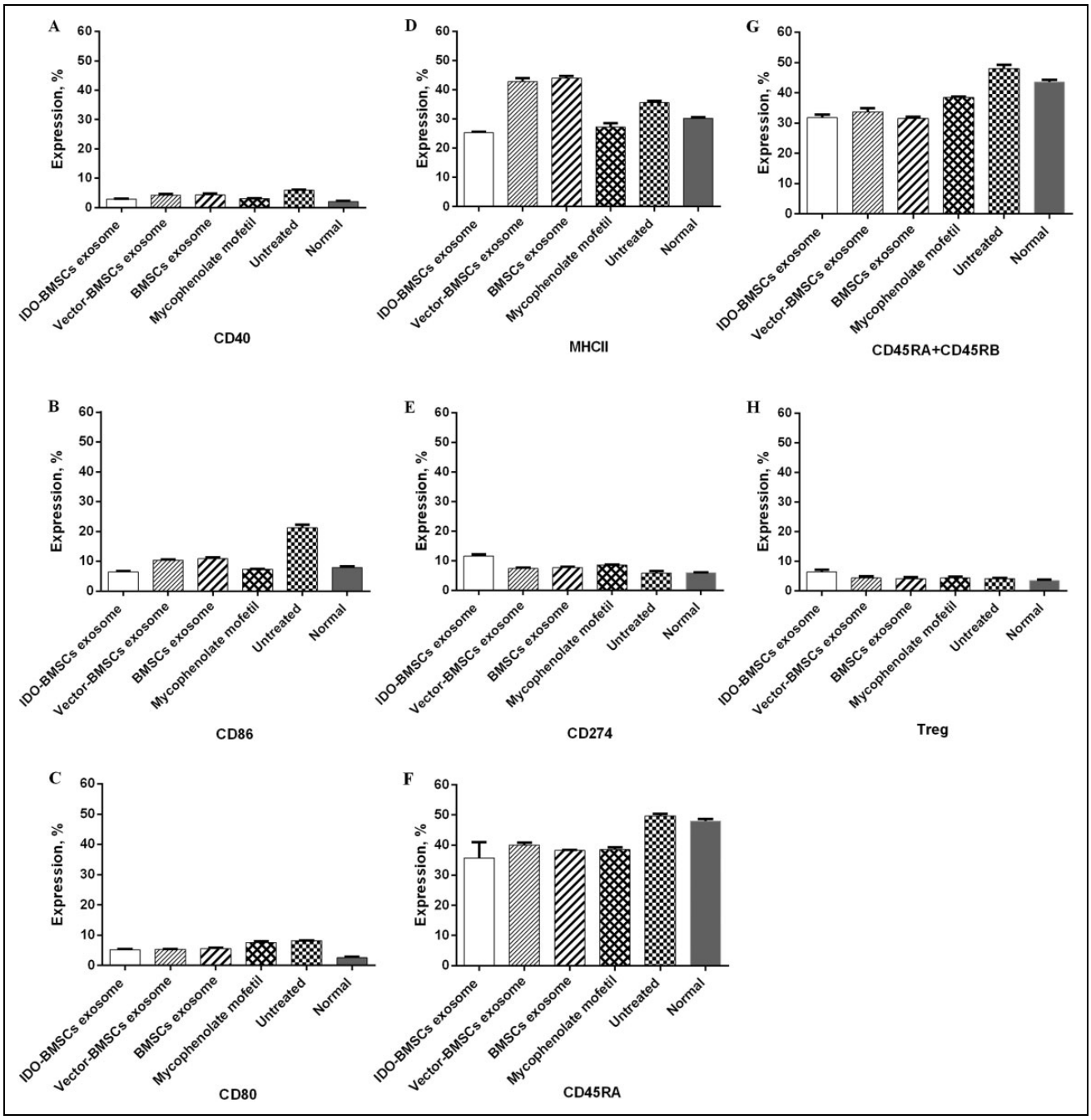

Figure 6. Surface marker expression at 72 hours (in-vivo experiments).

derived from IDO-BMSCs. Small RNA sequencing and TMT quantitative proteomics were used to identify exosome-mediated changes in miRNA expression. Our data showed that (1) Exosomes secreted by IDO-BMSCs regulated the activity of DCs, T-cells and cytokines to improve the survival of the transplanted heart. (2) Transplanted rats injected with exosomes secreted by IDO-BMSCs exhibited significantly lower infiltration of inflammatory cells compared with rats injected with exosomes from other groups. (3) Animals treated with IDO-BMSC-secreted exosomes had a significantly higher EF and FS. (4) Exosomes secreted by IDO-BMSCs exhibited upregulation of immunoregulatory protein FHL-1. (5) miR-540-3p was the most highly upregulated microRNA, and miR-338-5p was the most downregulated microRNA in exosomes secreted by IDO-BMSCs compared with the other groups of exosomes.

Donor-derived MSCs were previously shown to induce a profound T-cell hyporesponsiveness and to prolong survival of cardiac allografts in a mouse model via expansion of donor-specific Tregs, and inhibition of anti-donor Th1

