

Mesenchymal Stem Cells Secrete Immunologically Active Exosomes

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Mesenchymal stem cells (MSCs) have been shown to secrete exosomes that are cardioprotective. Here, we demonstrated that MSC exosome, a secreted membrane vesicle, is immunologically active. MSC exosomes induced polymyxin-resistant, MYD88-dependent secreted embryonic alkaline phosphatase (SEAP) expression in a THP1-Xblue, a THP-1 reporter cell line with an NFκB-SEAP reporter gene. In contrast to lipopolysaccharide, they induced high levels of anti-inflammatory *IL10* and *TGFβ1* transcript at 3 and 72 h, and much attenuated levels of pro-inflammatory *IL1B*, *IL6*, *TNFA* and *IL12P40* transcript at 3-h. The 3-h but not 72-h induction of cytokine transcript was abrogated by MyD88 deficiency. Primary human and mouse monocytes exhibited a similar exosome-induced cytokine transcript profile. Exosome-treated THP-1 but not MyD88-deficient THP-1 cells polarized activated CD4⁺ T cells to CD4⁺CD25⁺FoxP3⁺ regulatory T cells (Tregs) at a ratio of one exosome-treated THP-1 cell to 1,000 CD4⁺ T cells. Infusion of MSC exosomes enhanced the survival of allogenic skin graft in mice and increased Tregs.

Introduction

MESENCHYMAL STEM CELLS (MSCs) are multipotent fibroblast-like cells that could be easily isolated from adult tissues. Thus, their large ex vivo expansion capacity, multipotency, and immunosuppressive activity have made MSCs a popular experimental therapeutic agent for many diseases, including many autoimmune diseases as evidenced by the current 306 trials using MSC (<http://clinicaltrials.gov/>; accessed March 2013) and its recent approval as the first “off-the-shelf” stem cell-based pharmaceutical drug for the treatment of pediatric graft-versus-host disease (GVHD) in Canada and New Zealand.

The efficacy of MSCs against severe GVHD is best evidenced by a landmark multi-center non-randomized trial led by Katarina Le Blanc and colleagues in the European Group for Blood and Marrow Transplantation Mesenchymal Stem Cell Expansion Consortium. MSCs induced complete responses in 55% of 55 patients with acute GVHD grade 2–4, and this response was not dependent on the immune compatibility between the donor MSCs and the recipients [1]. Furthermore, clinical trial reports had consistently indicated that graft-versus-leukemia (GVL) reaction was not impaired, suggesting that MSCs do not cause systemic immunosuppression [2]. Despite this astounding clinical success, the underpinning mechanism for MSC immunomodulatory activity remains tenuous. A previous postulation

that MSC suppresses GVHD by inhibiting T-cell proliferation was not tenable, as there was no correlation between the ability to suppress T-cell proliferation and patient outcome [3]. Consistent with the increasingly popular hypothesis that MSC mediates its therapeutic efficacy through its secretion (reviewed [4]), it has recently been proposed that MSCs suppress GVHD by modulating regulatory T cells or Tregs, a subpopulation of T cells possibly through the secretion of soluble mediators known to enhance Treg expansion [eg, transforming growth factor-β (TGF-β), prostaglandin E2 (PGE2), human leukocyte antigen G (HLA-G), interleukin (IL)-10, and indoleamine 2,3-dioxygenase (IDO)] (reviewed [5]). However, MSC secretion is apparently not sufficient. It was observed that MSCs induced Treg expansion in a transwell system only in the presence of splenocytes or peripheral blood monocytes, but not with purified CD4⁺ T cells [6–8], suggesting that in addition to its secretion, MSCs require other mediator cells such as monocytes to induce Treg expansion.

Tregs are adaptive immune cells that are modulated by activated antigen presenting cells (APCs) such as dendritic cells, macrophages, and monocytes, and the activation of APCs, in turn, could be modulated by MSCs [9]. The activation of APCs is mediated by innate immune receptors, the most prominent of which is the Toll-like receptor (TLR) family. On binding ligands, most TLRs signal by recruiting MyD88, an adapter protein to initiate downstream signaling

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[10], leading to the activation of NF κ B and/or AP1 transcription factors and subsequent expression of inflammatory genes [11,12].

In this report, we evaluate whether MSC exosome has immunological activities and could contribute to the MSC-mediated immunosuppression of GVHD. Human MSC exosomes were first identified as the agents mediating MSC cardioprotective secretion [13–15]. Exosomes are 50–100 nm bi-lipid membrane vesicles with a protein- and RNA cargo, and they are actively secreted by many cell types [16,17]. They are considered as mediating intercellular communication by the transfer of proteins and RNA [18,19]. Exosomes have been implicated in many aspects of immune regulation such as stimulation of T-cell proliferation, B lymphocyte-mediated tumor suppression, induction of apoptosis in activated cytotoxic T cells, differentiation of monocytes into dendritic cells, and induction of myeloid-suppressive cells and T regulatory cells (review [20–23]). As such, exosome is a plausible candidate for the immunomodulatory factor in MSC secretion. Therefore, MSC exosomes were assessed for immunological properties such as cytokine induction in monocytes and the induction of Tregs through splenocytes or peripheral blood mononuclear cells (PBMCs) [6–8].

Materials and Methods

Approval for experiments using mouse and human samples

Mice were purchased from the Biological Resource Center (BRC), and the animal experiments were approved by the BRC Institutional Animal Care and Use Committee. The collection and use of human blood samples were approved by the Institutional Review Board of the National University of Singapore.

Preparation of exosomes

MSC exosomes were prepared from culture medium conditioned by huES9.E1 human embryonic stem cell (ESC)-derived MSCs. huES9.E1 MSCs were derived from huES9 human ESCs through spontaneous differentiation induced by repeated passaging in a feeder-free culture medium [24]. Briefly, MSC exosomes were prepared from a chemically defined culture medium that was conditioned by huES9.E1 MSCs for 3 days [13,25]. The conditioned medium was concentrated 50 \times by tangential flow filtration using a membrane with a 100 kDa MWCO (Sartorius), and then fractionated by high-performance liquid chromatography (TSK Guard column SWXL, 6 \times 40 mm and TSK gel G4000 SWXL, 7.8 \times 300 mm; Tosoh Corp.). The first eluted peak that contained the exosomes was concentrated using a 100 kDa MWCO filter (Sartorius) and assayed for protein using a NanoOrange Protein Quantification Kit (Life Technologies). The average exosome yield per liter of culture medium conditioned by 1 \times 10¹¹ cells was 1 mg. The exosome preparation was 0.22 μ m filtered and stored in –20°C freezer until use.

