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Immunosuppressive Actions of Embryonic Stem Cells and Mesenchymal Stem Cells in Alloimmune Response

동종면역반응에서 배아줄기세포와 중간엽줄기세포의 면역억제현상 규명

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Graduate School of Immunology

The Department of Medicine

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Immunosuppressive Actions of Embryonic Stem Cells and Mesenchymal Stem Cells in Alloimmune Response

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A thesis submitted to the Department of Medicine in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Graduate School of Immunology at Seoul National University College of Medicine

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ABSTRACT

Introduction: Embryonic stem cells (ESCs) and mesenchymal stem cells (MSCs) are known to have immunosuppressive effects. However, its immunosuppressive mechanisms still remain controversial. We therefore conducted this study to examine the immunosuppressive mechanisms of ESCs and MSCs in an *in vitro* environment and in an animal experimental model of the allograft transplantation of skin and pancreatic islet for *in vivo* environment.

Methods: To analyze the *in vitro* immunosuppressive effect of stem cells**,** naïve T cells or memory T cells from C57BL/6 mice and antigen presenting cells from BALB/c mice with ESCs or MSCs were co-cultured for 3 days and thymidine uptake, ELISA (enzyme-linked immunosorbent assay), CFSE (carboxyfluorescein succinimidyl ester) and 7-AAD staining were conducted. Transwell experiment and RT-PCR (reverse transcription-polymerase chain reaction) on immunosuppressive molecules such as PDL-1, Fas-L, CD30, CD70, perforin and granzyme B for the immunosuppressive mechanism were performed. For the functional assay, blocking antibodies or inhibitors on each molecule were treated. To analyze the *in vivo* immunosuppressive effect of both stem cells, we performed the allogeneic skin and pancreatic transplantation by intravenous injection or co-transplantation of stem cells with anti-CD40L antibodies or mycophenolate mofetil (MMF).

Results: ESCs or MSCs did not stimulate alloimmune response and synchronously suppressed the proliferation, survival, activation of naïve $CD4^+$ T cells in an *in vitro* alloimmune response. They also suppressed the release of cytokines such as IL-2, IL-12, IFN-γ, TNF-α, IL-4, IL-5, IL-1β and IL-10. But there were no significant changes in the expression of TGF-β or IDO (indoleamine 2,3-dioxygenase). In addition, they also suppressed $CD44^+$ memory T cells or T cell activation markers and induced regulatory T cells.

The transwell assay revealed that the immunosuppressive effects of both types of stem cells originated from the cell-to-cell contact. Both types of stem cells strongly expressed PDL-1, Fas-L, CD30, perforin and granzyme B, but weakly expressed CD70. When blocking agents on granzyme B of ESCs, the immunosuppressive effect of ESCs was significantly reduced. Both ESCs and MSCs by intravenous injection or co-transplantation did not strongly suppress the immune rejection of the allogeneic skin tissue and pancreatic islet. Despite the concomitant use of them with immunosuppressant, there was no additional effect. Significant immunological changes of immune cells by stem cells were not shown in transplanted recipients.

Conclusions: Murine ESCs or MSCs showed the low immunogenicity and the significant immunosuppressive effect such as the suppression of both proliferation and survival of naïve T cells and memory T cells, cytokine release reduction, the induction of Tregs in an *in vitro* environment. The main mechanism of immunosuppression was by cell-to-cell and granzyme B on ESCs played a key role. However, both stem cells did not suppress allogeneic rejection in the allogeneic skin and pancreatic islet transplantation. This discrepancy in the immunosuppressive effects of stem cells between an *in vitro* environment and an *in vivo* one showed that it has to be considered for the establishment of optimal transplantation condition and for the successful clinical application of stem cells in the field of organ transplantation.

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Keywords: embryonic stem cell, mesenchymal stem cell, T cell, allogeneic skin transplantation, allogeneic islet transplantation, immunosuppressive

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LIST OF ABBREVIATIONS

AP	Alkalin phosphatase
APC	Allophycocyanin
CD40L	CD40 ligand
CFSE	Carboxyfluorescein succinimidyl ester
CTLA-4	Cytotoxic T lymphocyte antigen 4
DC	dendritic cell
DMEM	Dulbecco's modified Eagle's minimal essential medium
ELISA	Enzyme-linked immunosorbent assay
ESC	Embryonic stem cell
FBS	Fetal bovine serum
FITC	Fluorescein isothiocynate
FoxP3	Forkhead box P3
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GM-CSF	Granulocyte macrophage colony-stimulating factor
HO	Heme oxygenase
HRP	Horseradish peroxide
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
IL	Interleukin
MHC	Major histocompatibility complex
MLR	Mixed lymphocyte reaction
MMF	Mycophenolate mofetil
MSC	Mesenchymal stem cell
NIH	National Institutes of Health
NO	Nitric oxide
Oct-4	Octamer-binding transcription factor 4
PCR	Polymerase chain reaction

PDL-1	Programmed death ligand-1
PE	Phycoerythrin
PEG	Polyethylene glycol
RT	Reverse Transcription
TGF	Transforming growth factor
TNF	Tumor necrosis factor
Sca-1	Stem cell antigen-1
SSEA-1	Stage-specific embryonic antigen-1
Sox-2	Sex determining region Y-box 2
Z-AAD-CMK	Z-Ala-Ala-Asp Chloromethyl Ketone

INTRODUCTION

It has been well known that immune modulating cell therapy can overcome the inevitable side effects of chronic immunosuppressant medication after transplantation. Although immunomodulatory cells such as regulatory T cells and tolerogenic dendritic cells have been studied, but it is practically difficult to isolate and expand these cells for clinical applications. As the alternative cells for this purpose, ESCs or MSCs are considered.

ESCs have a potential to differentiate into three different types of cells in the germ layer, and they are characterized by the indefinite proliferation in an *in vitro* environment (1). Based on these characteristics, ESCs are used as a key tool in the developmental biology research field. In addition, it has been accepted that ESCs have a potential to induce a recovery of the damaged tissue in patients with degenerative disease in cell therapy (2, 3, 4, 5). Due to the indefinite proliferation ability, however, ESCs also have a potential risk to differentiate into the tumor (6). Some research groups have reported that ESCs showed the ability to avoid the immune response and have the differentiation and indefinite proliferation ability (7, 8). These characteristics are due to a lower level of expression of MHC I in ESCs, a lack of the expression of MHC II and a lower level of expression of co-stimulators that are involved in the promotion of the activation of immune cells. Moreover, it has also been studied that ESCs have an inhibitory effect on the immune functions of various types of immune cells such as T cells, B cells, dendritic cells and natural killer cells (9). However, It has also been reported that the

degree of MHC I expression is increased when ESCs are differentiated into other cells or are stimulated by cytokines such as IFN-γ (7, 8). Due to these characteristics, ESCs would maintain the cell viability in both *in vivo* and *in vitro* environment. It can also enhance the graft survival by regulating immune rejection that might occur in tissue or organ transplantation recipients (2, 3, 4, 5). However, there are no consistent reports about the mechanisms of inhibitory effect of both stem cells on the immune responses; the cell-to-cell or soluble factors are involved in their inhibitory effects. With regard to cell-to-cell contact mechanism, Fas-L has been studied and TGF-β has been identified to be involved in and soluble factors (10, 11). To date, however, no substantial studies have been conducted on other immunosuppressive molecules on ESCs.

MSCs were first isolated and established from the bone marrow. Thereafter, they have also been isolated from various tissues including the adipose tissue, bone tissue, placenta and umbilical cord (12). MSCs are defined according to three characteristics that have been provided by the International Society for Cellular therapy (13). Firstly, MSCs are composed of a group of heterogeneous cells that can adhere to the floor of culture plate. Secondly, they express cell surface markers such as CD44, CD73, CD90 and CD105, but they do not CD11b, CD14, CD34 and CD45. Thirdly, MSCs are characterized by a potential to differentiate into the bone, fat and cartilage tissues. In addition, it has also been reported that MSCs express MHC I molecules at lower level, but they do not MHC II ones (14). They have been reported not to express co-stimulatory molecules such as CD40, CD40L,

CD86 and CD80 (14). It has also been revealed that MSCs have an inhibitory effect on the immune functions of various types of immune cells such as T cells, B cells, dendritic cells and macrophages (15, 16). But it remains controversial whether their immunosuppressive effects mainly originate from the cell-to-cell contact or soluble factors (17, 18). MSCs are expressed Fas-L and PDL-1 that are the representative immunosuppressive molecules involved in cell-to-cell contact. In addition, MSCs express the immunosuppressive soluble factors such as IDO, TGF-β, HO-1, NO and PEG-2 (19). Besides, there are some studies reporting that MSCs suppressed the proliferation of T cells (20). On the other hand, other contradictory reports have shown that they suppressed the survival of T cells (21). It has also been reported that MSCs induce the regulatory T cells (22). To date, it has been reported that memory T cells are the main obstacle on the regulation of graft rejection. But there are no effective immunomodulators. Regulatory T cells are effective for regulating allogeneic immuune response whose occurrence is triggered by naïve T-cells. But the degree of effect on memory T cells in alloimmune response is relatively lower (23). Previous studies have shown that MSCs can suppress the memory immune responses triggered by $CD8^+$ T cells in an *in vitro* stimulation of a minor mismatch barrier. This indicates that MSCs have a potential to inhibit memory immune responses (24). To date, however, no studies have clarified whether ESCs or MSCs can adjust the memory immune responses due to a major mismatch barrier. Therefore, as an alternative method for cell therapy to overcome this controversial situation, it is necessary to evaluate the immunosuppressive effect of ESCs and MSCs.

In an *in vivo* condition, the survival of cardiac graft tissue was enhanced following an intravenous injection of ESCs in allogeneic heart transplantation (25). Similarly, it has also been reported that MSCs enhanced the survival of cardiac graft tissue in allogeneic heart transplantation (26). Also in the allogeneic skin transplantation of nonhuman primates, MSCs enhanced the survival of allogeneic skin tissue for approximately four days (27). Type I diabetes mellitus is one of the most common autoimmune diseases, whose major etiology is based on insulin-secreting β-cell damages due to autoimmune responses. More definitely, its etiology is the T cell immune response against antigens in the pancreatic islet (28). According to a study about pancreatic islet transplantation by the methods of Edmonton, blood glucose could be efficiently controlled with no respect to the insulin injection in the early stage. Over time, however, insulin becomes requisite again (29, 30). As described here, as the causes of the decreased long term survival of allograft pancreatic islet, the activation of T cells against the alloantigen is presumed (30, 31).

To date, some reports have shown that the immunosuppressive responses could be controlled and the survival of allograft pancreatic islet could be improved by mixing the allograft tissue with stem cells (32). In an animal experimental model of allograft pancreatic islet using nonhuman primates, MSCs enhanced the survival of allograft (33). Also MSCs therapy showed an efficacy in clinical studies that have been conducted in patients with Type I diabetes mellitus (34, 35). On the other hand, MSCs were found to promote the formation of adjacent blood vessels for stable survival and engraftment of

the pancreatic islet following the transplantation of the mixture in immunodeficient mice (36). Moreover, following the infusion of a small amount of T cells in diabetic mice with a lack of immune cells which underwent transplantation of a mixture of allograft pancreatic islet with MSCs, the survival of allograft tissue was increased (37). Following the culture of MSCs with the pancreatic islet in an *in vitro* environment, the survival of pancreatic islet was increased. This was accompanied by the increased resistance to hypoxia-induced apoptosis and protection of pancreatic cells from the inflammatory cytokines (38, 39). But there are not still sufficient studies examining whether ESCs or MSCs could suppress the immune response of allogeneic pancreatic islet in mice with a normal immune cell functions.

Therefore, to elucidate these controversial results of previous studies, we attempted to analyze the immunological characteristics and the immunosuppressive effects of ESCs and MSCs in an *in vitro* allogeneic immune response, and to clarify the mechanisms by which they suppress the immune responses and evaluate whether ESCs and MSCs could suppress the immune rejection of allogeneic skin tissue and pancreatic islet and thereby enhance its survival.

MATERIALS AND METHODS

1. Experimental animals and preparation of ESCs and MSCs

Six-to-eight week old C57BL6/J (H-2^b) and BALB/C (H-2^d) mice were purchased from Orient Bio Inc. (Seongnam, Korea). FoxP3-GFP knock-in C57BL/6 mice were generously provided by Dr. AY Rudensky (University of Washington, Seattle, WA, USA). All experimental protocols were approved by the Animal Ethics Committee of Seoul National University College of Medicine and followed the NIH publication 'Principles of Laboratory Animal Care' (IACUC no. 08-0026, 10-0315, 12-0168). A murine ESC line from C57BL6/J mice (SCRC-1002) was purchased from ATCC (Manassas, VA, USA).). ESCs were cultured on a feeder layer of primary embryonic fibroblast in DMEM (Gibco Laboratories, Grand Island, NY, USA), supplemented with mouse leukemia inhibitory factor (LIF, 1000 U/ml, ESGRO-Chemicon Temecula, CA, USA) and 10% fetal bovine serum (FBS, Gibco Laboratories, Grand Island, NY, USA). Phenotypes of ESCs were investigated using immunocytochemical staining with anti-Sox-2, anti-Oct-4, anti-SSEA-1 antibody and alkaline phosphatase antibody (Abcam, Cambridge, UK). We isolated MSCs from the bone marrow of both BALB/C mice and C57BL6/J mice. Briefly, bone marrow cells were flushed out from tibia of mouse and incubated in DMEM/10% FBS for 3 days. Attached cells were cultured continuously and established. Phenotype of MSCs was investigated using flow cytometry with anti-CD106-PE, anti-Sca-1-PE, anti-CD44-APC, anti-CD34-FITC, anti-CD31-FITC, anti-CD11c-APC, anti-CD14-PE and anti-CD45-APC antibodies (BD biosciences, San Jose, CA, USA). For differentiation, MSCs were cultured under osteoid-differentiation inducing media containing glycerol-phosphate, ascorbic acid 2-phosphate and

dexamethasone or under adipocyte-differentiation media containing insulin and dexamethasone.