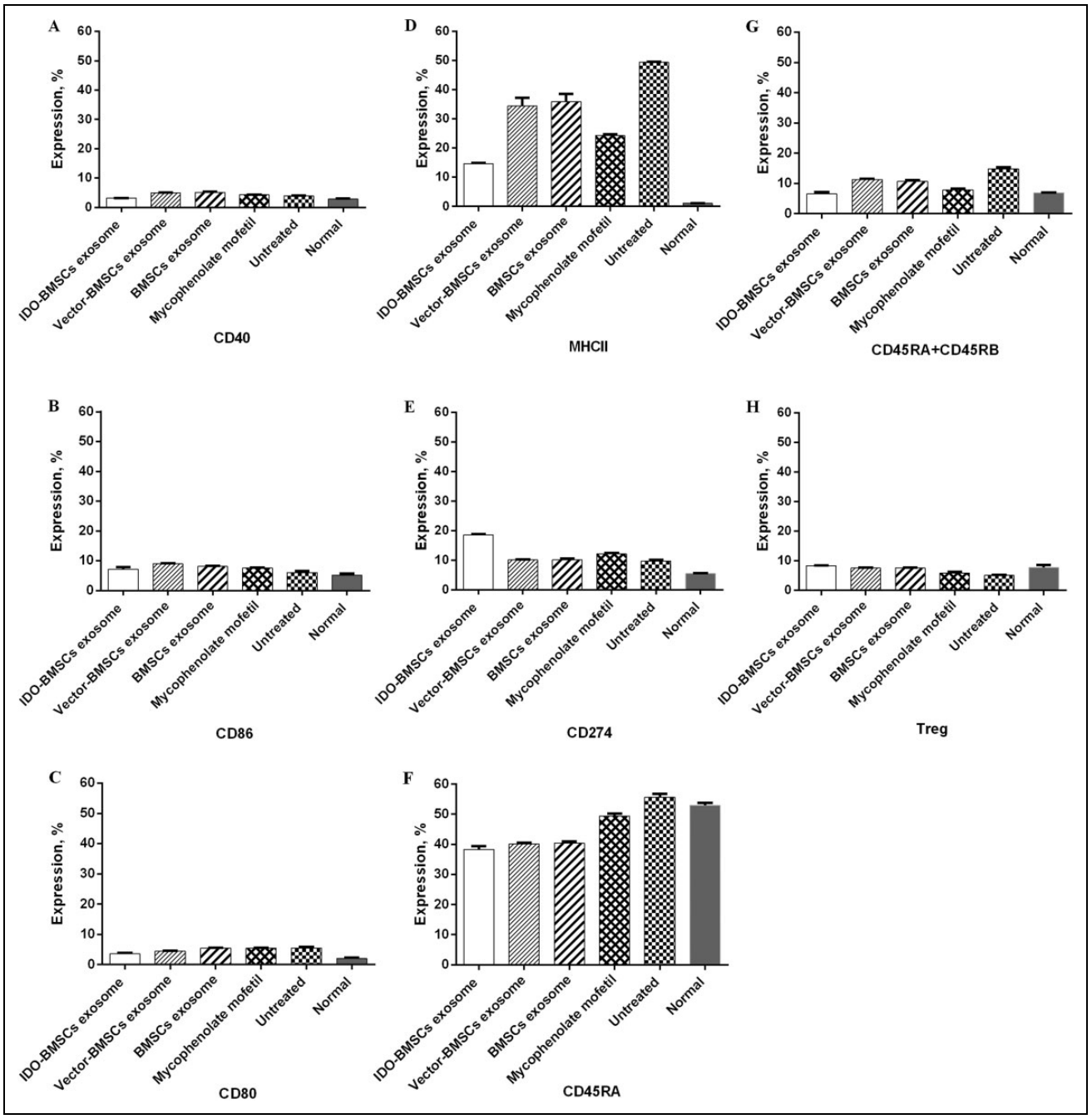

Figure 7. Surface marker expression at 96 hours (in-vivo experiments).

activity[35]. This immunoregulatory activity has been shown to be associated with a number of factors: (1) MSCs cannot activate heterologous[35] or allogeneic lymphocytes possibly because they do not express MHC-II and costimulatory molecules like CD80, CD86 and CD40[36]. These findings support the use of allogeneic MSCs (such as cord blood-derived MSCs) in the clinical treatment of diseases. (2) MSCs inhibit the activation and proliferation of T and B lymphocytes, which are mostly arrested in the G0/G1 phase[37,38]. MSCs also secrete soluble cytokines (such as IL-6 and macrophage-CSF) which interfere with the differentiation, maturation and function of DCs[39]. (3) MSCs release anti-inflammatory and anti-apoptotic molecules to repair the microenvironment of injured tissues and protect these tissues[40]. In addition to the treatment of graft-versus-host disease (GVHD), allogeneic stem cell transplantation with MSCs has been used to treat a number of immune diseases such as autoimmune type I diabetes[41], rheumatoid

Table 12. Surface Marker Expression at 96 Hours (In-Vivo Experiments).

		CD40	CD86	CD80	MHC-II	CD274	CD45RA	CD45RA+CD45RB	Treg
IDO-BMSCs exosome	mean	3.2[b,c,d,e,f]	7.2[b,c,e,f]	3.5[b,c,d,e,f]	14.7[b,c,d,e,f]	18.5[b,c,d,e,f]	38.3[b,c,d,e,f]	6.5[b,c,d,e]	8.3[b,c,d,e]
	SD	0.10	0.71	0.31	0.17	0.32	1.11	0.70	0.06
Vector-BMSCs exosome	mean	4.9[a,c,d,e,f]	9.0[a,c,d,e,f]	4.4[a,c,d,e,f]	34.4[a,d,e,f]	10.1[a,d,f]	40.1[a,d,e,f]	11.3[a,d,e,f]	7.5[a,d,e]
	SD	0.15	0.17	0.10	2.85	0.10	0.52	0.21	0.21
BMSCs exosome	mean	5.2[a,b,d,e,f]	8.1[a,b,e,f]	5.4[a,b,f]	35.8[a,d,e,f]	10.2[a,d,f]	40.4[a,d,e,f]	10.7[a,d,e,f]	7.6[a,d,e]
	SD	0.17	0.23	0.17	2.70	0.35	0.55	0.44	0.10
Mycophenolate mofetil	mean	4.3[a,b,c,e,f]	7.6[b,e,f]	5.4[a,b,f]	24.4[a,b,c,e,f]	12.2[a,b,c,e,f]	49.4[a,b,c,e,f]	7.8[a,b,c,e,f]	5.8[a,b,c,e,f]
	SD	0.10	0.10	0.12	0.42	0.32	0.84	0.47	0.40
Untreated	mean	3.9[a,b,c,d,f]	6.1[a,b,c,d,f]	5.4[a,b,f]	49.4[a,b,c,d,f]	9.7[a,d,f]	55.6[a,b,c,d,f]	14.8[a,b,c,d,f]	5.1[a,b,c,d,f]
	SD	0.21	0.40	0.42	0.10	0.40	1.14	0.61	0.10
Normal	mean	2.9[a,b,c,d,e]	5.2[a,b,c,d,e]	2.0[a,b,c,d,e]	1.1[a,b,c,d,e]	5.4[a,b,c,d,e]	52.9[a,b,c,d,e]	6.8[b,c,d,e]	7.7[a,b,c,d,e]
	SD	0.12	0.56	0.35	0.06	0.21	0.80	0.29	0.87
	p-value	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[a,b,c,d,e,f] Significantly different from
[a] IDO-BMSCs exosome,
[b] Vector-BMSCs exosome,
[c] BMSCs exosome,
[d] mycophenolate mofetil,
[e] untreated,
[f] normal.
BMSC: bone marrow mesenchymal stem cell; IDO: indoleamine 2,3-dioxygenase; MHC: major histocompatibility complex; SD: standard deviation; Treg: regulatory T-cell.