Activation of secreted embryonic alkaline phosphatase reporter cell lines

Four commercially available reporter cell lines with an optimized secreted embryonic alkaline phosphatase (SEAP)

reporter gene under the control of the NF- κ B promoter were used: (i) HEK-Blue-hTLR4, an HEK293 cell line stably co-transfected with human TLR4, MD2, and CD14; (ii) HEK-Blue-hTLR2, an HEK293 cell line stably co-transfected with human TLR2 and CD14; (iii) THP1-XBlue line derived from THP-1 human monocytic cell line; and (iv) THP1-XBlue-defMYD, a THP1-XBlue cell line that is deficient in MyD88 (InvivoGen). The unmodified THP-1 line was bought from ATCC. The HEK and THP-1 cells were maintained in Dulbecco's modified Eagle's medium and RPMI-1640 medium, respectively, with 10% fetal bovine serum (FBS; Life Technologies) and antibiotics as recommended by the manufacturer. To assess TLR activation by MSC exosomes, HEK and THP-1 cells were seeded in a 96-well plate at 1 \times 10⁴ and 1 \times 10⁵ cells/well, respectively and incubated for 24 h with 10 η g/mL *Escherichia coli* 026:B6 lipopolysaccharide (LPS; Sigma), 10 μ g/mL *Staphylococcus aureus* lipoteichoic acid (LTA; Sigma), or 100 η g/mL MSC exosomes, which were prepared as previously described [13]. SEAP secretion was assayed with 20 μ L of cell supernatant using the QUANTI-Blue kit as per the manufacturer's instructions (InvivoGen). IST-9, a mouse monoclonal antibody against human cFn-extradomain A (EDA; Abcam), and polymyxin B (Sigma), an antibiotic, were added to the culture medium to abrogate fibronectin and LPS activation, respectively. For gene expression studies, THP-1 cells were seeded at 1 \times 10⁶ cells/well in 24-well culture plates with either 10 η g/mL LPS or 100 η g/mL MSC exosomes for 0, 0.5, 1, 3, 6, 12, 24, 48, 72, 96, and 120 h. RNA was then purified and analyzed by real polymerase chain reaction (RT-PCR).

Western blot hybridization

Standard western blot analysis was performed. Briefly, the exosomes were denatured, resolved on 4–12% polyacrylamide gels, electroblotted onto a nitrocellulose membrane, probed with an antibody against human cellular fibronectin EDA [fibronectin 1 (FN1)-EDA] (clone DH1 Abnova), incubated with a horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (Santa Cruz), and visualized with an HRP-enhanced chemiluminescent substrate (Thermo Fisher Scientific).

Splenic lymphocyte proliferative assay

The inhibition of mitogen-activated splenic lymphocyte proliferation by MSC exosomes was assessed as previously described [26]. Briefly, mouse spleens were removed, minced in RPMI 1640 medium, and filtered through a cell strainer and residual erythrocytes were lysed in RBC lysis buffer (eBioscience). The splenocytes were pre-labeled with 2 mL of 10 μ M carboxyfluorescein succinimidyl ester (Molecular Probe) in phosphate-buffered saline (PBS) at 37°C for 15 min, seeded at 5 \times 10⁵ cells/mL, and incubated with 0.1, 0.5, 1, or 4 μ g/mL MSC exosomes in the presence or absence of either 100 η g/mL LPS or 5 μ g/mL ConA (Sigma) for 3 days. The number of fluorescent cells for each treatment group was quantitated by a BD FACS Calibur Flow Cytometer using Cell Quest software (Becton Dickinson), and the cell cycling time was calculated as previously described [27].

Real-time quantitative PCR

To quantify cytokine transcripts in THP-1 cells, RNA was extracted, reverse transcribed, and amplified using an RNeasy Mini kit (QIAGEN), a High-Capacity cDNA Reverse Transcription Kit (Life Technologies), and a Fast SYBR[®] Green Master Mix (Life Technologies), respectively. The amplification was performed on a StepOnePlus[™] Real-Time PCR Systems (Life Technologies) with a 10-min 95°C denaturation step, 40 cycles of 3-s 95°C denaturation, and 30-s 60°C annealing and elongation. The primers used were as follows: *IL1B* (FW: 5'-CCTGTCCTGCGTGTGAAAGA-3'; RV: 5'-GGGAAGTGGCAGACTCAA-3'), *TNFA* (FW: 5'-CCC CAGGACCTCTCTAATC-3'; RV: 5'-GGTTTGCTAC AACATGGGCTACA-3'), *IL6* (FW: 5'-TCGAGCCCACC GGAACGAA-3'; RV: 5'-GCAACTGGACCGAAGGCG CT-3'), *IL12P40* (FW: 5'-CATGGTGGATGCCGTTCA-3'; RV: 5'-ACCTCCACCTGCCGAGAAT-3'), *IL10* (FW: 5'-GTGATGCCCCAAGCTGAGA-3'; RV: 5'-CACGGCCTTG CTCTTGT-3'), *TGFβ1* (FW: 5'-CAGCAACAATTCCT GGCGATA-3'; RV: 5'-AAGGCGAAAGCCCTCAATTT-3'), and *GAPDH* (FW: 5'-GTCTTCACCACCATGGAGAAGG CT-3'; RV: 5'-CATGCCAGTGAGCTTCCCGTTCA-3'). Each sample was tested in triplicate. Data were analyzed using the comparative Δ CT method and Applied Biosystems StepOne software Version 2.0.1 according to the manufacturer's instructions.

Primary mouse and human monocyte experiments

Primary mouse monocytes were purified from bone marrow of the femurs of 6–8 week-old BALB/cJ mice using an EasySep mouse monocyte enrichment kit (Stem Cell Technologies), while primary human monocytes were isolated from the peripheral blood of healthy donors by Ficoll–Paque centrifugation (GE Healthcare Life Sciences) and then StemSep human monocyte enrichment Kit (Stem Cell Technologies). The mouse or human monocytes were cultured in RPMI-1640 medium containing 10% FBS at 5×10^5 cells/well in a 24-well plate with 10 η g/mL LPS or 100 η g/mL MSC exosomes for 0, 0.5, 1, 3, and 6 h before being harvested for an RT-PCR analysis of *IL1B*, *IL12P40*, and *IL10* transcript expression.