2. Flow cytometric analysis of immune cells

Anti-CD4-APC, anti-CD44-APC, anti-CD44-PE, anti-CD69-PE and anti-CD25-PE antibodies were purchased from BD biosciences (San Jose, CA, USA). Cultured cells were incubated with the flurochrome-labeled antibodies for 30 min at 4 °C and were washed twice. FoxP3 expression in $CD4^+$ T cells was analyzed by intracellular flowcytometric analysis using Anti-Mouse/Rat FoxP3 Staining Set FITC (e-Bioscience, San Diego, CA). Flow cytometric analysis was performed using FACSCalibur or FACSCanto (BD Biosciences, Immunocytometry Systems, San Jose, CA, USA). Granzyme B expression in both types of stem cells was analyzed by intracellular flow cytometric analysis using BD Cytofix/Cytoperm™ Fixation/Permeabilization Kit (BD Bioscience, San Diego, CA, USA) according to manufacturer's instructions. Briefly, the stem cells were incubated with fixation/permeabilization solution for 20 min at 4 °C. After washing with Perm/Wash™ buffer, the cells were incubated with PE-anti-granzyme B-antibodies (eBioscience, San Diego, CA, USA) for 30 min at 4 °C.

3. *In vitro* thymidine uptake-mixed lymphocyte reaction (MLR) assays

For analyzing the *in vitro* immunosuppressive effect of MSCs, irradiated (30 Gy) total splenocytes (8×10^5/well) were used as stimulators. Lymph node cells (2×10^5/well) were added as responders in the MLR. ESCs or MSCs were added to the MLR at a ratio of 1 to 10. Proliferation was measured after 72 h of co-culture using either thymidine uptake. Production of

IFN-γ was measured using the OptEIA ELISA kit (BD Bioscience, San Diego, CA). For analyzing the *in vivo* immunosuppressive effect of MSCs, spleen or lymph node was harvested from the recipient C57BL/6 mice with intravenous injection of MSCs after 12 days of allogeneic islet transplantation. Lymphocyte ($2x10^5$) as responder cell was co-cultured with allogeneic BALB/c splenocyte ($8x10^5$, 25Gy irradiated) of 3rd party as stimulator cell for 3 days. The proliferation of responder cell was analyzed by using thymidine uptake.

4. *In vitro* CFSE dilution- MLR assays

$CD4^+$ T cells were isolated from the spleen and lymph nodes from C57BL6/J mice using a $CD4^+$ T cell isolation kit (Miltenyi Biotec, Auburn, CA, USA). Splenic $CD11c^+$ dendritic cells were isolated from BALB/c mice using a CD11c dendritic isolation kit (Miltenyi Biotec, Auburn, CA, USA). C57BL/6 $CD4^+$ T cells (2×10^5) and BALB/c $CD11c^+$ (4×10^4) splenic dendritic cells were used as responders and stimulators, respectively. Anti-CD3/CD28 beads (8×10^4/2 ul, Invitrogen, Oslo, Norway) were also used as stimulators in some experiments. Fixed number (2×10^4) of irradiated (30 Gy) ESCs or MSCs were used in suppression assays, except dose-response experiments, where various numbers of the stem cells (2×10^3, 4×10^3, 2×10^4) were used. Proliferation and survival of $CD4^+$ T cells were simultaneously measured by CFSE (Molecular probe, Eugene, OR, USA) and 7-AAD (BD Bioscience, San Diego, CA, USA) staining. Both types of stem cells were pretreated for an hour at 4 °C with blocking antibodies for PDL-1, FasL, CD30, GM-CSF, IL-6 (10 ug/ml, eBioscience, San Diego, CA, USA), concanamycin A (100 ng/ml, Tocris, Bristol, UK) as a perforin inhibitor or Z-AAD-CMK (100 uM, BioVision, Mountain View, CA, USA) as granzyme B inhibitor before

suppression assays in order to investigate their rescue effects. For the secondary alloimmune response, T cells were isolated from the lymph nodes and spleens of either the naïve mice or sensitized mice using the pan-T-cell isolation kit (Miltenyi Biotec, Auburn, CA, USA) at 8 weeks after sensitization. Then, $CD44^+$ T cells were sorted from the sensitized mice and $CD44^-$ T cells were sorted from the naïve mice using FACSAria machine (BD Bioscience, San Diego, CA, USA) and used as responders in the MLR assay.

5. Measurement of cytokines and IDO activity

The level of TGF-β in culture supernatants of the MLR was measured by ELISA (R&D Systems, Minneapolis, MN). The other cytokines were measured by multiplex bead assay (BioRad Laboratories, Hercules, CA, USA). Levels of tryptophan and kynurenine in supernatant were measured using liquid chromatography/tandem mass spectrometry (LC/MS/MS; QTrap4000, Applied Biosystems, Foster City, CA) and the metabolic ratio of kynurenine to tryptophan was calculated as IDO activity in the MLR.

6. Classic and modified transwell experiments

C57BL/6 $CD4^+$ T cells and BABL/c $CD11c^+$ dendritic cells were co-cultured in the upper chamber of a 24-well transwell system with 0.4 μm-sized pores (Corning, NY, USA). MSCs or ESCs were added in the lower chamber in order to evaluate their suppressive activity across the membrane (Fig. 10A). Next, both $CD4^+$ T cells and $CD11c^+$ dendritic cells were added with MSCs or ESCs in the lower chamber. The upper chamber also contained $CD4^+$ T cells and $CD11c^+$ dendritic cells (Fig. 10B). This modified transwell system was designed to investigate whether soluble factors induced by cell-to-cell contact in the lower chamber can suppress the alloimmune response in the upper

chamber instead of the soluble factors from the stem cells themselves.

7. RT-PCR for immunosuppressive molecules

Both negative co-stimulatory molecules (PDL-1) and apoptosis-inducing molecules (CD30, CD70, Fas ligand, perforin and granzyme B) were screened for unstimulated ESCs and MSCs using RT-PCR. After 72 h of co-culture with $CD4^+$ T cells and allogeneic dendritic cells, ESCs or MSCs were isolated from the MLR using the $CD4^+$ T cell isolation kit and the $CD11c^+$ dendritic cell isolation kit. Expression of the surface molecules was also measured in these stimulated ESCs and MSCs. Primer sequences are summarized in Table 1.

8. Expansion of regulatory T cells

Total $CD4^+$ T cells or $CD4^+GFP^-$ T cells from Foxp3-GFP knock-in C57BL6/J mice were used as responders. $CD4^+GFP^-$ T cells were isolated using a FACSAria machine (BD Bioscience, San Diego, CA, USA). Expansion or induction of $CD4^+Foxp3^+$ T cells was stained with anti-CD4 and anti-CD25 antibody and analyzed by flow cytometric analysis.

9. Skin transplantation experiments

Full-thickness skin from the tails (0.5cm x 0.5cm) were transplanted onto the flanks of 6-8 week old recipient mice across both a major mismatch barrier (Balb/C to C57BL6/J) and a minor mismatch barrier (C57BL6/J male to female). Graft rejection was defined as complete loss of the skin graft. We adoptively transferred one million of ESCs or MSCs to the recipient mice through the tail vein, 1 week before the skin transplantation. As a control group, PBS was injected into the recipient mice. Anti-CD40L antibodies

(0.25mg, MR-1, BioExpress, West Labanon, NH, USA) were injected intraperitoneally on the same day of transplantation in some experiments using ESCs as immunnosuppressants.

10. Islet transplantation experiments

Pancreatic islets were isolated from BALB/c mice using collagenase P (0.8mg/ml, Roche Diagnotics, Milan, Italy) and were purified by Ficoll gradient (Biochrom, Berlin, Germany). Islets (500 IEQ) with or without ESCs ($1x10^4$, $2x10^5$, $3x10^5$) or MSCs ($2x10^5$, $5x10^5$, $1x10^6$) were transplanted beneath left renal capsule of streptozotocin induced (180 mg/kg, Sigma, St. Louis, MO, USA) C57BL/6 diabetic mice. MSCs ($1x10^6$) were injected into the tail vein of recipient mouse before 1 day of transplantation. MMF (40 mg/kg) was intravenously daily injected to the recipient for 14 day after transplantation. Blood glucose level was measured using a blood glucose meter (LifeScan Inc., Milpitas, CA, USA) every other day. Graft rejection was defined as blood glucose concentration higher than 300 mg/dL on two consecutive measurements.

11. Statistical Analysis

The proportions of proliferating cells, regulatory T cells and cytokine levels between the each group were compared using Student's t-test. P value less than 0.05 was considered to be a significant difference. Skin graft survival and islet graft survival rates were calculated by the Kaplan-Meier method and survival rates were compared by the Log-rank test. All analyses were performed using GraphPad Prism (Version 4.03, San Diego, CA, USA).

RESULTS

Characterization of both ESCs and MSCs

ESCs formed embryonic bodies successfully (Fig. 1A). They expressed alkaline phosphatase, SSEA-1, Oct-4 and Sox-2 (Fig.1B-E). These results indicated that these cells were ESCs with intact, undifferentiated characteristics. MSCs expressed CD106, Sca-1 and CD44, whereas they did not express CD34, CD31, CD11c, CD14, or CD45 (Fig. 2A-H). They were successfully differentiated to form either osteoid or adipocytes under conditions with differential differentiation media (Fig. 2I-J). These data confirmed the characteristics of bone marrow-derived MSCs.

In vitro immunosuppressive activity of ESCs and MSCs in primary alloimmune responses

In order to assess whether ESCs themselves induce alloimmune responses, irradiated ESCs from C57BL6/J mice were co-cultured with the responders from the BALB/C mice. The proliferation response of the MLR with allogeneic ESCs was weaker, compared to the allogeneic splenocytes from the C57BL6/J mice (4928 ± 594 vs. $42{,}827 \pm 4088$ CPM, $p = 0.003$, Fig. 3A). IFN-γ production was also very low, when ESCs were co-cultured with allogeneic responders (81.3 ± 3.3 pg/mL, Fig. 3D). Next, we investigated whether ESCs could suppress alloimmune responses *in vitro*. They suppressed proliferation of both syngeneic and allogeneic responders in the primary MLR ($p = 0.015$ with syngeneic responders, Fig. 3B, $p = 0.003$ with allogeneic responders, Fig. 3C). They also suppressed IFN-γ production in the MLR ($p = 0.036$, Fig. 3D). These results demonstrated that ESCs could suppress the *in vitro* primary alloimmune responses of syngeneic and allogeneic responders

and that their own immunogenicity was low. MSCs from BALB/C mice induced neither proliferation (153 ± 49, 163 ± 74 CPM, Fig. 4A) nor IFN-γ production (9.5 ± 0.6 pg/mL, Fig. 4D) of allogeneic responders and irrespective of irradiation. MSCs suppressed proliferation and IFN-γ production by both allogeneic ($p = 0.009$ for proliferation, Fig. 4C, $p = 0.000$ for IFN-γ, Fig. 4D) and syngeneic lymphocytes ($p = 0.003$, for proliferation, Fig. 4B, $p = 0.001$, for IFN-γ, Fig. 4E) in the primary MLR. Taken together, these data showed that both ESCs and MSCs can suppress the primary alloimmune response *in vitro*, whereas they did not induce significant alloimmune responses by themselves.

In vitro immunosuppressive activity of ESCs and MSCs in secondary alloimmune responses

$CD44^+$ T cells were sorted from the sensitized mice eight weeks after sensitization in order to obtain a population of resting memory T cells (Fig. 5A). Greater proliferative response of $CD44^+$ T cells compared with $CD44^-$ T cells from naïve mice, confirmed successful sensitization to alloantigens. Both ESCs and MSCs suppressed proliferation of $CD44^+$ T cells as well as $CD44^-$ T cells in the MLR (Fig. 5B). Both stem cells also suppressed interferon-γ production in the secondary MLR ($p = 0.000$ in ESCs, $p = 0.000$ in MSCs, Fig. 5C), although the secondary MLR induced secretion of much more interferon-γ compared to primary MLR (17036 ± 1060 pg/mL in memory, 1313 ± 95 pg/mL in naïve, $p = 0.000$, Fig. 5C). Overall, these results showed that both ESCs and MSCs could suppress secondary alloimmune responses as well as primary alloimmune responses *in vitro*.

ESCs/MSCs suppressed both proliferation and survival of T cells in alloimmune responses

Simultaneous staining of CFSE and 7-AAD demonstrated that both ESCs and MSCs suppressed not only the proliferation but also the survival of $CD4^+$ T cells in response to stimulation by allogeneic dendritic cells (Fig. 6A-C). They also suppressed both proliferation and survival of $CD4^+$ T cells in response to nonspecific anti-CD3/CD28 stimuli (Fig. 6D). ESCs (Fig. 6E) and MSCs (Fig. 6F) suppressed $CD4^+$ T cells in a dose-dependent manner.

ESCs/MSCs suppressed cytokine production in alloimmune responses

Both types of stem cells suppressed Th1/Th2 cytokines in the MLR (Fig. 7 and Fig. 8, IL-2, IFN-γ; IL-4, IL-10). They also suppressed proinflammatory monokines (IL-12p70 and TNF-α). However, they did not influence either the TGF-β level or IDO activity (Fig. 7H-I, Fig. 8H-I). Interestingly, IL-6 (Fig. 7G, $p = 0.0265$), secretion was increased and GM-CSF (Fig. 8J, $p = 0.1045$), maintained by adding the MSCs whereas it was decreased by adding ESCs (Fig. 7J, $p = 0.0088$). Treatment of blocking anti-GM-CSF or IL-6 antibody on MLR did not showed any significant change (Fig. 9A, B).