arthritis (RA)[42], systemic lupus erythematosus[43] and multiple sclerosis (MS)[44,45]. BMSCs have been shown to exert their therapeutic effects via leukocyte-induced immune tolerance mediated by $CD4^+/CD25^{+++}$Tregs and $CD8^+/CD28^-$Tregs, and are increasingly being used in clinical practice[46].

Our present in-vitro data showed that (1) exosomes from IDO-BMSCs incubated with DCs regulated DC activity by downregulation of CD40, CD86, CD80, MHC-II, CD45RA, CD45RA+CD45RB and OX62 and upregulation of CD274 expression (2) exosomes from IDO-BMSCs incubated with T-cells increased the number of Tregs and decreased the number of CD8+ T-cells, although the number of CD4+ T-cells remained unchanged (3) exosomes from IDO-BMSCs incubated with DCs and T-cells together downregulated CD40, CD86, CD80, MHC-II, CD45RA, CD45RA+CD45RB and OX62, upregulated CD274 expression, increased the number of Tregs, and decreased the number of CD8+ T-cells, although the number of CD4+ T-cells remained unchanged. (4) Expression of IDO RNA was highest in the group where exosomes from IDO-BMSCs were incubated with DCs and T-cells. (5) Exosomes from IDO-BMSCs incubated with DCs and T-cells had significantly lower levels of pro-inflammatory cytokines such as IL-1α, IL-4, IL-1β, IL-2, IFNγ and IL-18, but significantly higher levels of anti-inflammatory cytokines such as IL-10, TGFβ1, TGFβ2 and TGFβ3 compared with the other groups. Our data agreed with recent data which showed that exosomes derived from MSCs of healthy donors suppressed the levels of pro-inflammatory TNFα and IL-1β, increased the levels of anti-inflammatory TGFβ, and increased the number of Tregs during in-vitro culture[47].

In our present study, rats which underwent ectopic heart transplantation were injected with exosomes from the different BMSC groups. Our data showed that (1) EF and FS were improved significantly in rats injected with exosomes from IDO-BMSCs. (2) Transplanted rats injected with exosomes from IDO-BMSCs had significantly lower levels of CD40, CD86, CD80, MHC-II, CD45RA and CD45RA+CD45RB, significantly higher levels of CD274, and significantly higher numbers of Tregs compared with other groups. (3) Transplanted rats injected with exosomes from IDO-BMSCs had significantly lower levels of serum IL-1α, IL-4, IL-1β, IL-2, IFNγ and IL-18, and significantly higher levels of serum IL-10, TGFβ1, TGFβ2 and TGFβ3 compared with the other groups. (4) Transplanted rats injected with exosomes from IDO-BMSCs had significantly lower numbers of infiltrated inflammatory cells compared with the other groups. Our data suggested that exosomes from IDO-BMSCs regulated DCs, T-cells and cytokine secretion to improve survival of transplanted heart. Our data were consistent with a previous mouse study which showed that exosomes from IDO-overexpressing DCs inhibited the progression of collagen-induced arthritis, and inhibited the DTH response, and these effects were partially dependent on B7-1 and B7-2[48]. Our data also validated previous findings

Table 13. Expression Levels of IL-1α, IL-4, IL-1β and IL-2 at 48, 96, and 168 h (In-Vivo Experiments).