Differentiation of Treg and Th17 cells from CD4⁺ T cells

CD4⁺ T cells were isolated using EasySep FITC Selection Kit (Stem Cell Technologies) after incubating mouse splenocytes with anti-mouse CD16/CD32 (2.4G2; BD Pharmingen) for 15 min at 4°C and then fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 antibody (GK1.5; BD Pharmingen) for 30 min. The cells were plated at 1×10^6 per well in 24-well plates that were pre-coated with 4 μ g/mL anti-CD3 mAb (145-2C11; eBioscience) with 5 μ g/mL soluble anti-CD28 mAb (37.51; eBioscience) in the absence or presence of 100 η g/mL MSC exosomes or exosome-treated THP-1 cells. For Treg cell control, CD4⁺ T cells were polarized with 10 η g/mL recombinant human transforming growth factor-beta1 (rhTGF- β 1, eBioscience) and for Th17 cell control, CD4⁺ T cells were polarized with 20 η g/mL recombinant human interleukin-6 (rhIL-6, eBioscience) and 1 η g/mL rhTGF- β 1.

The cells were grown in RPMI-1640 medium containing 10% FBS for 6 days. Treg cells were stained with FITC-conjugated anti-mouse CD4 (RM4-5), APC-conjugated anti-mouse CD25 (PC61.5), and PE-conjugated anti-mouse Foxp3 Ab (FJK-16s) mAbs using the Mouse Regulatory T-Cell Staining Kit (eBioscience). Th17 cells were stained with FITC-conjugated anti-mouse CD4 (RM4-5) and PE-Cy7-conjugated anti-mouse IL-17A mAb (eBio17B7; eBioscience). Fluorescence-activated cell sorting analysis was performed on a BD[™] LSR II flow cytometer (BD Biosciences).

Allogeneic skin grafting

Tail skins from C57BL/6J mice were grafted onto BALB/cJ mice using a modification of a previously described technique [28,29]. Briefly, 0.6 \times 0.8 cm tail skins from 8- to 12 week-old female C57BL/6J mice were grafted onto the dorsum of 6–8 week-old female BALB/cJ mice. 0.3 μ g exosomes in 50 μ L PBS ($n=10$) or 50 μ L PBS ($n=10$) were subcutaneously injected into each graft recipient mouse every day for 4 days and then, every other day for 15 days. Another two groups of ungrafted 8 week-old female BALB/cJ mice were similarly treated with either exosomes ($n=10$) or PBS ($n=10$). Images of the grafts were captured every other day after removal of bandages on the seventh day. Graft rejection was quantitated using a previously described scoring system [30]. Fifteen days after transplantation, splenic T cells were purified from recipient mice and assayed for Tregs.

Intrasplenic injection of exosomes

A median incision was made on a 6–8 week-old female BALB/cJ mouse that was anesthetized with 0.016 mL 2.5% avertin/g body weight, and 0.3 μ g MSC exosomes in 50 μ L PBS or 50 μ L PBS were injected directly into the spleen (10 mice per group). At 0, 3, 6, and 9 days, the spleens were isolated and assayed for CD4⁺CD25⁺Foxp3⁺ Treg cells.

Statistical analyses

GraphPad Prism 5 software was used for the analysis of all data. The graft rejection scores data were analyzed by the analysis of variance between groups. All other data were analyzed using unpaired one-tailed Student's *t*-test.

Results

MSC exosome-activated APCs via an MyD88-dependent, polymyxin B-resistant TLR signaling

Since MSCs have been widely reported to inhibit the proliferation of mitogen activate lymphocytes (reviewed [31]), we first investigated the effect of MSC exosomes on the proliferation of LPS- or conA-stimulated mouse splenocytes (Fig. 1A). No inhibitory effect was observed at 100–1,000 η g/mL exosomes. An inhibitory effect on proliferation was observed only at a relatively high concentration of 4,000 η g/mL exosomes.

We next tested whether MSC exosomes activate monocytes, a major cell component of PBMCs using THP1-XBlue cells as surrogates for human monocytes. THP1-XBlue cells are a TLR reporter cell line derived from THP1 human acute

monocytic leukemia cell line. Activation of TLR in this cell line induced a proportional increase in SEAP expression. At 100 $\eta\text{g/mL}$, exosomes activated TLR signaling in THP1-XBlue cells and secreted the same level of SEAP reporter as 10 $\eta\text{g/mL}$ LPS (Fig. 1B). Similar to LPS, this induction was dependent on MyD88 as evidenced by abrogation of SEAP secretion in MyD88-deficient THP1-XBlue cells, THP1-XBlue-defMYD (Fig. 1B). However, unlike LPS, exosome induction of SEAP was polymyxin B resistant (Fig. 1B).

To identify some of the TLR targets of MSC exosomes, MSC exosomes were incubated with HEK-Blue-hTLR4 or

HEK-Blue-hTLR2 cells that secrete SEAP on activation of TLR4 or TLR2 signaling, respectively. At 100 $\eta\text{g/mL}$, MSC exosomes induced a five-fold increase in SEAP activity in HEK-Blue-hTLR4 cells but not HEK-Blue-hTLR2 cells (Fig. 1C). SEAP activity in HEK-Blue-hTLR2 cells remained low and increased by <2-fold over baseline with a 20-fold increase in exosomes to 2,000 $\eta\text{g/mL}$ (Fig. 1D), indicating that MSC exosomes were very weak activators of TLR2. Activation of TLR4 signaling by MSC exosomes unlike LPS activation was not abrogated by polymyxin B (Fig. 1C). Notably, 100 $\eta\text{g/mL}$ MSC exosomes had the same potency as 10 $\eta\text{g/mL}$ LPS in the induction of SEAP in