Role of cell-to-cell contact in the immunosuppressive activity of ESCs/MSCs

When ESCs were separated from the responders and stimulators by a transwell system (Fig. 10A), their suppressive activity dramatically decreased (Fig. 10C–E). There was a possibility that cell-to-cell contact between the stem cells and other cells induced soluble mediators at the first step and then contact-induced soluble factors mediated immunosuppression by the stem

cells at the second step (Fig. 10B). However, ESCs also failed to significantly suppress alloimmune responses in the upper chamber of the modified transwell system (Fig. 10C–E). Although both contact with the MSCs and soluble mediators from MSCs played a significant role in suppression of T cell proliferation, suppression of T cell survival decreased dramatically by loss of cell-to-cell contact (Fig. 10F–H). In summary, the cell-to-cell contact was the main mechanism of immunosuppression by both ESCs and MSCs.

Roles of surface molecules on ESCs/MSCs in their immunosuppressive activity

Both ESCs and MSCs expressed PDL-1, Fas-L, CD30, perforin and granzyme B (Fig. 11A). Expression of CD70 was very weak at basal status, and increased in response to stimulation (Fig. 11A). Granzyme B expression was confirmed in both ESCs and MSCs by FACS (Fig. 11B). Anti-PDL-1, anti-Fas-L, and anti-CD30 blocking antibodies failed to abrogate immunosuppression by ESCs (Fig. 12) and MSCs (Fig. 13). Although inhibition of perforin did not abrogate immunosuppression, inhibition of granzyme B attenuated immunosuppression by ESCs (Fig. 11C). Neither perforin nor granzyme B was involved in suppression by the MSC (Fig. 11D). Overall, granzyme B was involved in the immunosuppression by ESCs in a perforin-independent manner, whereas none of the above molecules played a significant role in the immunosuppression by the MSCs.

Impacts of ESCs/MSCs on regulatory T cells

Both ESCs and MSCs reduced expression of CD44, an activation marker of effector $CD4^+$ T cells ($p < 0.0001$, Fig. 14A). They also suppressed expression of CD69, an early T cell activation marker ($p = 0.0007$, ESCs; $p = 0.0005$,

MSCs, Fig. 14B). Interestingly, both types of stem cells increased the relative proportion of $CD25^{+}Foxp3^{+}$ cells among the $CD4^{+}$ T cells ($p = 0.0077$, ESCs; $p = 0.0333$, MSCs, Fig. 14C), although absolute numbers of both total $CD4^{+}$ T cells and $CD25^{+}Foxp3^{+}$ cells decreased. When $Foxp3^{-}$ non-regulatory $CD4^{+}$ T cells were co-cultured with the stem cells, both type of stem cells also induced Foxp3 expression ($p < 0.0001$, Fig. 14D). These data suggest that the expansion of $Foxp3^{+}CD4^{+}$ regulatory T cells might be one of the mechanisms of immunosuppression by stem cells.

In vivo immunosuppressive effect of intravenous injection of ESCs or MSCs in allogeneic skin transplantation

We investigated immunosuppressive activities of ESCs and MSCs *in vivo* using adoptive transfer and skin transplantation models. All BALB/c skin grafts in both ESC and MSC group (recipient C57BL6/J) were rejected on day 10, when the skin grafts were rejected in the control group (Fig. 15A). The combination of ESCs and low dose anti-CD40L therapy prolonged graft survival slightly compared to the control group (Fig. 15A, mean survival time 13.2±0.3 days vs. 10.0±0.0 days, p=0.003); however, the combination therapy also failed to induce skin graft tolerance. When donor skin from male C57BL6/J mice was transplanted onto the female C57BL6/J mice, ESCs prolonged skin graft survival slightly compared to the control group; however, the difference was not statistically significant (Fig. 15B, mean survival days 40.7 ± 3.4 days vs. 33.7 ± 1.6 days, p = 0.102). MSCs also failed to significantly prolong skin graft survival (Fig. 15B, mean survival time 38.0 ± 4.5 days vs. 33.7 ± 1.6 days, p= 0.191). Taken together, neither ESCs nor MSCs suppressed graft rejection in the stringent skin transplantation models.

In vivo immunosuppressive effect of co-transplantation with ESCs or MSCs in allogeneic islet transplantation

When ESCs were transplanted together with BALB/c islet into the diabetic C57BL/6 mice, islet graft survival was not prolonged compared to the control group without ESCs (Fig. 16A, mean survival time 16.0±2.6 days vs. 14.7±2.1 days, p=0.4737). Teratoma formation was found in transplanted kidney after 3 week of transplantation (Fig. 16B). Next, co-transplantation of MSCs also failed to prolong islet graft survival compared to the control group without MSCs (Fig. 16C, 14.4±0.6 days vs. 13.75±1.5 days, p=0.7597). When different numbers of ESCs (islet only, $1x10^4$, $2x10^5$, $3x10^5$) were transferred to the islet graft site, the graft survival was not enhanced according to the changed stem cell number (Fig. 18A, mean survival time 14.3±0.6 days vs. 13.7±0.6 days vs. 14.0±0.0 days vs. 8.7 days±0.6, p=0.0295 vs. p=0.3173 vs. p=0.0295). MSCs (islet only, $2x10^5$, $5x10^5$, $1x10^6$) showed the same results (Fig. 18B, mean survival time 14.3±1.2 days vs. 13.7±1.2 days vs. 13.3±1.5 days vs. 10.3 days±1.5, p=0.4561 vs. p=0.3701 vs. p=0.0246). When anti-CD154 antibodies were injected with MSCs, there was no additive suppressive effect between anti-CD154 antibodies and MSCs (Fig. 16D, mean days 64.7±26.5 days vs. 50.8±41.4 days, p=0.9886). Infiltration of immune cells such as $CD4^+$ T cells, $CD8^+$ T cells, B cells, macrophages in the graft site was not changed by co-transplantation with allogeneic islet and MSCs (Fig. 20G-K). These data indicates that co-transplantation of allogeneic islet and ESCs or MSCs did not prolong the graft survival.

In vivo immunosuppressive effect of intravenous injection with MSCs in allogeneic islet transplantation

When MSCs ($1x10^6$) were injected into the tail vein of diabetic C57BL/6 mice

before 1 day of allogeneic islet transplantation, islet graft survival was not prolonged compared with the control group (Fig. 19, mean survival time 12.8±0.5 days vs. 14.71±3.3, p=0.2003). Combination of MSCs and MMF (40 mg/kg for 14day after transplantation) did not have an effect on the graft survival compared with MSC i.v group (Fig. 19, mean survival time 14.71±3.3 days vs. 13.75±3.0, p=0.4154). This indicates that synergistic effect was not worked in combination of MSCs and MMF in allogeneic islet transplantation. Infiltration of immune cells such as $CD4^+$ T cells, $CD8^+$ T cells, B cells, macrophages in the graft site were not changed by intravenous injection of MSCs in allogeneic islet transplantation (Fig. 20L-P). Each immune cells such as $CD4^+$ T cells, $CD8^+$ T cells and $CD4^+FoxP3^+$ T cells in spleen or draining renal lymph node were not significantly changed by MSCs (Fig. 21-22). When *in vitro* MLR was conducted by co-culturing lymphocyte from the transplanted recipient with allogenic antigen presenting cells of 3rd party, proliferation of lymphocyte was not suppressed (Fig. 23, p=0.1360). As a result, these data indicates that intravenous injection of MSCs did not significantly prolong the graft survival in allogeneic islet transplantation.

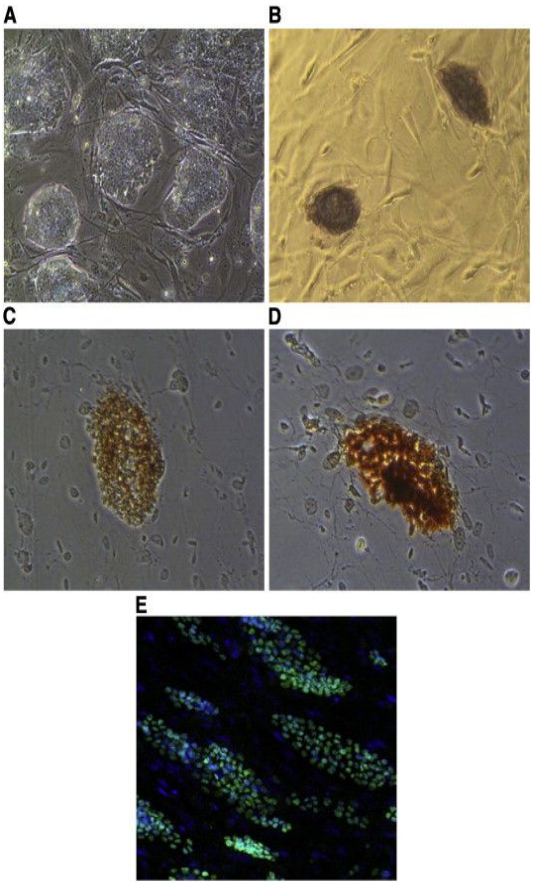

Figure 1. ESCs were characterized by specific phenotype markers.

(A) ESCs formed embryonic colonies successfully. ESCs expressed alkaline phosphatase (B), SSEA-1 (C) and Oct-4 (D) in immunocytochemical staining (×200 magnification). They also expressed Sox-2 (E) in immunofluorescent staining (×200 magnification).

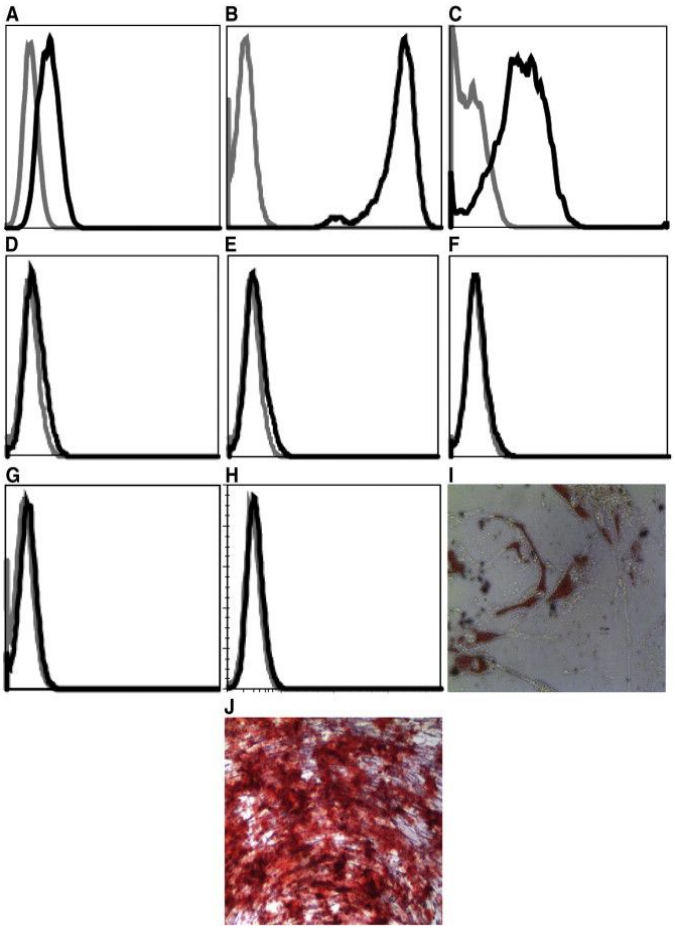

Figure 2. MSCs were characterized by specific phenotype markers.

MSCs expressed CD106 (A), Sca-1 (B) and CD44 (C), whereas they did not express CD34 (D), CD31 (E), CD11c (F), CD14 (G), and CD45 (H) in the FACS analysis. Gray lines and black lines represented isotype control antibody staining and anti-marker antibody staining, respectively. MSCs were successfully differentiated to form either osteoid (Alizarin red staining, I) or adipocytes (oil red staining, J) under conditions with differential differentiation media.

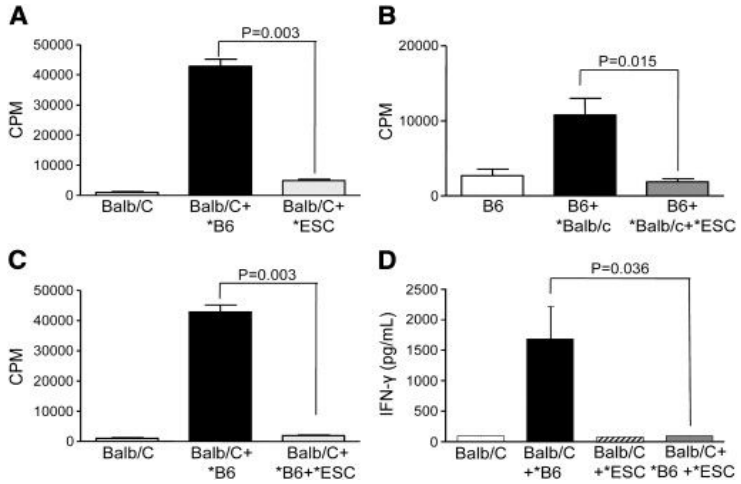

Figure 3. ESCs could suppress the *in vitro* primary allogeneic immune responses.

(A) ESCs did not induce proliferation of allogeneic responders in contrast to splenocytes (A). (A–B) ESCs suppressed proliferation of syngeneic (B) and allogeneic (C) responders in response to allogeneic stimuli. (D) ESCs suppressed interferon-γ production in the mixed lymphocyte reaction. Values are shown as the mean with standard deviation in triplicate. Asterisk mark (*) indicates irradiated status. ESCs, embryonic stem cells. p values were calculated using Student's t-test.