	IL-1α	IL-4	IL-1β	IL-2
2 days				
IDO-BMSC-exosome	63.5 (1.88)[b,c,d,e,f]	7.4 (0.27)[b,c,d,e,f]	125.3 (1.53)[b,c,d,e,f]	64.0 (2.10)[b,c,d,e,f]
Vector-BMSC-exosome	75.4 (1.69)[a,d,e,f]	8.4 (0.46)[a,e]	178.7 (0.58)[a,c,e,f]	75.4 (3.82)[a,e,f]
BMSC-exosome	75.8 (3.48)[a,d,e,f]	8.1 (0.03)[a,d,e,f]	171.4 (1.04)[a,b,d,e,f]	77.5 (3.02)[a,e,f]
Mycophenolate mofetil	82.5 (2.12)[a,b,c,e,f]	8.7 (0.31)[a,c,f]	184.6 (4.42)[a,c,e,f]	77.5 (3.00)[a,e,f]
Untreated	98.2 (0.92)[a,b,c,d,f]	9.1 (0.00)[a,b,c,f]	275.8 (6.64)[a,b,c,d,f]	97.6 (1.53)[a,b,c,d,f]
Normal	30.4 (1.77)[a,b,c,d,e]	4.5 (0.13)[a,b,c,d,e]	135.2 (1.68)[a,b,c,d,e]	48.4 (4.33)[a,b,c,d,e]
p-value	<0.0001	<0.0001	<0.0001	<0.0001
4 days				
IDO-BMSC-exosome	62.7 (1.62)[b,c,d,e,f]	7.0 (0.03)[c,d,e,f]	118.3 (2.08)[b,c,d,e,f]	62.7 (2.58)[b,c,d,e,f]
Vector-BMSC-exosome	74.1 (1.53)[a,d,e,f]	7.1 (0.06)[c,d,e,f]	166.7 (0.58)[a,d,e,f]	74.7 (3.15)[a,e,f]
BMSC-exosome	74.8 (3.60)[a,d,e,f]	7.5 (0.32)[a,b,d,e,f]	168.5 (0.98)[a,d,e,f]	76.7 (2.55)[a,e,f]
Mycophenolate mofetil	83.0 (0.80)[a,b,c,e,f]	8.8 (0.25)[a,b,c,e,f]	187.3 (3.03)[a,b,c,e,f]	78.6 (2.52)[a,e,f]
Untreated	106.1 (2.14)[a,b,c,d,f]	9.3 (0.13)[a,b,c,d,f]	274.3 (10.31)[a,b,c,d,f]	96.7 (1.15)[a,b,c,d,f]
Normal	30.5 (1.93)[a,b,c,d,e]	4.8 (0.15)[a,b,c,d,e]	134.9 (1.30)[a,b,c,d,e]	49.3 (6.98)[a,b,c,d,e]
p-value	<0.0001	<0.0001	<0.0001	<0.0001
7days				
IDO-BMSC-exosome	61.4 (1.77)[b,c,d,e,f]	6.5 (0.28)[b,c,d,e,f]	115.9 (3.36)[b,c,d,e,f]	61.8 (2.17)[b,c,d,e,f]
Vector-BMSC-exosome	72.8 (1.13)[a,d,e,f]	7.0 (0.09)[a,c,d,e,f]	164.8 (0.81)[a,d,e,f]	73.1 (3.51)[a,d,e,f]
BMSC-exosome	74.0 (3.45)[a,d,e,f]	7.4 (0.28)[a,b,d,e,f]	167.1 (1.01)[a,d,e,f]	75.6 (2.59)[a,e,f]
Mycophenolate mofetil	85.4 (1.69)[a,b,c,e,f]	8.8 (0.22)[a,b,c,e,f]	184.7 (2.08)[a,b,c,e,f]	80.2 (2.34)[a,b,e,f]
Untreated	107.5 (1.41)[a,b,c,d,f]	9.4 (0.12)[a,b,c,d,f]	275.8 (10.41)[a,b,c,d,f]	97.6 (1.20)[a,b,c,d,f]
Normal	30.4 (1.37)[a,b,c,d,e]	4.9 (0.13)[a,b,c,d,e]	134.1 (0.90)[a,b,c,d,e]	48.5 (5.41)[a,b,c,d,e]
p-value	<0.0001	<0.0001	<0.0001	<0.0001

BMSC: bone marrow mesenchymal stem cell; IDO: indoleamine 2,3-dioxygenase; IL: interleukin.

Table 14. Expression levels of IL-10, IFNγ and IL-18 at 48, 96, and 168 h (In-Vivo experiments).

	IL-10	IFNγ	IL-18
2 days			
IDO-BMSC-exosome	434.0 (4.36)[b,c,d,e,f]	67.2 (2.14)[b,c,d,e,f]	143.7 (0.58)[b,c,d,e]
Vector-BMSC-exosome	399.7 (4.93)[a,d,e,f]	75.3 (2.26)[a,e,f]	172.0 (1.99)[a,e,f]
BMSC-exosome	390.8 (3.02)[a,d,e,f]	77.1 (2.42)[a,e,f]	175.3 (2.52)[a,e,f]
Mycophenolate mofetil	370.0 (2.00)[a,b,c,e,f]	74.0 (1.52)[a,e,f]	187.0 (2.00)[a,e,f]
Untreated	271.7 (9.29)[a,b,c,d,f]	93.6 (2.19)[a,b,c,d,f]	246.7 (25.50)[a,b,c,d,f]
Normal	241.1 (14.38)[a,b,c,d,e]	32.1 (4.91)[a,b,c,d,e]	152.0 (3.04)[b,c,d,e]
p-value	<0.0001	<0.0001	<0.0001
4 days			
IDO-BMSC-exosome	476.0 (8.66)[b,c,d,e,f]	66.2 (2.66)[b,c,d,e,f]	143.2 (0.59)[b,c,d,e]
Vector-BMSC-exosome	402.0 (6.08)[a,d,e,f]	74.0 (2.31)[a,e,f]	164.6 (1.13)[a,d,e]
BMSC-exosome	392.2 (3.65)[a,d,e,f]	76.2 (2.85)[a,e,f]	166.3 (0.91)[a,d,e]
Mycophenolate mofetil	371.4 (2.23)[a,b,c,e,f]	75.1 (3.28)[a,e,f]	188.2 (1.87)[a,b,c,e,f]
Untreated	271.7 (8.43)[a,b,c,d,f]	95.0 (0.41)[a,b,c,d,f]	258.1 (26.33)[a,b,c,d,f]
Normal	240.9 (14.81)[a,b,c,d,e]	31.8 (3.83)[a,b,c,d,e]	151.7 (3.66)[d,e]
p-value	<0.0001	<0.0001	<0.0001
7 days			
IDO-BMSC-exosome	484.3 (4.93)[b,c,d,e,f]	65.1 (2.81)[b,c,d,e,f]	141.7 (0.85)[b,c,d,e]
Vector-BMSC-exosome	411.2 (7.81)[a,c,d,e,f]	73.0 (2.11)[a,e,f]	163.4 (1.11)[a,d,e]
BMSC-exosome	387.7 (3.06)[a,b,d,e,f]	75.5 (2.67)[a,e,f]	165.3 (0.48)[a,d,e]
Mycophenolate mofetil	371.3 (1.15)[a,b,c,e,f]	75.6 (3.07)[a,e,f]	189.3 (2.13)[a,b,c,e,f]
Untreated	270.6 (9.21)[a,b,c,d,f]	96.0 (0.39)[a,b,c,d,f]	259.6 (25.09)[a,b,c,d,f]
Normal	242.3 (15.53)[a,b,c,d,e]	32.1 (5.02)[a,b,c,d,e]	150.6 (3.88)[d,e]
p-value	<0.0001	<0.0001	<0.0001