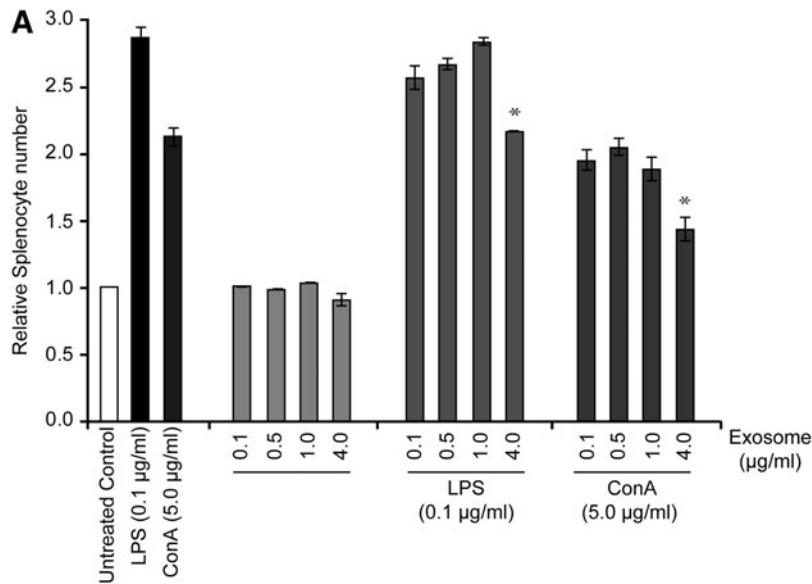


FIG. 1. Mesenchymal stem cell (MSC) exosome-mediated activation of antigen presenting cells (APCs) and the role of Toll-like receptor (TLR) signalling. **(A)** Effect of MSC exosomes on mitogen-stimulated splenocyte proliferation. Mouse splenocytes were harvested, labeled with carboxyfluorescein succinimidyl ester, and plated at a density of 5×10^5 cells/mL with 0.1, 0.5, 1, and 4 $\mu\text{g/mL}$ of MSC exosomes in the presence or absence of either lipopolysaccharide (LPS, 0.1 $\mu\text{g/mL}$) or ConA (5 $\mu\text{g/mL}$) for 3 days. Cellular proliferation was assessed by fluorescence-activated cell sorting (FACS), normalized to the untreated control, and presented as mean [\pm standard deviation (SD)] of triplicate samples, $*P < 0.01$. **(B)** Activation of TLR signaling in THP-1 human monocytic cell line. Two THP-1 reporter cell lines: THP1-XBlue, which is a THP-1 reporter line with a secreted embryonic alkaline phosphatase (SEAP) reporter gene under the control of an NF- κ B promoter and THP1-XBlue-defMYD, an MyD88-deficient THP1-XBlue line, were used. 24 hours after seeding at 10^5 cells per well in a 96-well plate with 0.01 $\mu\text{g/mL}$ LPS or MSC exosomes (0.05, 0.1, and 0.2 $\mu\text{g/mL}$) with or without polymyxin B (100 $\mu\text{g/mL}$), an antibiotic that neutralizes LPS activity, SEAP secretion into the culture medium was assayed. Each bar represents the mean (\pm SD) of three independent assays. Each assay was performed in triplicate, $*P < 0.001$. **(C)** Effect of MSC exosomes on TLR-4 signaling pathway. Cells from HEK-Blue-hTLR4 reporter cell line were seeded in a 96-well plate at 10^4 cells per well and incubated with LPS (0.01 $\mu\text{g/mL}$) or MSC exosomes (0.1 $\mu\text{g/mL}$) for 24 h. HEK-Blue-hTLR4 cells have a stably transfected TLR4 and an optimized SEAP reporter gene under the control of the NF- κ B promoter. SEAP activity in each well was determined using a Quanti-Blue assay kit. Results were normalized to “No treatment control.” Data were the mean (\pm SD) of three independent experiments, $*P < 0.001$. **(D)** Effect of MSC exosomes on TLR-2 signaling pathway. HEK-Blue-hTLR2 reporter cell line was incubated with either LTA (10 $\mu\text{g/mL}$) or MSC exosomes (0.1, 0.5, 1.0, and 2.0 $\mu\text{g/mL}$) for 24 h. HEK-Blue-hTLR2 cells resembled HEK-Blue-hTLR4 except that they have a stably transfected TLR2 instead of TLR4. SEAP activity and data analysis were performed as for **(C)**, $*P < 0.01$. **(E)** A list of candidate endogenous TLR2 and TLR4 ligands based on our previously published proteomic analysis of the MSC exosomes. **(F)** Western blot analysis for the presence of extracellular matrix protein 1 (EDA-FN) in MSC exosome. Proteins in MSC exosome and MSC were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, electroblotted onto nitrocellulose membrane, and the membrane was probed with an antibody that was specific for EDA-FN. **(G)** Activation of TLR4 by exosome EDA-FN. HEK-Blue-hTLR4 cells were seeded in a 96-well plate at 10^4 cells per well and incubated for 24 h with 0.1 $\mu\text{g/mL}$ MSC exosomes and 100 $\mu\text{g/mL}$ Polymyxin B in the presence of different concentrations (0, 5, 10, 20, and 40 $\mu\text{g/mL}$) of IST-9 Ab, an EDA-FN neutralizing antibody. As reference controls, the cells were treated with 0.01 $\mu\text{g/mL}$ LPS with or without IST-9 Ab and Polymyxin B, or IST-9 Ab alone. SEAP activity was determined using Quanti-Blue, normalized to the “No treatment” control, and expressed as a mean (\pm SD) of three independent experiments, $*P < 0.001$.