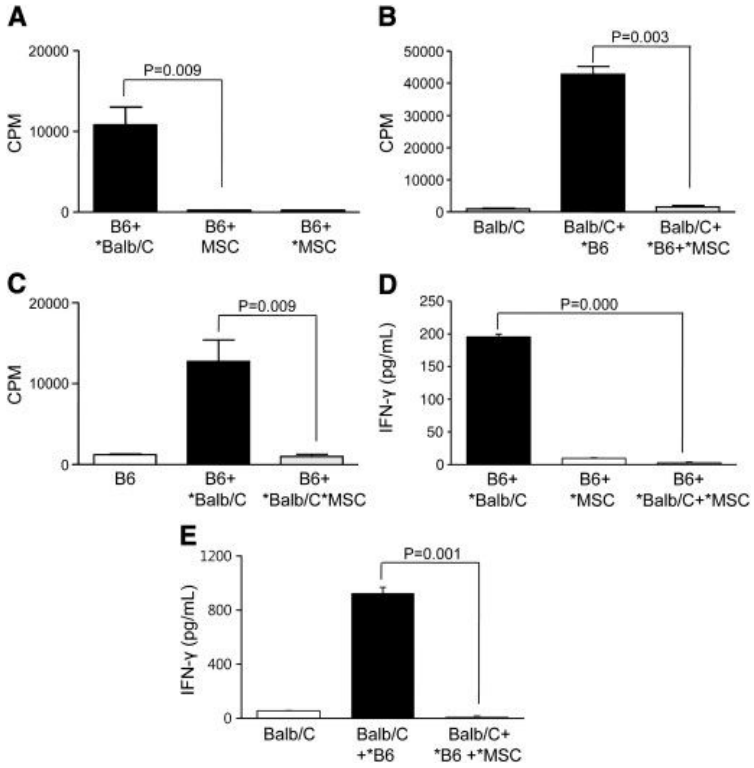

Figure 4. MSCs could suppress the *in vitro* primary allogeneic immune responses.

(A) MSCs did not induce proliferation of allogeneic responders in contrast to splenocytes. (B–C) MSCs suppressed proliferation of syngeneic (B) and allogeneic (C) responders in response to allogeneic stimuli. (D–E) MSCs suppressed interferon-γ production by allogeneic (D) and syngeneic (E) responders in the mixed lymphocyte reaction. Values are shown as the mean with standard deviation in triplicate. Asterisk mark (*) indicates irradiated status. MSCs, mesenchymal stem cells. p values were calculated using Student's t-test.

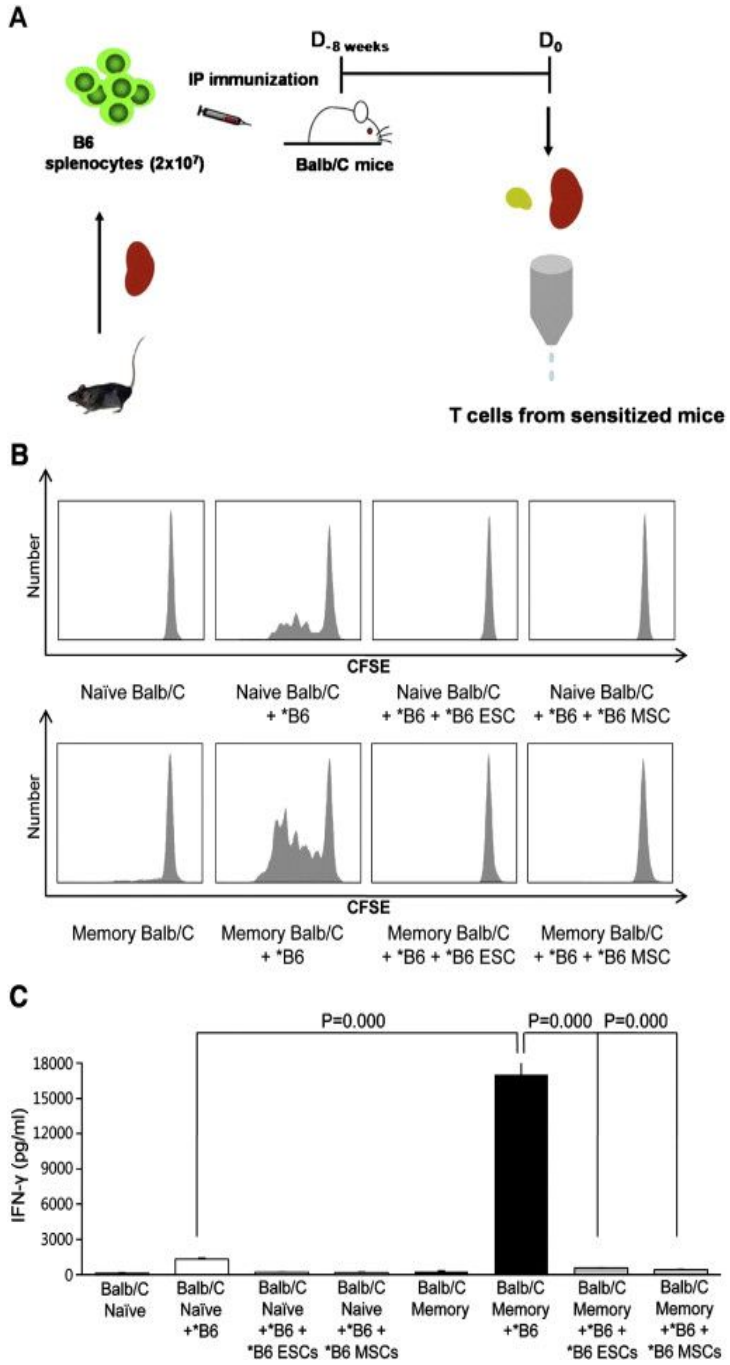

Figure 5. ESCs/MSCs could suppress the *in vitro* secondary alloimmune responses.

(A) C57BL6/J splenocytes were injected into BALB/C mice and T cells were isolated from secondary lymphoid organs at eight weeks after sensitization. (B)

Both ESCs and MSCs suppressed T cell proliferation in secondary alloimmune responses by $CD44^+$ memory T cells as well as in primary alloimmune response by $CD44^-$ naïve T cells. CFSE histograms are representative of three independent experiments. (C) Both ESCs and MSCs suppressed interferon-γ production in secondary alloimmune responses as well as in primary alloimmune responses. Values are shown as the mean with standard deviation in triplicate. Asterisk mark (*) indicates irradiated status. ESCs, embryonic stem cells. MSCs, mesenchymal stem cells. p values were calculated using T-test.

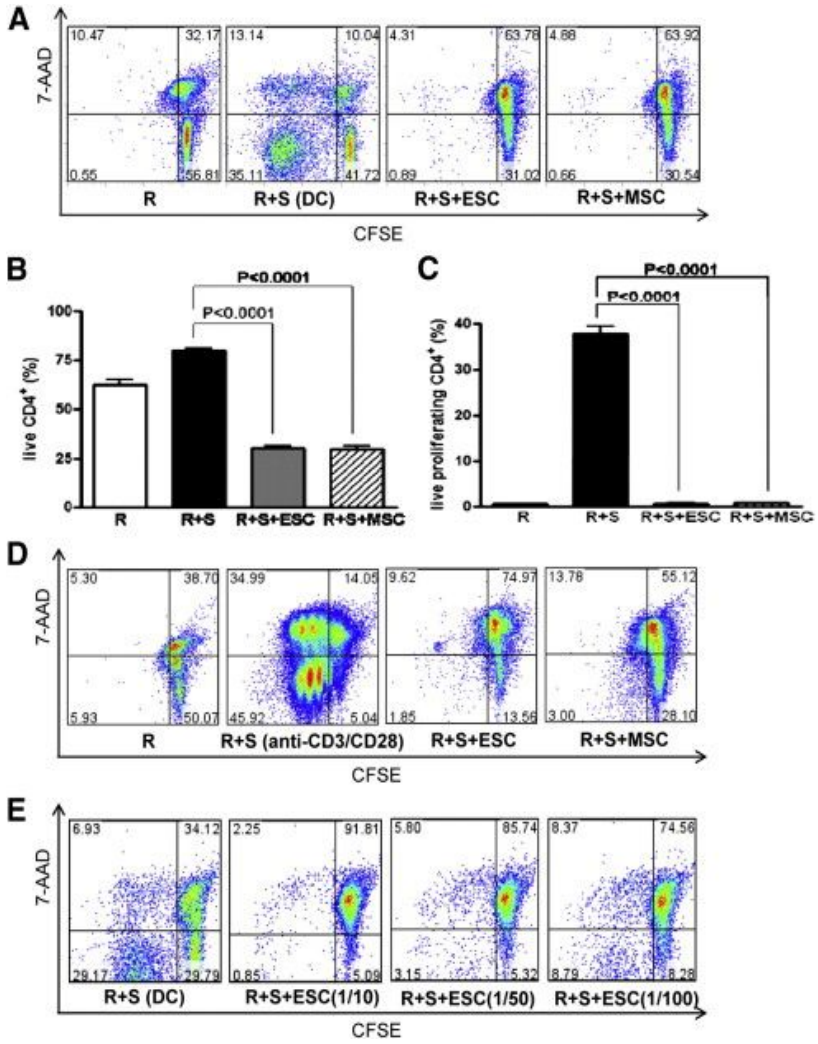

Figure 6. ESCs/MSCs could suppress not only the proliferation but also the survival of T cells in an *in vitro* alloimmune response.

(A) When the ESCs or MSCs were co-cultured with C57BL/6 $CD4^+$ T cells and BALB/c splenic DCs for 3 days, both the proliferation and survival of $CD4^+$ T cells were strongly suppressed. (B) The proportion of live $CD4^+$ T cells decreased significantly by either ESCs or MSCs ($p < 0.0001$; $p < 0.0001$). (C) The proportion of live proliferating $CD4^+$ T cells decreased significantly by either ESCs or MSCs ($p < 0.0001$; $p < 0.0001$). (D) Both ESCs and MSCs also suppressed the proliferation and survival of $CD4^+$ T cells in response to

anti-CD3/CD28 stimulation. When the ratio of responders to ESCs (E) or MSCs (F) was changed, the suppressive effects of ESCs or MSCs were dose-responsive. Mean value with standard deviation among the triplicates was displayed in each bar graph. These results were representative of more than 3 iterations of the experiments. ESCs, embryonic stem cells; MSCs, mesenchymal stem cells; R, responders; S, stimulators; DC, dendritic cells

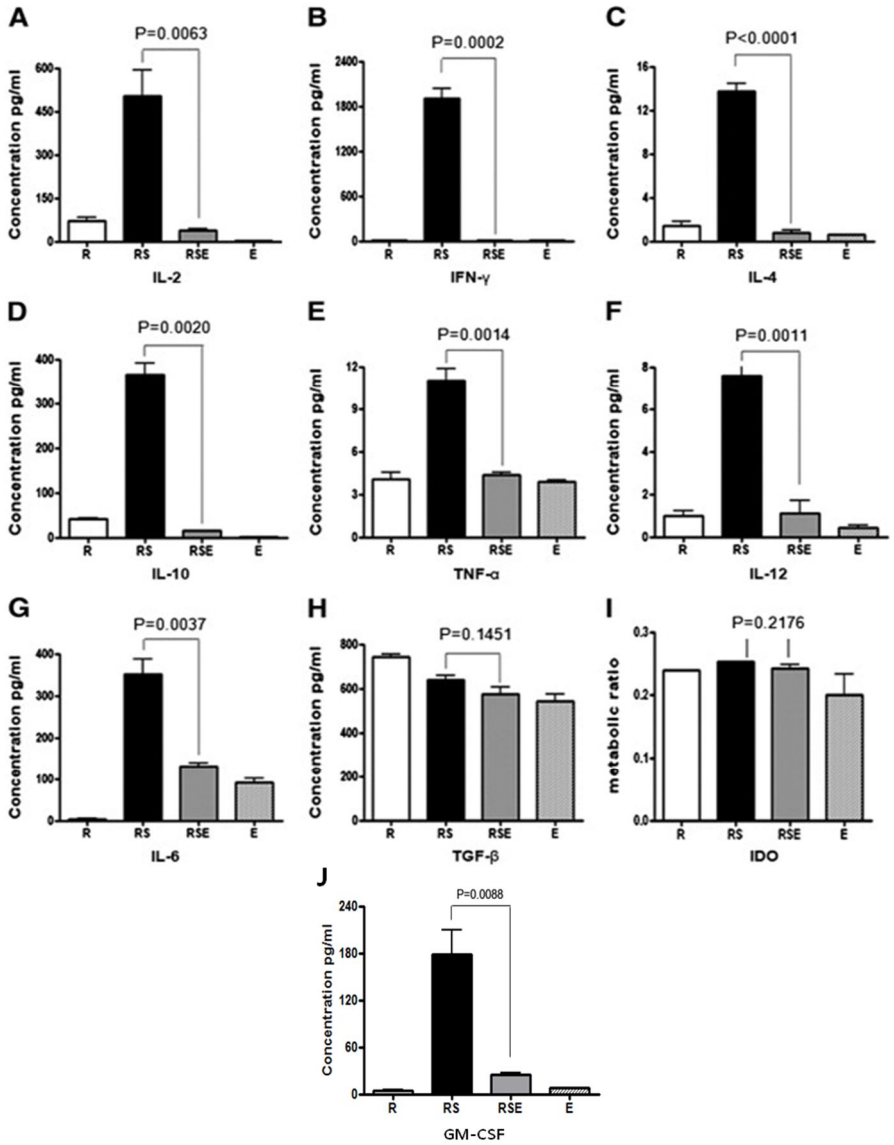

Figure 7. ESCs could suppress cytokine production in an *in vitro* alloimmune response.

After ESCs were co-cultured with C57BL/6 $CD4^+$ T cells and BALB/c splenic dendritic cells for 3 days, the cultured supernatant was harvested and cytokine levels were measured. ESCs suppressed secretion of IL-2 (A), IFN-γ (B), IL-4 (C), IL-10 (D), TNF-α (E), IL-12 (F), and IL-6 (G). However, there was no significant change in the levels of TGF-β ($p = 0.1451$, H) or IDO activity ($p =$

0.2176, I). Experiments in each group were performed in triplicate. Mean value with standard deviation among the triplicates was displayed in each bar graph. These results were representative of 2 experiments. E, embryonic stem cells; R, responders; S, stimulators.