§ Value were summarized as mean (SD); Unit: pg/ml.
[a,b,c,d,e,f] Significantly different from
[a]IDO-BMSCs exosome,
[b]Vector-BMSCs exosome,
[c]BMSCs exosome,
[d]mycophenolate mofetil,
[e]untreated,
[f]normal.
BMSC: bone marrow mesenchymal stem cell; IDO: indoleamine 2,3-dioxygenase; IFN: interferon; IL: interleukin; SD: standard deviation.

Table 15. Expression Levels of TGFβ1, TGFβ2 and TGFβ3 at 48, 96, and 168 h (In-Vivo Experiments).

	TGFβ1	TGFβ2	TGFβ3
2 days			
IDO-BMSC-exosome	120,612.0 (4101.59)[b,c,d,e,f]	5098.7 (102.26)[b,c,d,e,f]	66.7 (0.00)[b,c,d,e,f]
Vector-BMSC-exosome	78,600.7 (1786.68)[a,d,e,f]	2553.3 (25.32)[a,c,d,e,f]	43.1 (0.00)[a,d,e,f]
BMSC-exosome	79,978.7 (1585.51)[a,d,e,f]	2303.0 (16.70)[a,b,d,e,f]	45.5 (2.03)[a,d,e,f]
Mycophenolate mofetil	53,717.3 (20.13)[a,b,c,e,f]	1827.7 (12.70)[a,b,c,e,f]	48.2 (1.91)[a,b,c,e,f]
Untreated	38,809.3 (595.69)[a,b,c,d,f]	916.0 (1.73)[a,b,c,d,f]	35.3 (1.90)[a,b,c,d,f]
Normal	23,709.0 (1240.02)[a,b,c,d,e]	527.3 (24.68)[a,b,c,d,e]	29.7 (0.00)[a,b,c,d,e]
p-value	<0.0001	<0.0001	<0.0001
4 days			
IDO-BMSC-exosome	145,572.7 (2532.86)[b,c,d,e,f]	5253.0 (44.24)[b,c,d,e,f]	78.7 (1.73)[b,c,d,e,f]
Vector-BMSC-exosome	85,269.0 (260.32)[a,c,d,e,f]	2906.6 (59.28)[a,c,d,e,f]	44.7 (0.00)[a,c,e,f]
BMSC-exosome	89,234.3 (1388.87)[a,b,d,e,f]	2559.7 (4.51)[a,b,d,e,f]	47.7 (0.86)[a,b,e,f]
Mycophenolate mofetil	52,235.7 (65.68)[a,b,c,e,f]	1744.3 (25.01)[a,b,c,e,f]	46.2 (0.85)[a,e,f]
Untreated	38,656.3 (286.31)[a,b,c,d,f]	858.3 (42.34)[a,b,c,d,f]	34.5 (1.17)[a,b,c,d,f]
Normal	23,992.3 (1513.4)[a,b,c,d,e]	518.9 (0.00)[a,b,c,d,e]	29.2 (1.00)[a,b,c,d,e]
p-value	<0.0001	<0.0001	<0.0001
7 days			
IDO-BMSC-exosome	155,415.7 (2013.15)[b,c,d,e,f]	5397.7 (63.67)[b,c,d,e,f]	88.3 (1.53)[b,c,d,e,f]
Vector-BMSC-exosome	86,600.7 (284.50)[a,c,d,e,f]	2971.3 (14.57)[a,d,e,f]	45.4 (0.30)[a,c,e,f]
BMSC-exosome	90,301.3 (552.47)[a,b,d,e,f]	2962.0 (14.71)[a,d,e,f]	49.8 (0.30)[a,b,d,e,f]
Mycophenolate mofetil	51,585.0 (58.92)[a,b,c,e,f]	1660.7 (43.75)[a,b,c,e,f]	43.5 (1.25)[a,c,e,f]
Untreated	37,780.0 (16.82)[a,b,c,d,f]	833.9 (16.75)[a,b,c,d,f]	32.8 (1.53)[a,b,c,d,f]
Normal	24,418.0 (1255.7)[a,b,c,d,e]	521.8 (13.16)[a,b,c,d,e]	30.2 (1.00)[a,b,c,d,e]
p-value	<0.0001	<0.0001	<0.0001

§ Value were summarized as mean (SD); Unit: pg/ml.
[a,b,c,d,e,f] Significantly different from
[a]IDO-BMSCs exosome,
[b]Vector-BMSCs exosome,
[c]BMSCs exosome,
[d]mycophenolate mofetil,
[e]untreated,
[f]normal.
Functional Classification of Differentially Quantified Proteins
GO Classification of Terms Level 2
Based on GO annotation information of identified proteins, we calculated the number of differentially expressed proteins in each GO term of level 2 (IDO1/NC-BMSC).
BMSC: bone marrow mesenchymal stem cell; GO: gene ontology; IDO: indoleamine 2,3-dioxygenase; IFN: interferon; IL: interleukin; SD: standard deviation; TGF: transforming growth factor.

that showed that exosomes derived from MSCs reduced inflammation and improved heart function in a rat myocardial infarction model, and this effect was superior to that seen with MSCs alone[49].

We used proteomics with TMT-labeled quantification of peptides to show that FHL-1 was the most highly upregulated protein in exosomes from IDO-BMSCs. FHL-1 has been reported to inhibit proliferation and migration of cancer cells[50], inhibit IGF / PI3 K signal transduction, and activate endoplasmic reticulum (ER) signal transduction, leading to the inhibition of downstream Akt activation[51]. The resulting inhibition of mammalian target of rapamycin (mTOR) is thought to mediate immunotolerance after transplantation.