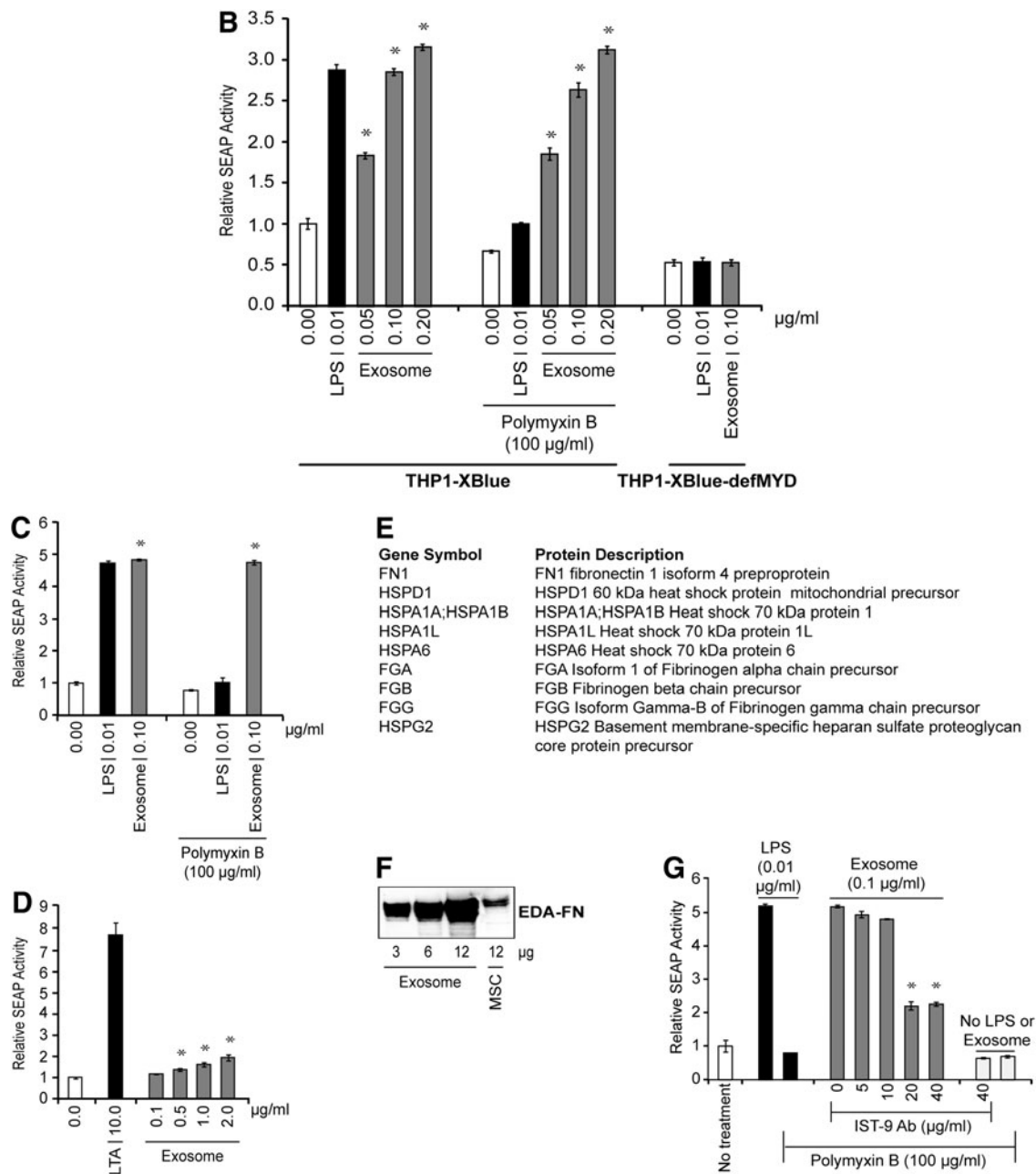


FIG. 1. (Continued).

HEK-Blue-hTLR4 or THP1-XBlue reporter cell lines. These observations suggest that MSC exosomes have TLR4 but not TLR2 ligands.

Consistent with its capacity to activate TLR4, our previously published proteomic analysis of the MSC exosomes [4,32] revealed the presence of several candidate endogenous TLR4 ligands in MSC exosomes, namely FN1, the heat shock proteins, and fibrinogens (Fig. 1E). Incidentally, TLRs that are present in MSCs [33] were absent in the exosomes. FN1 is a family of high-molecular-weight alternatively spliced glycosylated products of a single gene. Plasma FN1s are produced by liver cells, while cellular FN1s are produced by many cell types in response to injury. Cellular FN1 containing a specific alternatively spliced

domain known as EDA is the first and best characterized endogenous TLR4 ligand [34]. We confirmed that MSC exosomes have FN1 with EDA using an EDA-specific antibody (Fig. 1F). In addition, IST-9, an EDA-neutralizing monoclonal antibody [35], abolished 60% of exosome-induced SEAP in HEK-Blue-hTLR4 (Fig. 1G), indicating that 60% of TLR4 activation by exosomes was mediated by EDA-containing FN1 and the remaining 40% by other endogenous ligands such as the heat shock proteins or fibrinogens.

Together, these experiments demonstrated that MSC exosomes do not inhibit proliferation of mitogen-activated lymphocytes, but they could activate MyD88-dependent nuclear translocation of NFκB by a polymyxin B-resistant

pathway through TLR4 and, possibly, other remaining nine human TLRs except TLR2.

MSC exosomes induced an anti-inflammatory M2 phenotype in monocytes

Since 100 ng/mL MSC exosomes and 10 ng/mL LPS induced similar levels of SEAP activity in HEK-Blue-hTLR4 and THP1-XBlue reporter cell lines (Fig. 1B, C), we compared the expression kinetics of pro- and anti-inflammation cytokine genes in 100 ng/mL exosome- versus 10 ng/mL LPS-treated THP-1 cells. In contrast to LPS induction, MSC exosomes induced a much lower level of pro-inflammatory cytokine genes, namely *IL1B*, *IL6*, *IL12P40*, and *TNFA*, and a higher level of anti-inflammatory *IL10* (Fig. 2A). Interestingly, *TGFB1*, which is known to have both anti- and pro-inflammatory activities [36], was induced to the same level by both LPS and MSC exosomes. Another notable difference was the monophasic LPS induction at 3 h versus the biphasic exosome induction at 3 and then 72–96 h. The failure of LPS to induce *IL10* was not unexpected, as LPS was previously reported to elicit *IL10* gene expression in monocytes only at a high concentration of 1,000 and not 10 ng/mL as used in this experiment [37]. The contrast in the induction of *IL10* and *IL12P40* genes by exosomes and LPS suggested that LPS induced an M1 macrophage-like phenotype, while MSC exosomes activated an M2 macrophage-like phenotype [38,39]. The 3-h induction of cytokines by LPS and exosomes was attenuated by MYD88 deficiency in THP1-XBlue-defMYD (Fig. 2B) but not the 72-h induction of anti-inflammatory *IL10* by exosomes (Fig. 2B). Therefore, the first phase of induction at 3 h by both LPS and MSC exosomes was TLR dependent, and the second phase of induction 72–96 h by MSC exosomes was TLR independent. The pattern of cytokine induction in THP-1 cells by MSC exosomes and LPS was mirrored in mouse and human primary monocytes with MSC exosomes inducing high *IL10* expression and low *IL1B* and *IL12P40* expression, while LPS induced the opposite (Fig. 2C). However, unlike THP-1, the induction in the primary monocytes was faster at 1 h instead of 3 h.

MSC exosomes required monocyte to mediate differentiation of CD4⁺ T cells to Treg

Since MSCs could induce Treg expansion in a transwell system only when co-incubated with splenocytes or peripheral blood monocytes but not purified CD4⁺ T cells [6–8], we rationalized whether MSC exosomes were the soluble mediators of MSC induction of Tregs; then, exosomes would also require other mediator cells to induce Treg expansion. Consistent with this, co-incubation of MSC exosomes with CD4⁺ T cells activated with anti-CD3 mAb and anti-CD28 mAb did not induce the differentiation of CD4⁺CD25⁺FoxP3⁺ Treg cells (Fig. 3A). Incidentally, MSC exosomes also cannot induce the differentiation of Th17 cells (Fig. 3B).

Since we had shown that MSC exosomes could modulate monocytes toward an M2-like phenotype (Fig. 2) and macrophage colony-stimulating factor polarized M2 monocytes could induce Tregs [40,41], we next investigated whether MSC exosome-activated M2-like monocytes could induce Treg polarization. THP-1 cells that had been exposed to 100

ng/mL MSC exosomes or 10 ng/mL LPS for either 3 or 72-h were incubated with activated CD4⁺ T cells in a ratio of 1:100, 1:1,000, and 1:10,000. At 1:1,000 or more, exosome-treated but not LPS-treated or untreated THP-1 cells could induce the differentiation of CD4⁺CD25⁺FoxP3⁺ Treg cells (Fig. 3C; Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/scd). THP-1 cells exposed to exosomes for 3 h could not induce the differentiation of CD4⁺CD25⁺FoxP3⁺ Treg cells. THP-1 cells that had been exposed for 24 h to exosomes could activate CD4⁺ T cells (Fig. 3D). This capacity peaked at 48 h and was dependent on MYD88, as exosome-treated THP1-XBlue-defMYD88 cells could not induce Treg differentiation (Fig. 3D). Together, these observations suggested that the capacity to induce Tregs was acquired by 48 h after exposure to exosomes.