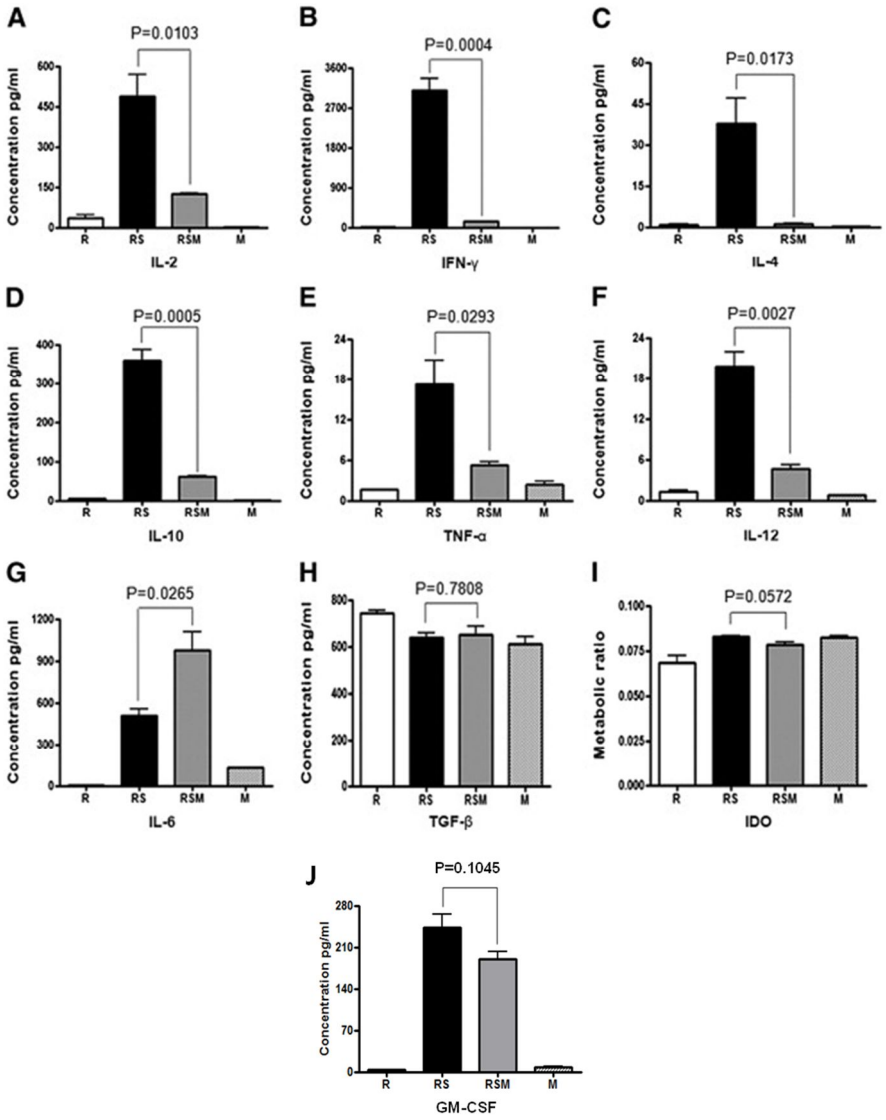

Figure 8. MSCs could suppress cytokine production in an *in vitro* alloimmune response.

After the MSCs were co-cultured with C57BL/6 $CD4^+$ T cells and BALB/c splenic dendritic cells for 3 days, the cultured supernatant was harvested and cytokine levels were measured. The MSCs suppressed secretion of IL-2 (A), IFN-γ (B), IL-4 (C), IL-10 (D), TNF-α (E), and IL-12 (F). Interestingly, the MSCs increased the IL-6 level ($p = 0.0265$, G). The levels of TGF-β ($p = 0.7808$, H) and IDO activity ($p = 0.0572$, I) were unchanged. Experiments in

each group were performed in triplicate. Mean value with standard deviation among the triplicates was displayed in each bar graph. These results were representative of 2 experiments. M, mesenchymal stem cells; R, responders; S, stimulators.

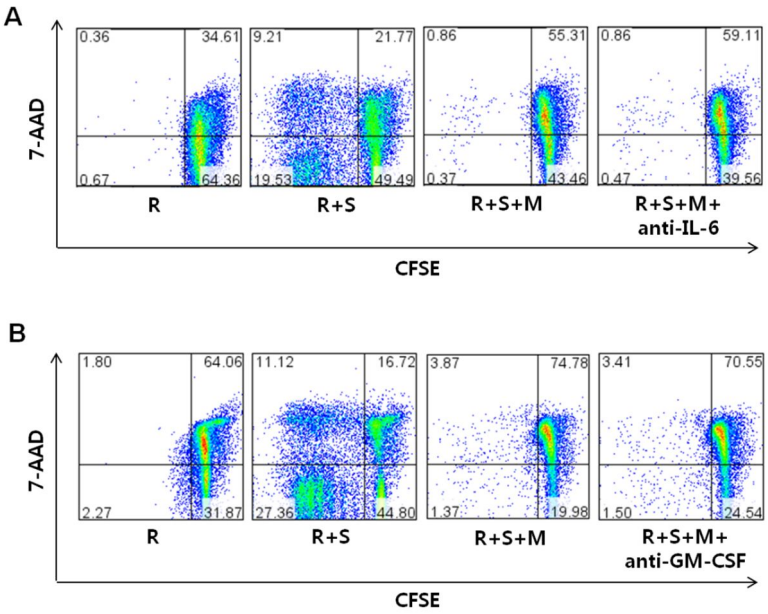

Figure 9. IL-6 and GM-CSF on MSCs did not show the immunosuppressive effect in an *in vitro* alloimmune response.

(A) Anti-IL-6 could not reduce immunosuppressive effect of MSCs on allogeneic T cell proliferation and survival. (B) Anti-GM-CSF could not reduce immunosuppressive effect of MSCs on allogeneic T cell proliferation and survival.

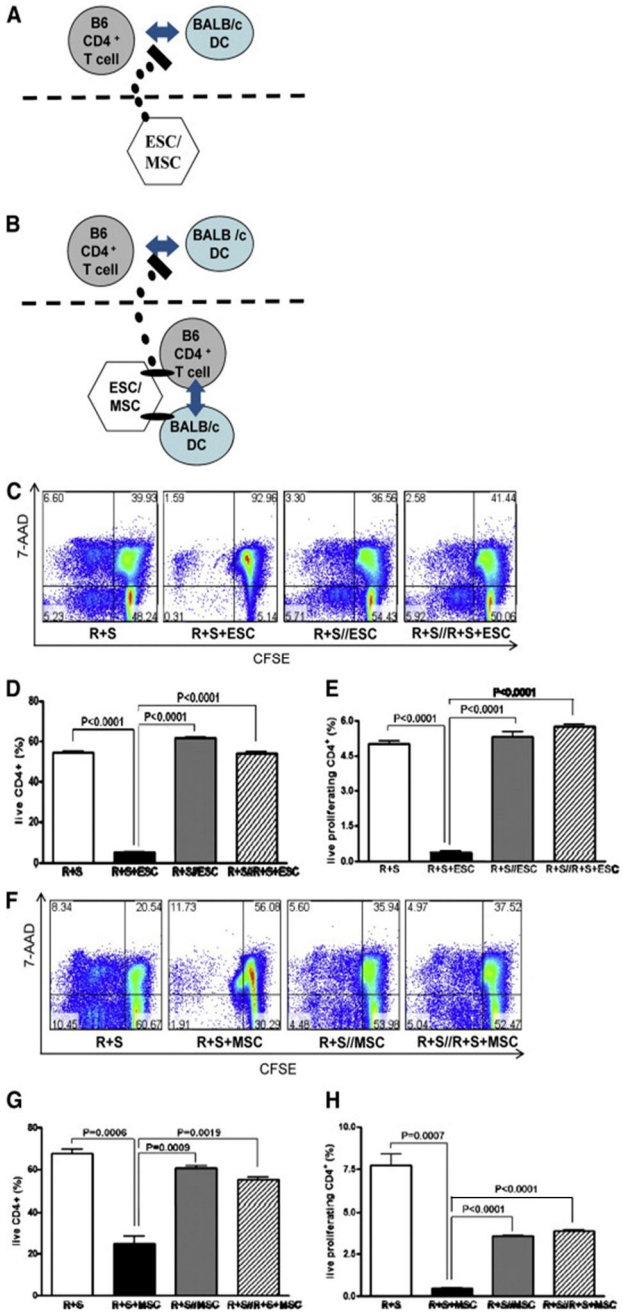

Figure 10. ESCs/MSCs could not suppress the proliferation and survival of T cells mainly through cell-to-cell contact dependent mechanisms in an *in vitro* alloimmune response.

(A) ESCs or MSCs were added in the lower chamber, while C57BL/6 (B6) $CD4^+$ T cells and BALB/c splenic dendritic cells (DC) were co-cultured in the

upper chamber of the transwell plate. (B) In a modified transwell system, ESCs or MSCs were co-cultured with C57BL/6 (B6) $CD4^+$ T cells and BALB/c DCs in the lower chamber, while C57BL/6 $CD4^+$ T cells and BALB/c DCs were co-cultured in the upper chamber. (C–E) Suppression by ESCs dramatically decreased in both classic ($p < 0.0001$, R + S//E) and modified transwell systems ($p < 0.0001$, R + S//R + S + E). (F–H) Suppression by the MSCs also significantly decreased in both classic ($p = 0.0009$ for survival, $p < 0.0001$ for proliferation, R + S//M) and modified transwell systems ($p = 0.0019$ for survival, $p < 0.0001$ for proliferation, R + S//R + S + M). Mean value with standard deviation among the triplicates was displayed in each bar graph. These results were representative of 3 experiments. ESCs, embryonic stem cells, MSCs, mesenchymal stem cells; R, responders; S, stimulators, DC, dendritic cells.

Molecules	Direction	Sequence
PDL-1	Forward	TTA CTG CTG CAT AAT CAG CTA CG
	Reverse	CGT CTC CTC GAA TTG TGT ATC AT
Fas-L	Forward	GCC CAT GAA TTA CCC ATG TC
	Reverse	GTT CTG CCA GTT CCT TCT GC
CD30	Forward	CAA CCC TGG CTG AGT TAC TC
	Reverse	AGC GGC AGG TTC TTC AGG TA
CD70	Forward	GTA GCG GAC TAC TCA GTA AG
	Reverse	CAA GGG CAT ATC CAC TGA AC
Perforin	Forward	ACA GCT GAT GAG GTA GGA GAC TG
	Reverse	TGT AGT CTC CCC ACA GAT GTT CT
Granzyme B	Forward	TCC TTA TTC GAG AGG ACT TTG TG
	Reverse	GGA GGT GAA CCA TCC TTA TAT CC
GAPDH	Forward	TGC TGA GTA TGT CGT GGA GTC TA
	Reverse	GAT GTC ATC ATA CTT GGC AGG TT

Table 1. Primer sequences of immunosuppressive molecules

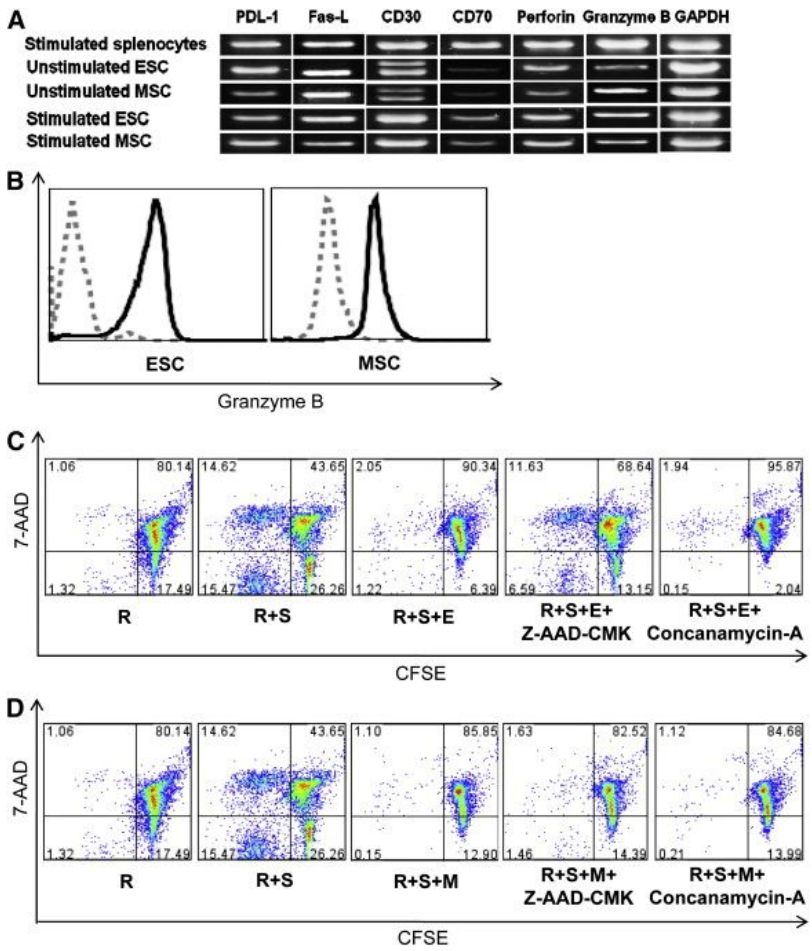

Figure 11. Granzyme B on ESCs could suppress the *in vitro* alloimmune responses.

(A) Unstimulated ESCs and MSCs expressed PDL-1, Fas-L, CD30, perforin and granzyme B in RT-PCR. Expression of these molecules in the stimulated stem cells was similar, except induction of CD70 by stimulation. Splenocytes that were stimulated by allogeneic splenocytes were used as control cells. (B) Both ESCs and MSCs expressed granzyme B in intracellular flowcytometric analysis. A gray dotted line indicated isotype control antibody staining and black; a solid line indicated anti-granzyme B staining. (C) Granzyme B inhibition by Z-AAD-CMK abrogated suppression by ESCs, whereas perforin inhibition by concanamycin-A failed to abrogate the suppression. (D)

Inhibition of neither perforin nor granzyme B abrogated suppression by the MSCs. These results were representative of 3 experiments. ESCs or E, embryonic stem cells, MSCs or M, mesenchymal stem cells; R, responders; S, stimulators.