We used small RNA sequencing to detect immune-related microRNAs in exosomes from IDO-BMSCs. We found that miR-540-3p was the most highly upregulated microRNA, and miR-338-5p was the most highly downregulated microRNA in these exosomes compared with exosomes from the other groups. Previous studies reported that upregulation of miR-338-5p inhibited the proliferation, metastasis and invasion, and promoted apoptosis in a number of cancer cells[52–54]. Although our present study showed a significant downregulation of miR-338-5p expression in exosomes from IDO-BMSCs, the entry of miR-338-5p into receptor cells actually increased the concentration of miR-338-5p in receptor cells (data not shown). Gene prediction data showed that RAG2 is a downstream target gene which is negatively regulated by miR-338-5p[55]. RAG2 encodes a protein involved in the initiation of V(D)J recombination during the development of B-cells and T-cells. Although our data suggested that miR-338-5p could downregulate RAG2 in order to mediate immunotolerance, it is important for future studies to investigate in greater detail the role of

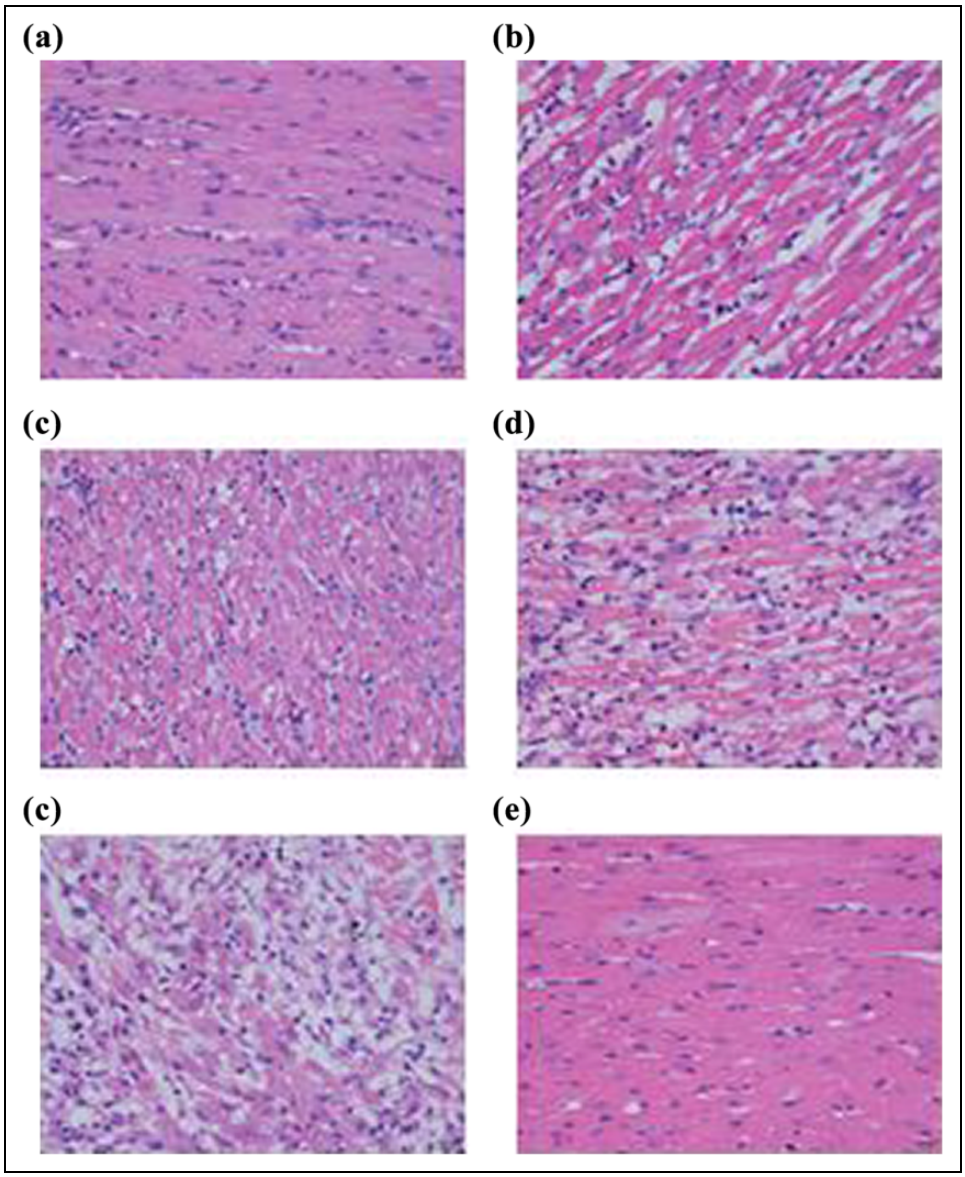

Figure 8. Representative images of H&E staining of heart tissue from transplanted animals 2 days after treatment with different exosome groups. (a) IDO-BMSC-exosomes: myocardial cells had edema. Some infiltration of inflammatory cells. (b) Vector-BMSC-exosome: myocardial cells had edema. Inflammatory cell infiltration between cells was greater than the IDO-BMSC-exosome group. (c) BMSC-exosome: myocardial cells had edema. Inflammatory cell infiltration was more than the IDO-BMSC-exosome group and similar to the vector-BMSC-exosome group. (d) Mycophenolate mofetil: myocardial cells had edema. Inflammatory cell infiltration was more than the IDO-BMSC-exosome group and similar to the vector-BMSC-exosome group. (e) Untreated: myocardial cells had edema. Inflammatory cell infiltration was more than the IDO-BMSC-exosome, vector-BMSC-exosome, and BMSC-exosome groups. There were some dead myocardial cells. (f) Normal: myocardial cells arranged in a regular shape and no edema seen.
BMSC: bone barrow mesenchymal stem cell; H&E: hematoxylin and eosin; IDO: indoleamine 2,3-dioxygenase.

miR-338-5p in immunomodulation after heart transplantation. Gene prediction also showed that JAK3 is a downstream target of miR-540-3p. JAK3 protein is expressed in hematopoietic cells and epithelial cells and is thought to be an immune activator[56]. Our data suggested that high expression of miR-540-3p in exosomes from IDO-BMSCs could exert a negative regulatory effect on JAK3 to induce tolerance. Interestingly, it was previously reported that although exosomes derived from MSCs had a mostly similar miRNA profile as that of the MSCs, the expression of some miRNAs were significantly different, and this difference was thought to explain the superiority of therapeutic benefit seen in exosomes over MSCs[49]. It will be interesting to further analyze differences in miRNA expression profiles in our study and correlate the differences with therapeutic benefits.