MSC exosomes enhanced allogeneic skin graft

Based on the enhancement of Treg polarization by MSC exosomes, we hypothesized that MSC exosomes could delay allogeneic skin graft rejection with a concomitant increase in Tregs in the recipient mice. Tail skins from C57BL/6J mice were grafted on BALB/cJ recipients, and followed by subcutaneous injections of either 0.3 μg exosomes in 50 μL PBS or 50 μL PBS per mouse every day for 4 days and then every other day for 15 days. Using a score of 4 as a criterion for rejection as in a previously described skin rejection scoring system [30], exosome- and PBS-treated mice took 13 and 11 days to reject the grafts, respectively (Fig. 4A, B). Therefore, MSC exosome administration significantly improved skin allograft survival ($P < 0.001$). To determine whether this delayed graft rejection was due to increased Treg polarization, the spleens of the mice were harvested on day 15 and assayed for Tregs. The level of Tregs was significantly higher in exosome-treated graft recipient animals ($P < 0.0005$) (Fig. 4C; Supplementary Fig. S2). Interestingly, Treg induction was not observed in the spleens of non-graft recipient mice that had the same exosome treatment regimen or intrasplenic injection with exosomes ($P > 0.5$) (Fig. 4C, D). A possible explanation is that MSC exosomes induce Tregs only when the immune system is activated.

Discussion

In this report, we demonstrated that MSC exosome is immunologically active. Using a THP-1 cell line with a reporter gene for TLR activation, 100 ng/mL MSC exosomes was shown to be as potent as 10 ng/mL LPS in inducing expression of the reporter gene, and this induction by both exosome and LPS was abrogated by MYD88 deficiency, demonstrating that MSC exosomes could activate TLR signaling. However, exosome potency but not LPS potency was resistant to polymyxin B, an antibiotic that binds LPS and neutralizes its activity [42,43], indicating that this exosome potency was not due to endotoxin contamination. Using HEK reporter cell lines transfected with a TLR2 or TLR4 reporter gene system, we further demonstrated that MSC exosomes have ligands for at least one TLR, namely TLR4 and not TLR2. We further demonstrated using a neutralizing antibody that EDA-containing FN1 was the major TLR4 ligand in the MSC exosome, contributing to