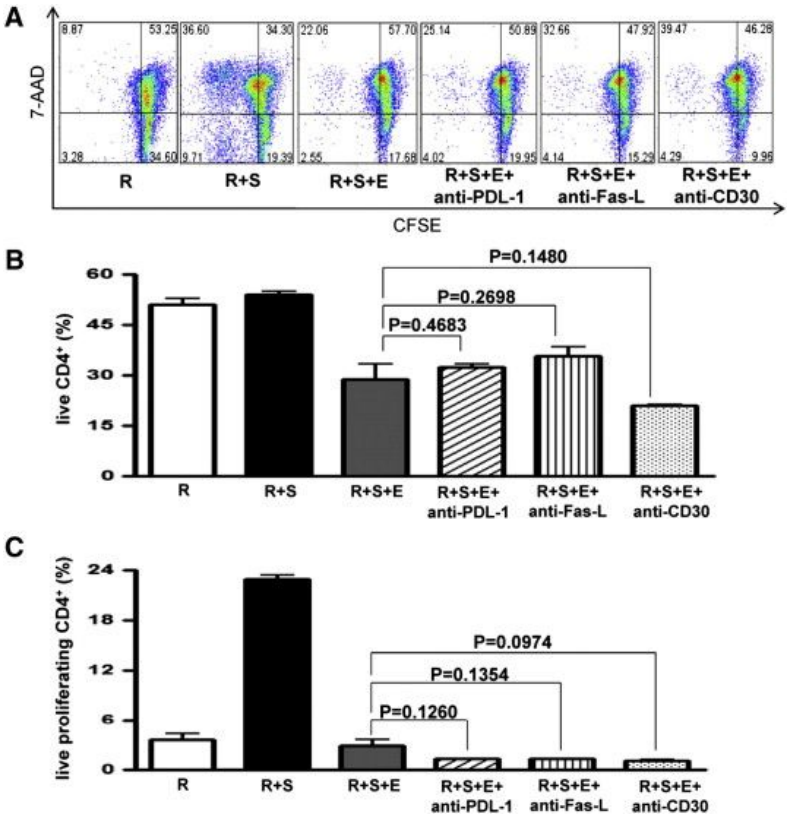

Figure 12. PDL-1, Fas-L, and CD30 on ESCs did not show the immnosuppressive effect in an *in vitro* alloimmune response.

(A) Anti-PDL-1, anti-Fas-L, and anti-CD30 blocking antibodies failed to abrogate suppression by ESCs. (B) Anti-PDL-1, anti-Fas-L, and anti-CD30 blocking antibodies failed to abrogate ESC's suppression on T cell survival ($p = 0.4683$; $p = 0.2698$; $p = 0.1480$). (C) Anti-PDL-1, anti-Fas-L, and anti-CD30 blocking antibodies failed to abrogate ESC's suppression on T cell proliferation ($p = 0.1260$; $p = 0.1354$; $p = 0.0974$). Mean value with standard deviation among the triplicates was displayed in each bar graph. These results were representative of 3 iterations of the experimental protocols. E, embryonic stem cells, R, responders; S, stimulators

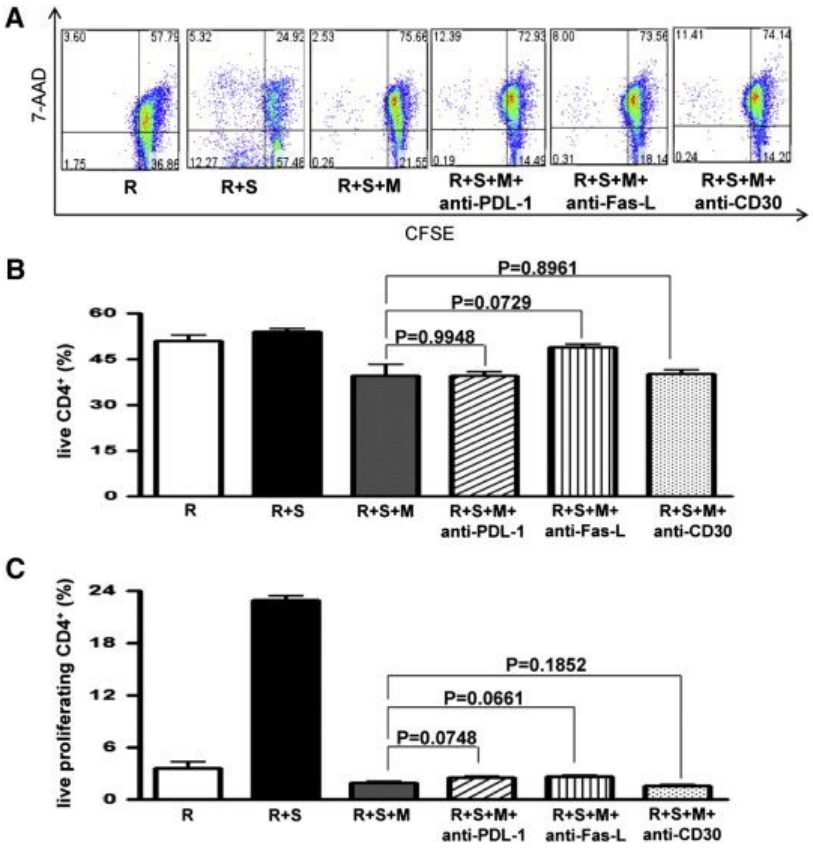

Figure 13. PDL-1, Fas-L, and CD30 on MSCs did not show the immnosuppressive effect in an *in vitro* alloimmune response.

(A) Suppression by MSCs was not abrogated by anti-PDL-1, anti-Fas-L, or anti-CD30 blocking antibodies. (B) Anti-PDL-1, anti-Fas-L, and anti-CD30 blocking antibodies failed to abrogate ESC's suppression on T cell survival ($p = 0.9948$; $p = 0.0729$; $p = 0.8961$). (C) Anti-PDL-1, anti-Fas-L, and anti-CD30 blocking antibodies failed to abrogate ESC's suppression on T cell proliferation ($p = 0.0748$; $P = 0.0661$; $p = 0.1852$). Mean value with standard deviation among the triplicates was displayed in each bar graph. These results were representative of 3 iterations of the experimental protocols. M, mesenchymal stem cells; R, responders; S, stimulators.

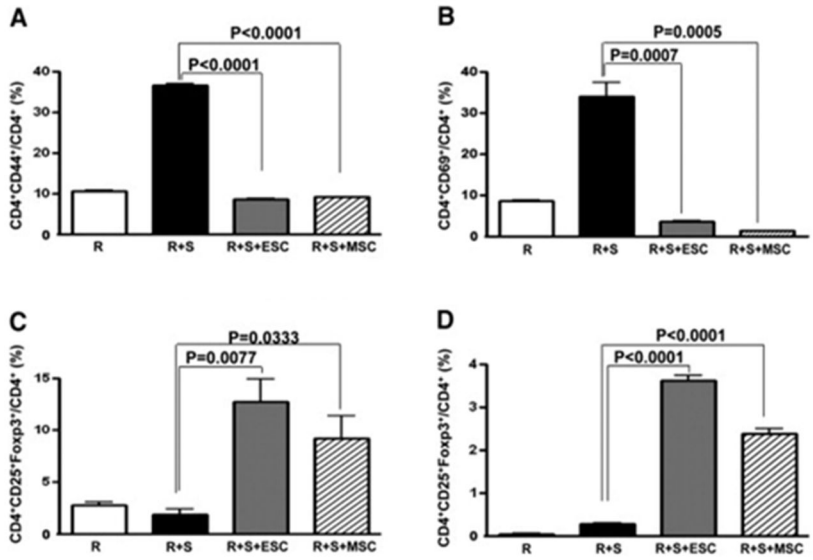

Figure 14. ESCs/MSCs could suppress the activation of $CD4^+$ T cells and induce the expansion of regulatory T cells in an *in vitro* alloimmune response.

(A) After 3 days of co-culture with C57BL/6 $CD4^+$ T cells and BALB/c $CD11c^+$ DCs, ESCs/MSCs reduced the expression of CD44, a T cell activation marker on $CD4^+$ T cells ($p < 0.0001$). (B). ESCs/MSCs also reduced the expression of CD69, another activation marker of $CD4^+$ T cells ($p = 0.0007$, 0.0005). (C) Both ESCs and MSCs increased the proportion of CD4+CD25+Foxp3+ T cells after 3 days of co-culture ($p = 0.0077$, ESCs; $p = 0.0333$, MSCs). (D) Both ESCs and MSCs induced $CD4^+CD25^+Foxp3^+$ T cells from $CD4^+Foxp3^-$ T cells after 3 days of co-culture of $CD4^+Foxp3^-$ T cells with ESCs/MSCs ($p < 0.0001$). Experiments in each group were performed in triplicate. Mean value with standard deviation among the triplicates was displayed in each bar graph. ESCs, embryonic stem cells, MSCs, mesenchymal stem cells; R, responders; S, stimulators.

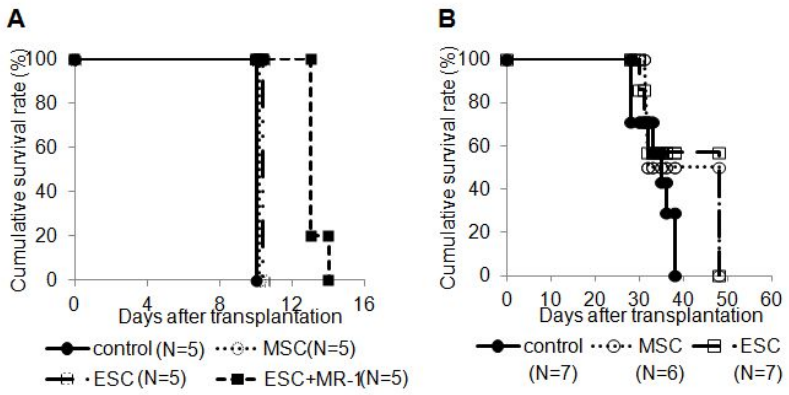

Figure 15. Intravenous injection of ESCs or MSCs could not prolong allogeneic skin graft survival.

(A) Neither ESCs nor MSCs prolonged skin graft survival across a major mismatch barrier (Balb/C to C57BL6/J). A low dose of MR-1 combined with ESCs also failed to induce skin graft tolerance. (B) Neither ESCs nor MSCs prolonged skin graft survival significantly across a minor mismatch barrier (C57BL6/J male to female). ESCs, embryonic stem cells. MSCs, mesenchymal stem cells.

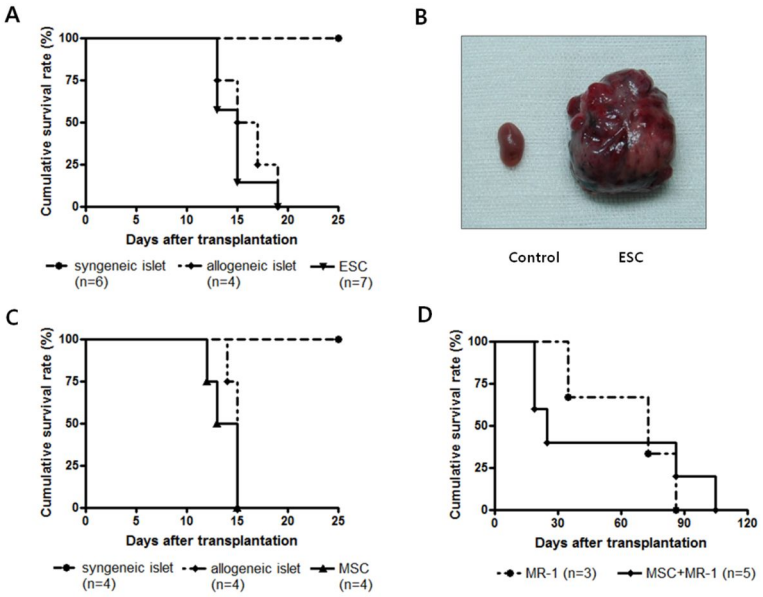

Figure 16. Co-transplantation of ESCs or MSCs could not prolong allogeneic islet graft survival.

(A) ESCs did not prolong islet allograft survival across a major mismatch barrier (BALB/C to C57BL6/J). (B) ESCs generated teratoma at the graft site in 3 weeks after transplantation. (C) MSCs also failed to prolong islet allograft survival. (D) When MSCs were combined with 0.25mg of anti-CD40L antibodies, there was no additive immunosuppressive effect between MSCs and anti-CD40L antibodies. P values were calculated using Log-rank test. ESCs, embryonic stem cells; MSCs, mesenchymal stem cells.

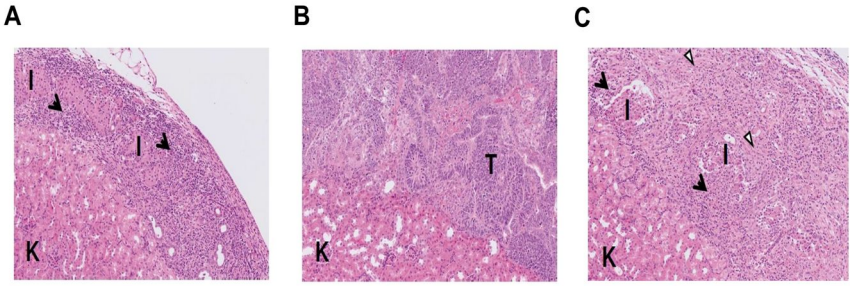

Figure 17. *In vivo* histology in the graft site of co-transplantation with ESCs or MSCs.

Immune cell infiltration was not suppressed in co-transplantation group (C) with MSCs, compared with islet only group (A). Especially, co-transplantation group (B) with ESCs showed the teratoma (H&E, 100 x). I, islet; K, kidney; T, teratoma; black arrow, immune cells; white arrow, MSCs; ESCs, embryonic stem cells; MSCs, mesenchymal stem cells.