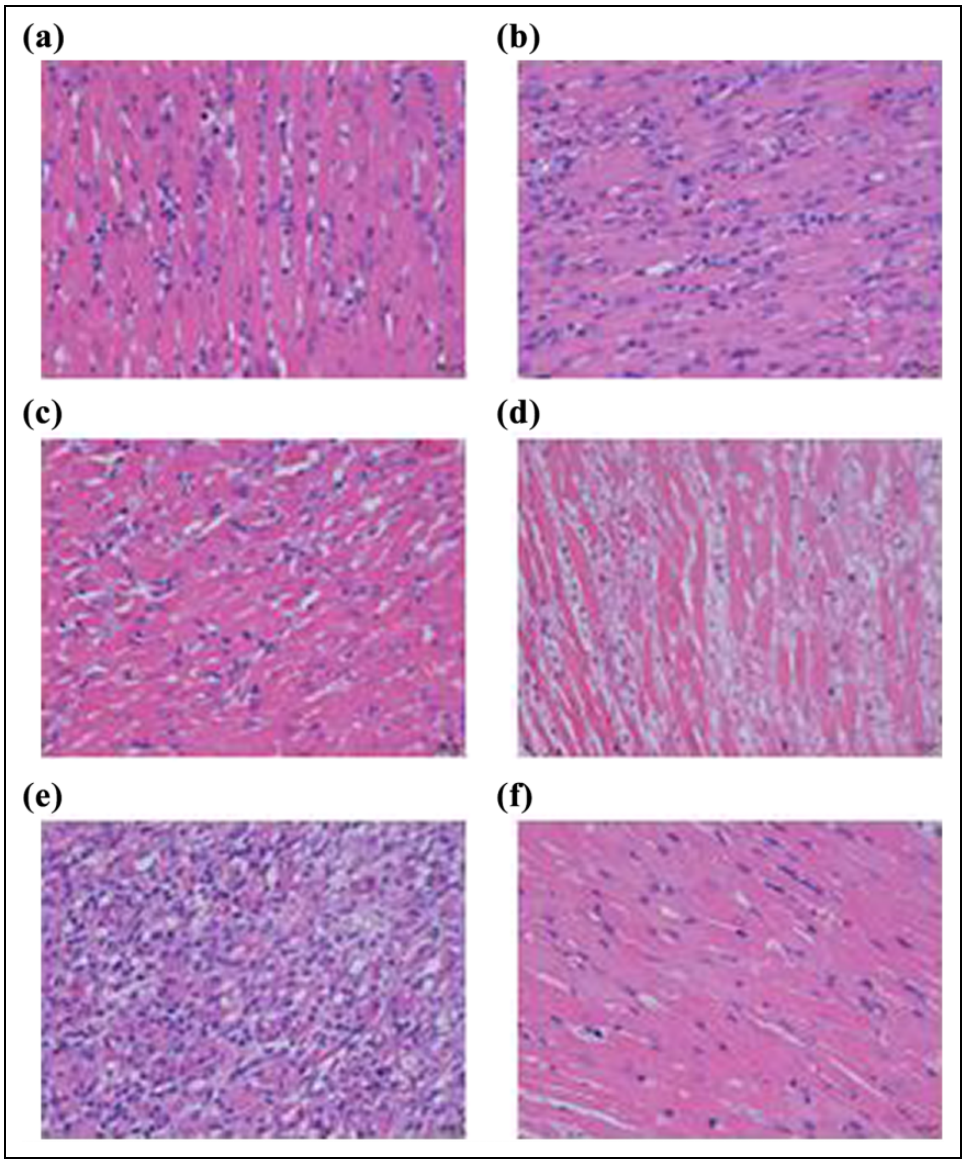

Figure 9. Representative images of H&E staining of heart tissue from transplanted animals 4 days after treatment with different exosome groups. (a) IDO-BMSC-exosomes: myocardial cells had edema. Some infiltration of inflammatory cells. (b) Vector-BMSC-exosome: myocardial cells had edema. Inflammatory cell infiltration between cells was greater than the IDO-BMSC-exosome group. (c) BMSC-exosome: myocardial cells had edema. Inflammatory cell infiltration was more than the IDO-BMSC-exosome group and similar to the vector-BMSC-exosome group. (d) Mycophenolate mofetil: myocardial cells had edema. Inflammatory cell infiltration was more than the IDO-BMSC-exosome group and similar to the vector-BMSC-exosome group. There were some dead myocardial cells. (e) Untreated: myocardial cells had edema. Inflammatory cell infiltration was more than the IDO-BMSC-exosome, vector-BMSC-exosome, and BMSC-exosome groups. There were many dead myocardial cells. (f) Normal: myocardial cells arranged in a regular shape and no edema seen.
BMSC: bone barrow mesenchymal stem cell; H&E: hematoxylin and eosin; IDO: indoleamine 2,3-dioxygenase.