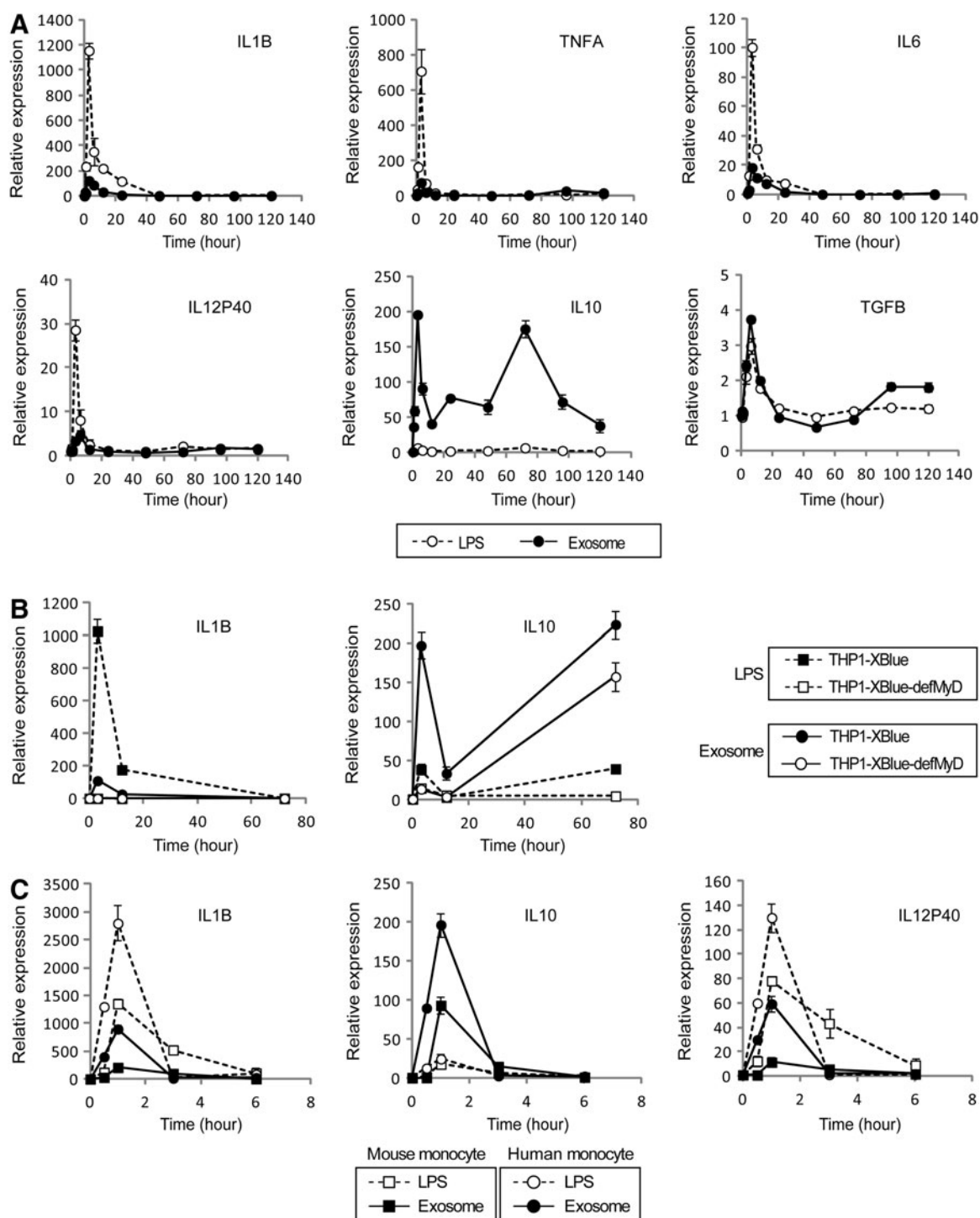


FIG. 2. Induction of monocytic cytokines by MSC exosomes. **(A)** Induction kinetics of cytokines by MSC exosomes. THP-1 monocytic cells were incubated with 0.1 $\mu\text{g}/\text{mL}$ MSC exosomes or 0.01 $\mu\text{g}/\text{mL}$ LPS. At 0, 0.5, 1, 3, 6, 12, 24, 48, 72, 96, and 120 h, cells were harvested, RNA extracted, and a quantitative real time polymerase chain reaction (RT-PCR) for *IL1B*, *TNFA*, *IL6*, *IL12P40*, *IL10*, and *TGFB1* transcript levels were performed. The transcript level for each gene was normalized to its level at 0 h. The data represent the mean (\pm SD) of three independent assays, and each assay was performed in triplicate. **(B)** The role of MYD88 in MSC exosome-mediated induction of cytokine. The experiment in **(A)** was repeated using THP-1XBlue cells and MYD88-deficient THP-1XBlue cells, and *IL1B* and *IL10* transcript analysis was performed at 0, 3, 12, and 72 h. The transcript level was normalized to that at 0 h. Each data point represents the mean (\pm SD) of three independent assays performed in triplicate. **(C)** Effect of MSC exosomes on cytokine induction in primary human and mouse monocytes. About 10 $\eta\text{g}/\text{mL}$ LPS or 100 $\eta\text{g}/\text{mL}$ MSC exosomes were incubated with 5×10^5 primary human and mouse monocytes/well in a 24-well plate. At 0, 0.5, 1, 3, and 6 h, cells were harvested, RNA extracted, and a quantitative RT-PCR for *IL1B*, *IL12p40*, and *IL10* was performed. Each data point represents the mean (\pm SD) of three independent assays performed in triplicate.

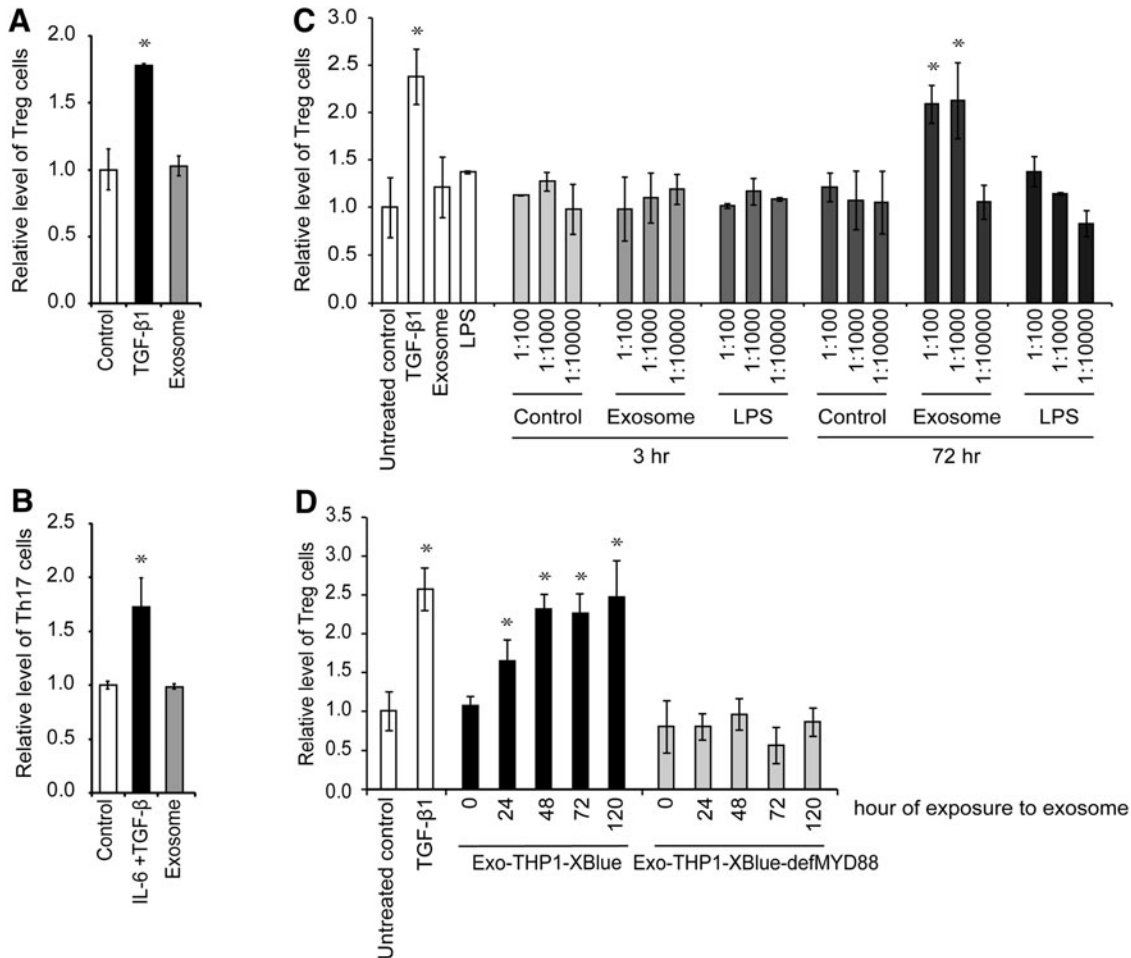


FIG. 3. Treg differentiation by MSC exosome. **(A, B)** Incubation of MSC exosomes with CD4⁺ T cells. CD4⁺ T cells were purified from mouse spleens and incubated with MSC exosomes (100 ng/mL), recombinant human transforming growth factor-beta1 (rhTGF-β1) (10 ng/mL) for CD4⁺CD25⁺Foxp3⁺ Treg cells, or recombinant human interleukin-6 (rhIL-6) (20 ng/mL) and rhTGF-β1 (1 ng/mL) for CD4⁺IL-17⁺ Th17 cells in the presence of anti-CD3 mAb (4 μg/mL) and anti-CD28 mAb (5 μg/mL) for 6 days. The cells were then harvested and analyzed by FACS for the presence of CD4⁺CD25⁺Foxp3⁺ Treg cells or CD4⁺IL-17⁺ Th17 cells. The number of CD4⁺CD25⁺Foxp3⁺ Treg cells or CD4⁺IL-17⁺ Th17 cells was normalized to that of CD4⁺ T cells exposed to only anti-CD3 mAb (4 μg/mL) and anti-CD28 mAb (5 μg/mL) (control). The positive controls for Tregs were TGF-β1-treated CD4⁺ T cells and for Th17 cells, they were IL-6- and TGF-β1-treated CD4⁺ T cells. Each bar represents the mean (±SD) of three independent assays performed in triplicate. **P* < 0.01. **(C)** Effect of exosome-treated monocyte on Treg differentiation. THP-1 cells were incubated for 3 or 72-h with 100 ng/mL MSC exosomes, 10 ng/mL LPS, or without exosomes or LPS (control). The treated THP-1 cells were then incubated with CD4⁺ T cells activated with anti-CD3 mAb (4 μg/mL) and anti-CD28 mAb (5 μg/mL) at a ratio of 1:100, 1:1,000, or 1:10,000. After 6 days, the cells were harvested and analyzed by FACS for the presence of CD4⁺CD25⁺Foxp3⁺ Treg cells. The “untreated control” was anti-CD3- and anti-CD28-activated CD4⁺ T cells; “TGF-β1” were anti-CD3- and anti-CD28-activated CD4⁺ T cells treated with 10 ng/mL rhTGF-β1; “exosome” and “LPS” were anti-CD3- and anti-CD28-activated CD4⁺ T cells treated with 100 ng/mL MSC exosomes and 10 ng/mL LPS, respectively. The number of CD4⁺CD25⁺Foxp3⁺ Treg cells was normalized to that of “untreated control,” **P* < 0.01. **(D)** Role of MYD88 in mediating Treg induction by exosome-activated monocytes. THP-1 and THP-1XBlue-defMYD88 cells were incubated with 100 ng/mL MSC exosomes for 0, 24, 48, 72, and 120 h before incubating with activated CD4⁺ T cells at a ratio of 1:1,000. After 6 days, the cells were harvested and analyzed by FACS for the presence of CD4⁺CD25⁺Foxp3⁺ Treg cells. The “untreated control” was anti-CD3- and anti-CD28-activated CD4⁺ T cells, and “TGF-β1” was anti-CD3- and anti-CD28-activated CD4⁺ T cells treated with 10 ng/mL rhTGF-β1. The number of CD4⁺CD25⁺Foxp3⁺ Treg cells was normalized to that of untreated control. Each bar represents the mean (±SD) of triplicate samples with at least three independent assays, **P* < 0.01.