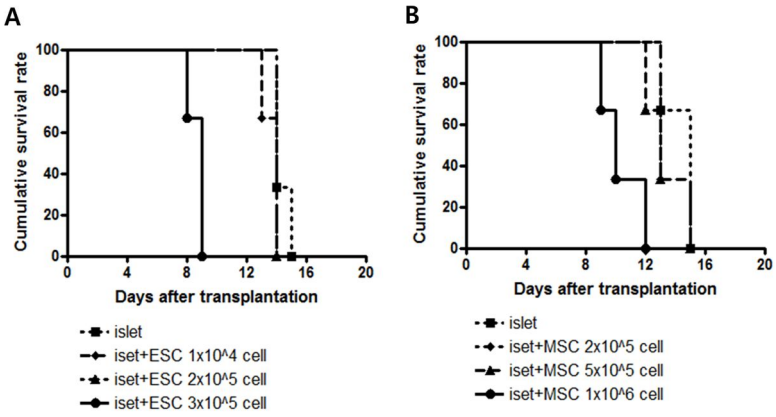

Figure 18. Allogeneic islet graft survival was not prolonged by the increased cell number ratio of ESCs or MSCs.

(A) When more ESCs were co-transplanted, conversely the graft survival rate was reduced. (B) Although co-transplanted MSCs' cell number was increased, the graft survival rate was not enhanced.

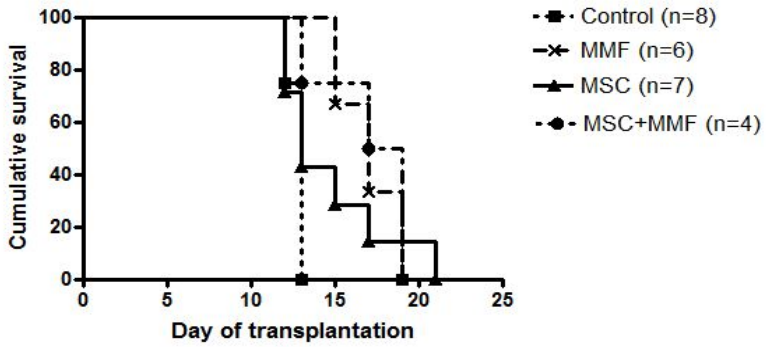

Figure 19. Intravenous injection of MSCs could not prolong allogeneic islet graft survival.

MSCs could not prolong islet allograft survival across a major mismatch barrier (BALB/C to C57BL6/J). Combination of MSCs and MMF (40 mg/kg for 14day after transplantation) did not show the effect on the graft survival compared with only MSC i.v. group.

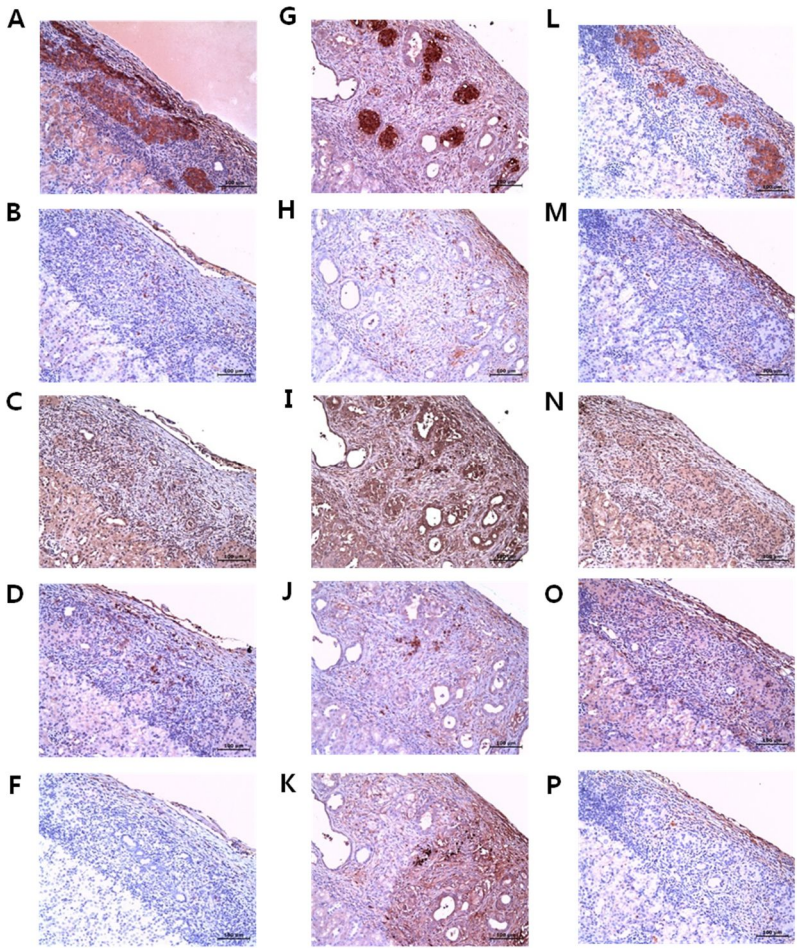

Figure 20. MSCs by co-transplantation or intravenous injection did not affect the infiltration of immune cells in the allogeneic islet graft site.

Infiltration of immune cells among islet only group (A, B, C, D, E, F), co-transplantation group (G, H, I, J, K) and intravenous injection of MSCs group were not significantly changed (L, M, N, O, P). A, G, L, insulin; B, H, M, $CD4^+$ T cell ; C, I, N, $CD8^+$ T cell; D, J, O, B cell; F, K, P, macrophage (100 x).

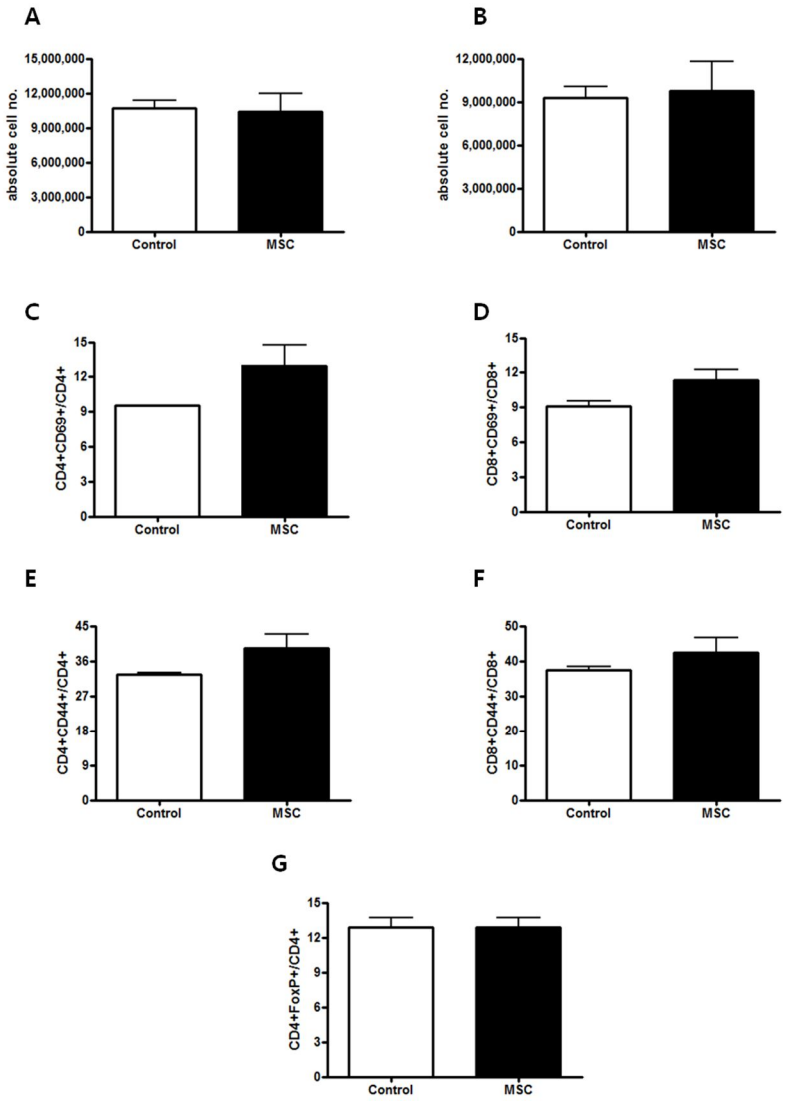

Figure 21. Intravenous injection of MSCs did not affect T cell population in the spleen of transplanted recipients.

(A, B) Absolute cell number of $CD4^+$ T cells and $CD8^+$ T cells between control and MSCs group was not changed significantly ($CD4^+$ T cells, p=0.8728; $CD8^+$ T cells, p=0.8390). (C, E) $CD4^+CD69^+$ T cell and $CD4^+CD44^+$ T cell population between control and MSCs group was not changed significantly (p=0.1323, p=0.1293). (D, F) $CD8^+CD69^+$ T cell and $CD8^+CD44^+$ T cell population between control and MSCs group was not

changed significantly (p=0.0932, p=0.3112).

(G) $CD4^{+}FoxP3^{+}$ T cell population between control and MSCs group was not changed significantly (p=1.0000).

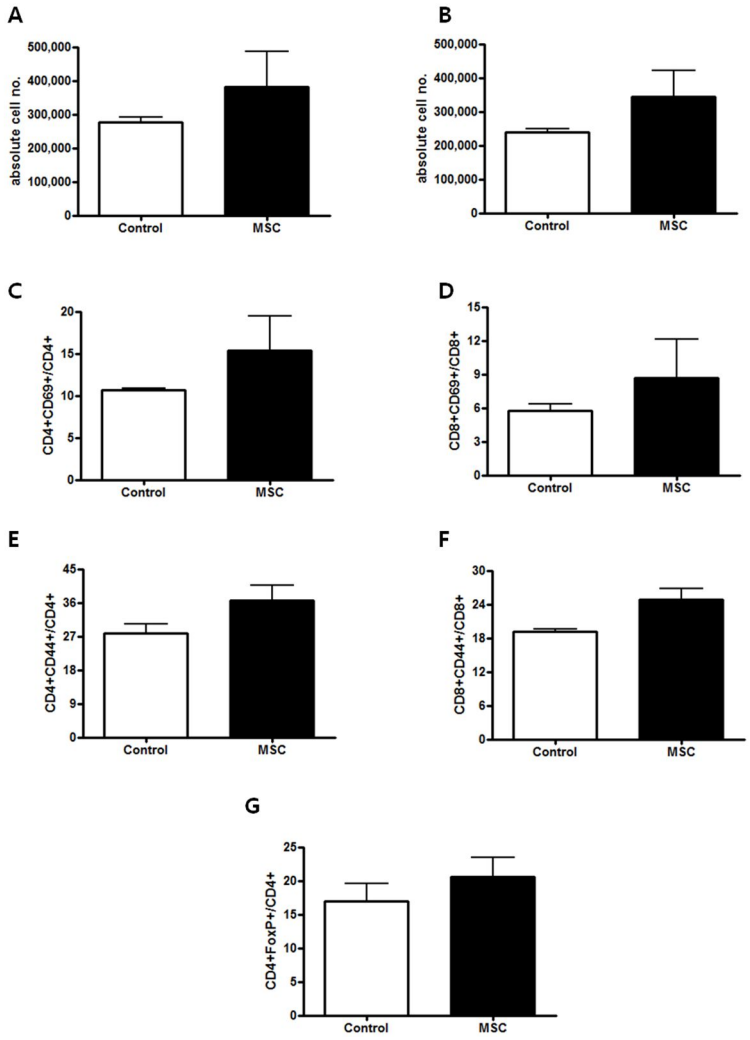

Figure 22. Intravenous injection of MSCs did not affect T cell population in the renal lymph node of transplanted recipients.

(A, B) Absolute cell number of $CD4^+$ T cells and $CD8^+$ T cells between control and MSCs group was not changed significantly ($CD4^+$ T cells, p=0.3795; $CD8^+$ T cells, p=0.2518). (C, E) $CD4^+CD69^+$ T cell and $CD4^+CD44^+$ T cell population between control and MSCs group was not changed significantly (p=0.3076, p=0.1372). (D, F) $CD8^+CD69^+$ T cell and $CD8^+CD44^+$ T cell population between control and MSCs group was not changed significantly (p=0.4492, p=0.0540). (G) $CD4^+FoxP3^+$ T cell

population between control and MSCs group was not changed significantly ($p=0.4124$).

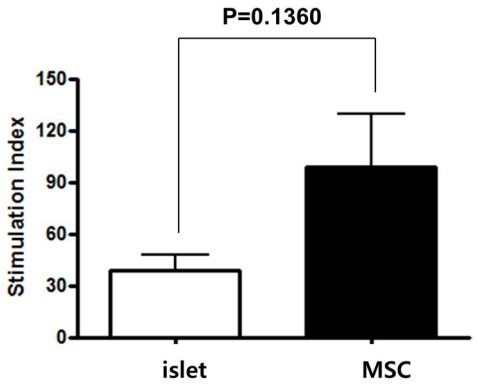

Figure 23. MSCs by intravenous injection could not show the immunosuppressive effect in the lymph node of transplanted recipients.

Proliferation of lymphocyte by allogeneic stimulators of 3rd party was not significantly suppressed in the MSCs' group (p=0.1360) compared with the control group.

DISCUSSION

Even though several immunosuppressve effects of ESCs or MSCs have been studied, there have been controversial results and interpretations depending on the each research group. In the current studies, it has been shown that ESCs and MSCs are not triggering the occurrence of alloimmune response. In addition, their immunosuppressive effects on alloimmune response have been demonstrated in an *in vitro* experiment whereas not in an *in vivo* transplantation model such as allogeneic skin or pancreatic islet.