Conclusion

In this study, we established a rat heart transplantation model in which transplanted animals were injected with exosomes derived from different groups of BMSCs. We showed that exosomes secreted by IDO-BMSCs mediated a (1) decrease in the serum levels of pro-inflammatory cytokines such as IL-1α, IL-4, IL-1β, IL-2, IFNγ, and IL-18; (2) an increase in the serum levels of anti-inflammatory cytokines such as IL-10, TGFβ1, TGFβ2, and TGFβ3; (3) an improvement in EF and FS; and (4) a decrease in infiltration of inflammatory cells compared with exosomes from other groups of BMSCs. Our data demonstrated the potential therapeutic use of exosomes derived from IDO-BMSCs, which can be used as a cell-free approach to promote immunotolerance and prolong the survival of cardiac allografts.

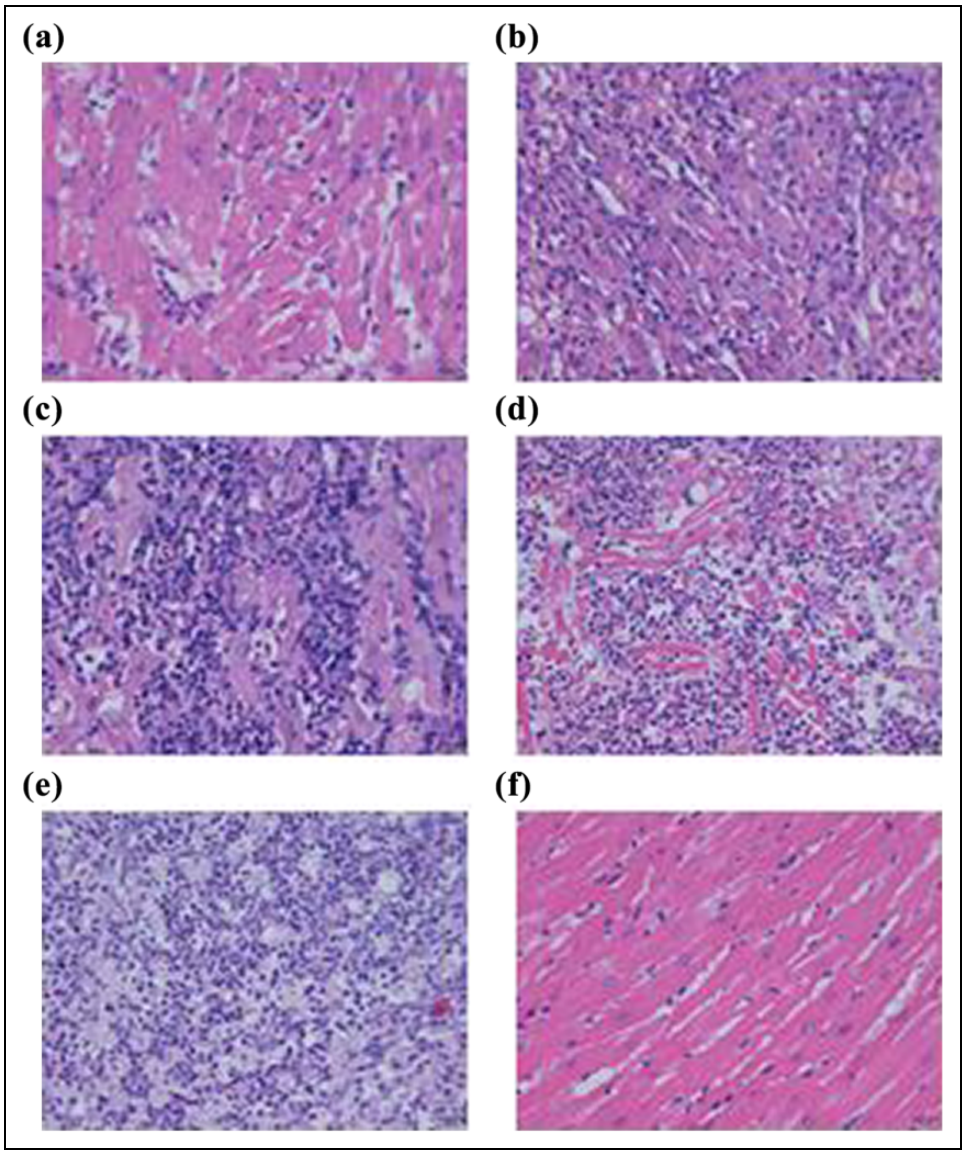

Figure 10. Representative images of H&E staining of heart tissue from transplanted animals 4 days after treatment with different exosome groups. (a) IDO-BMSC-exosomes: myocardial cells had edema. Some infiltration of inflammatory cells. (b) Vector-BMSC-exosomes: myocardial cells had edema. Inflammatory cell infiltration was more than the IDO-BMSC-exosome group. Some dead myocardial cells were seen. (c) BMSC-exosomes: myocardial cells had edema. Inflammatory cell infiltration was more than the IDO-BMSC-exosome group and similar to the vector-BMSC-exosome group. Some dead myocardial cells were seen. (d) Mycophenolate mofetil: myocardial cells had edema. Inflammatory cell infiltration was more than the IDO-BMSC-exosome group and similar to the vector-BMSC-exosome group. Many dead myocardial cells were seen. (e) Untreated: myocardial cells had edema. Inflammatory cell infiltration was more than the IDO-BMSC-exosome group, the vector-BMSC-exosome group, and the BMSC-exosome group. (f) Normal: myocardial cells arranged in a regular shape and no obvious inflammatory cell infiltration seen.
BMSC: bone barrow mesenchymal stem cell; H&E: hematoxylin and eosin; IDO: indoleamine 2,3-dioxygenase.

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Ethical Approval

All animal studies were approved by the Animal Care and Use Committee of the First People's Hospital of Yunnan Province, China.

Statement of Human and Animal Rights

All animal studies were approved by the Animal Care and Use Committee of the First People's Hospital of Yunnan Province, China and were performed according to Good Laboratory Practice.

Statement of Informed Consent

Statement of Informed Consent is not applicable for this article.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

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