Not triggering the alloimmune response by both stem cells in an *in vitro* is based on the previous findings that both ESCs and MSCs have a lower potential to express MHI I molecules, key immunological markers for antigen-presenting cells, do not express MHC II molecules and have a low expression on co-stimulatory molecules such as CD80 and CD86. In particular, it has been reported that MSCs do not express pro-inflammatory cytokines, and these include IL-2, IL-3, IL-4 and IL-5 (40, 41). Previous reports have shown that ESCs release TGF-β, one of the representative immunosuppressive molecules, and thereby show an immunosuppressive effect (11). Likewise, MSCs have also been reported to express immunosuppressive molecules such as PDL-1, Fas-L, IDO, IL-6, IL-10, PGE-2 and IL-10 (19, 42, 43, 44). As the mechanisms by which ESCs or MSCs show an immunosuppressive effect, there have been demonstrated by two different pathways due to the cell-to-cell contact or soluble factors in each research group (10, 11, 19). Besides, there are also contradictory reports that they also have an immunosuppressive effect on the proliferation or survival of T-cells (20, 21). The results of thymidine uptake, predominantly used in previous studies, remain problematic in that it is also subject to change depending on the proliferation or survival of T cells, both factors cannot be

separately considered and thymidine can also be consumed by the proliferated stem cells. Therefore, in the current study, for the purposes of simultaneous analyzing the proliferation and survival of T-cells, we stained $CD4^+$ T cells as responder with CFSE and 7-AAD. As a result, we found that both types of stem cells suppressed both proliferation, survival and the major immunosuppressive mechanisms of both types of stem cells are based on the cell-to-cell by transwell experiment in the alloimmune response. As reported to be soluble factors with an immunosuppressive effect by ESCs and MSCs, however TGF-β and IDO showed no specific changes in the alloimmune response. In particular, in association with IL-6 and GM-CSF produced with the addition of MSCs, there were no immunosuppressive effects. The difference in the degree of IL-6 expression between the two types of stem cells was in agreement with previous reports (45). Moreover, unlike previous reports, there were no immunosuppressive effects following the blocking of Fas-L and PDL-1 on both stem cells in an *in vitro* allogeneic MLR. These phenomena were also seen in the treatment anti-CD30 blocking antibody and perforin inhibitor in the same experiment. In the previous study, MSCs which expressed Fas-L showed immnosuppressive efficacy in an *in vivo* mouse model, in addition, showed an immunosuppressive effect in clinical studies that have been conducted in patients with immune related diseases (46). The differences between previous studies and these studies include the experimental conditions such as the number of responsive cells and the types of stimuli in an *in vitro* MLR. Furthermore, because a multiple immunosuppressive molecules released from stem cells can be involved, it is probable that the immunosuppressive functions of specific molecules might not be confirmed. It is noteworthy that granzyme B in ESCs had an immunosuppressive effect on the proliferation and survival of T cells by

perforin independent pathway in alloimmune response. Previous studies have also shown that granzyme B had an immunosuppressive effect in plasmacytoid dendritic cells and regulatory T cells via a signal pathway that is independent from perforin (47, 48). The uptake of granzyme B into T cells occurs via a receptor-mediated endocytosis or fluid-phase one independently from perforin pathway by cell-to-cell contact (48, 49, 50, 51). This eventually induces the apoptosis of T cells and lowers the activity of intracellular molecules that are associated with the proliferation of T cells (52, 53, 54). The proliferation of regulatory T cells was also induced in ESCs as well as MSCs. This indicates that the induction of regulatory T cells might be another mechanism by which both types of stem cells regulate the immune responses.

To elucidate the immunosuppressive effects of both type of stem cells in an *in vivo* environment, we analyzed whether ESCs and MSCs could enhance the survival of allogeneic skin tissue and pancreatic islet. Memory T-cells have been well known that it can be an obstacle to the survival and function sustainability of the tissue graft by their strong survival, a rapid reactivation, a variety of immune functions and responsiveness to donor-specific antigens. In the adjustment of these memory T-cells, strong immunosuppressive agents are essential. Because there are limitations of the immunosuppressive agents in that they produce such side effects as infection and tumor, the cell therapy agents with an immunosuppressive ability can be alternatively used. More than anything else, there are limitations in that regulatory T cells cannot regulate the immune responses of memory T cells if they are induced in an *in vitro* environment (23). In the current study, both type of stem cells could significantly suppress naïve and memory T cell in an *in vitro* alloimmune response. However, both types of stem cells did not significantly enhance the survival of the allograft skin tissue in both the major mismatch and minor

mismatch barrier. These results suggest that it is inefficient to regulate memory T cells with both types of stem cells in an *in vivo* allogeneic skin and pancreatic islet transplantation. In addition, we also attempted to obtain the additional effects in enhancing the survival of the allograft skin tissue with a concomitant use of immunomodulators such as anti-CD40L antibodies, but failed to obtain the additional effects that are statistically significant. For the further study, it will be required to develop the optimal condition for the immunosuppressive protocol with stem cells and immnosuppressants in order to regulate memory T cells in an *in vivo* environment.

There are also contradictory reports in an *in vivo* environment. ESCs with an immunosuppressive ability showed the increased immunogenicity when they were differentiated into other types of cells (55) and showed the immune rejection in the xenotransplantation (56). It has also been proposed that they can be used as a tool to increase the immune responses against cancer (57). In addition, it has also been reported that MSCs also have an immunogenicity when they are infused in an *in vivo* environment (58). In organ transplantation, previous reports have shown that ESCs of the rats enhanced the survival of allograft cardiac tissue. However, murine ESCs had no such effects (59, 60). As shown herein, there might be a difference in the degree of immune modulation between the species of stem cells. There are also several reports that MSCs also had an immunosuppressive effect in an animal experimental model of skin transplantation using nonhuman primates (27). But their results could not be are limited because of a small number of experimental animals. There are also contradictory reports that the degree of graft rejection was increased in an animal experimental model of allograft heart transplantation using mice (60). With regard to the difference in the degree of immunosuppressive activity of stem cells between an *in vitro* environment

and an *in vivo* one in the current study, it is probable that the number of infused stem cells is insufficient to adjust the powerful allogeneic rejection and an intravenous injection cannot achieve an cell-to-cell contact between the stem cells and the immune cells. One the other hand, it is also probable that ESCs or MSCs would have a immunogenicity if they are differentiated into other cells in an *in vivo* environment. When we transplanted allograft pancreatic islet mixed with MSCs into the subrenal capsule or infused the stem cells into the venous vessels of recipient tail prior to the transplantation, we failed to achieve a significantly increased survival of the allograft pancreatic islet despite a concomitant use of MMF. According to the previous studies, following the infusion of a small amount of T-cells in immunodeficient mice with co-transplantation of MSCs to the allograft pancreatic islet, the survival of allograft pancreatic islet was increased as compared with the only islet transplantation group (37). But we used a much greater number of T-cells in wild-type mouse recipient model compared with animal experiments attempted in the previous studies. By this reason, we speculate that the it would be insufficient to regulate immune rejection and prolong the graft survival in our *in vivo* environment system. On the other hand, in the presence of the increased number of MSC cells, an animal experimental model of subrenal capsular co-transplantation of the allograft pancreatic islet with MSCs showed the decreased survival ratio compared with control group. MSCs are present with heterogeneous cells (61), and they can differentiate into various types of cells in an *in vivo* environment. Besides, they can undergo a rapid proliferation even in an *in vivo* environment. It is therefore probable that they might interfere with the engraftment of transplanted pancreatic islets. Contrast to our study, following the co-transplantation of the allogeneci pancreatic islet with adipose-derived stem

cells in the subrenal capsule, the infiltration of the immune cells in the adjacent areas to the pancreatic islet was reduced and synchronously the formation of blood vessels adjacent graft was induced (32). Bone marrow-derived MSCs used in the current study and adipose-derived stem cells have been reported to have differences in the degree of proliferation and immunosuppressive effect (62). It is probable that these causes might have induced the differences between previous reports and the current study. On the other hand, following the co-transplantation of ESCs mixed with the allograft pancreatic islet in the subrenal capsule, the teratoma was formed. These findings might originate from the indefinite proliferation ability and the differentiation ability of ESCs despite the co-transplantation of ESCs at a decreased number of ESCs of up to $1x10^4$. In the current study, following the intravenous infusion of MSCs in mice, the survival of the allograft tissue was not increased and the degree of the infiltration of immune cells was not reduced in the adjacent areas to the graft stie. Moreover, unlike *in vitro* immunosuppressive effects, there were no alterations in the activity of immune cells that are present in the spleen and renal lymphatic ducts. Furthermore, the regulatory T-cell was not also significantly changed. Presumably, these contradictory results might arise from the differences in types of experimental animals, the number of infused stem cells, infusion method of stem cells and the types of immunosuppressants. In the previous report, MSCs suppressed the proliferation of T-cells in an *in vitro* environment. And following the infusion of MSCs in an *in vivo* environment, however, they did not suppress the proliferation of T-cells (63). This was also accompanied by a lack of the recovery of arthritic symptoms. As shown in the current study, the immunosuppressive effects of stem cells were notable in an *in vitro* environment but did not appear in an *in vivo* environment. This might be

because the number of MSCs might not have been intravenously infused to the spleen or lymphatic enough to show the immunosuppressive effect in an *in vivo* environment.

To summarize, both types of stem cells synchronously inhibited the proliferation and survival of T cells mainly via cell-to-cell contact in the alloimmune response. In addition, they suppressed the release of inflammatory cytokines and memory T cells and induced the regulatory T cells. Particularly in ESCs, granzyme B molecule played a major role in suppressing the immune responses by cell-to-cell contact via perforin independent pathway. Unlike a higher degree of *in vitro* immunosuppressive effect, however, following both the allogeneic skin tissue and pancreatic islet transplantation with stem cells, the survival of the allograft tissue was not significantly increased and there were no additional effects of the immunosuppressants. Therefore, further studies will be to identify the causes that are responsible for the discrepancy in the immunosuppressive effects of stem cells between an *in vitro* and an *in vivo* environment and the different immunosuppressive effect and the experimental conditions have to be considered have to be considered to develop the optimal condition with stem cells and immunosuppressants for the successful clinical application in the field of organ transplantation.

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국문 초록

서론: 배아줄기세포 (ESC)와 중간엽줄기세포 (MSC)는 면역억제효과가 있는 것으로 알려져 있지만, 면역억제기작은 여전히 논란이 있다. 따라서 본 연구에서는 배아줄기세포와 중간엽줄기세포에 대해 생체 외 면역억제 기작과 동종피부 및 췌도이식 동물 실험모델을 이용하여 생체 내 면역억제 효능을 분석하고자 한다.

방법: 생체 외에서 두 줄기세포의 면역억제효과를 분석하기 위해서, C57BL/6 마우스로부터 분리된 naïve T cells 또는 memory T cells 그리고 BALB/c 마우스로부터 분리된 항원제시세포를 ESC 또는 MSC와 함께 3일 동안 혼합 공동배양하였고, thymidine 섭취, 효소면역측정법 (ELISA) 그리고 CFSE와 7-AAD 염색방법을 시행하였다. 두 줄기세포의 면역억제 기작을 분석하기 위해 transwell 실험과 PDL-1, Fas-L, CD30, CD70, perforin 그리고 granzyme B와 같은 면역억제유전자발현확인을 위한 역전사효소 중쇄연쇄반응 실험 (RT-PCR)을 수행하였다. 유전자의 기능분석을 위해서 각 유전자들에 대한 차단 항체와 저해제를 처리하였다. 두 줄기기세포의 생체 내 면역억제효과를 분석하기 위해서 동종의 피부 또는 췌도 이식모델에서, 정맥혈관 또는 공동이식으로 줄기세포를 주입하였고 면역억제제인 항-CD40L와 MMF와 함께 사용하였다.

결과: ESC 또는 MSC 는 생체 외에서 동종면역반응을 유발하지 않았으며, naïve $CD4^+$ T 세포의 증식과 생존을 동시에 억제하였고 IL-2, IL-12, IFN-γ, TNF-α, IL-4, IL-5, IL-1β, IL-10 등의 싸이토카인 발현을

억제하였다. 그러나 TGF-β 또는 IDO 발현에는 특별한 변화가 나타나지 않았다. 뿐만 아니라, 두 줄기세포는 $CD44^+$ memory T 세포의 증식을 억제하였고, naïve T 세포의 활성화 인자를 억제하였으며, 면역조절 T 세포를 유도하였다. Transwell 실험 결과, 두 줄기세포의 면역억제 효과는 세포간 접촉이 주요한 기작이었다. 두 줄기세포는 PDL-1, Fas-L, CD30, perforin 그리고 granzyme B 를 강하게 발현시켰지만, CD70 는 약하게 발현되었다. granzyme B 에 대한 저해제를 ESC 처리한 결과, ESC 의 면역억제효과가 현저히 감소하였다. 생체 내에서는 두 줄기세포의 정맥혈관주입 또는 공동이식방법으로 동종피부 또는 췌도이식의 면역거부반응을 억제하지 못하였다. 두 줄기세포와 면역억제제와의 부수적인 사용에도 불구하고, 부가적인 효과는 나타나지 않았다. 이식된 개체에서 두 줄기세포에 의한 면역세포들의 유의성있는 면역학적 변화는 나타나지 않았다.

결론: 마우스 배아줄기세포와 중간엽 줄기세포는 생체 외에서 낮은 면역원성을 나타내었고, naïve T 세포의 증식과 생존억제, memory T 세포 면역반응 억제, 싸이토카인 분비 억제, Treg 의 유도와 같은 유의성있는 면역억제효과를 나타내었다. 주 면역억제 기작은 세포간 접촉이었으며, ESC 에서 발현되는 granzyme B 가 주요한 역할을 하였다. 그러나 두 줄기세포는 생체 내 동종피부와 췌도이식에서 면역거부반응을 억제하지 못하였다. 생체 외 및 생체 내에서의 두 줄기세포의 이러한 상반되는 결과는 장기이식분야에서 최적의 이식조건을 확립과 줄기세포의 성공적 적용을 위해서 반드시 고려되어야 함을 보여준다.

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주요어 : 배아줄기세포, 중간엽줄기세포, T 세포, 동종 피부이식, 동종 췌도이식, 면역억제

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