60% of TLR4 ligand activity in the exosomes. The presence of ligands for the other TLRs is presently unknown. Our proteomic analysis revealed that MSC exosomes do not have TLRs, despite having a membrane derived from the TLR-containing plasma membrane of MSCs.

The equivalent potency of 100 ng/mL exosomes and 10 ng/mL LPS in activating a THP-1 TLR-reporter cell lines did not result in equivalent cytokine induction. Unlike LPS, MSC exosomes induced an attenuated pro-inflammatory cytokine response but a much enhanced anti-inflammatory

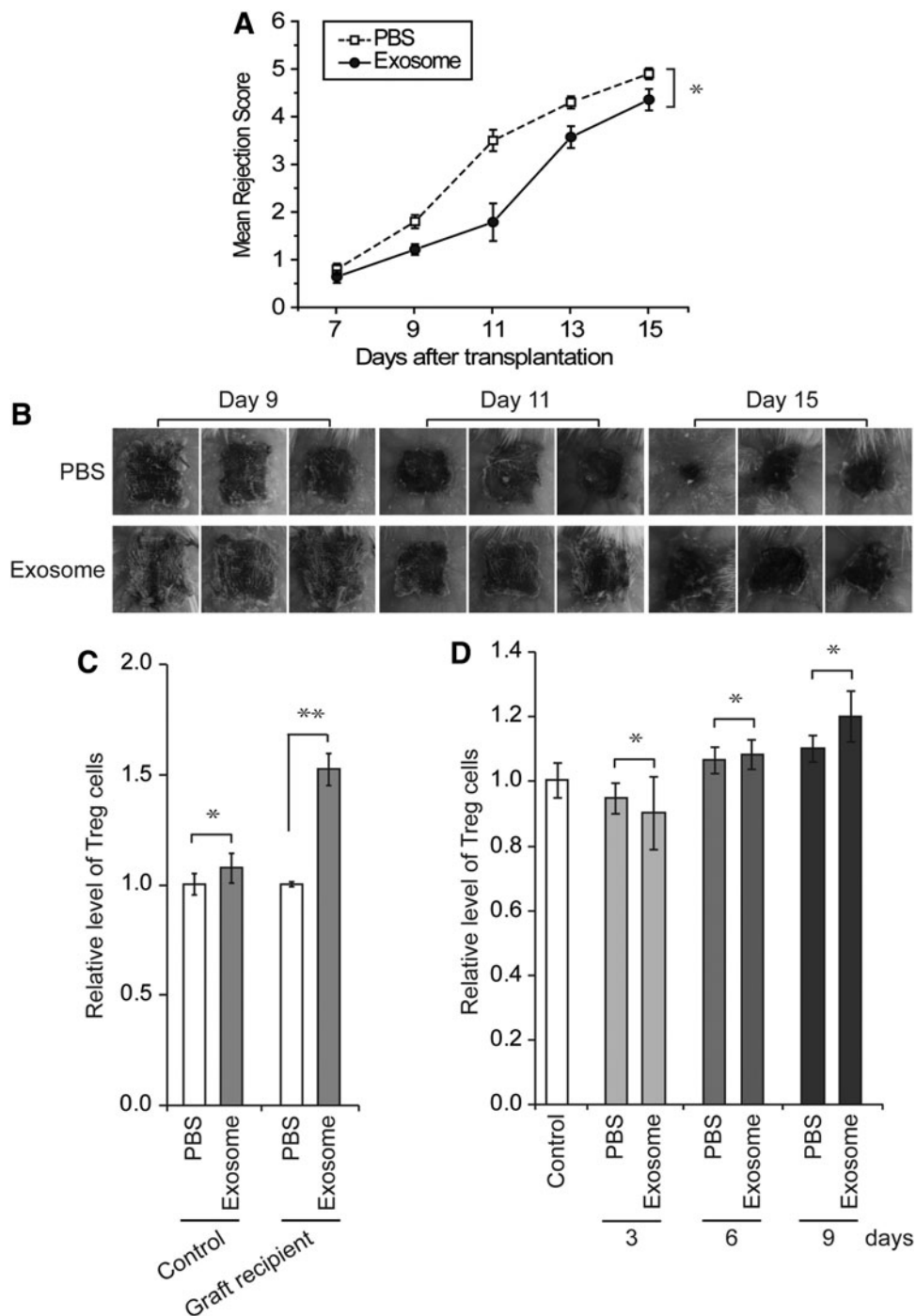


FIG. 4. MSC exosomes and allogeneic skin graft survival. **(A)** Tail skins from C57BL/6 mice were grafted onto BALB/cJ mice. 0.3 μ g exosomes in 50 μ L phosphate-buffered saline (PBS) or 50 μ L PBS were injected subcutaneously into each recipient mouse every day for 4 days and then every other day for 15 days. At day 7 when the dressing was removed, the graft was scored for rejection every 2 days and photographed every other day. Two independent experiments, each consisting of 10 grafted and 10 non-grafted mice in the exosome-treated group, and 10 grafted and 10 non-grafted mice in the PBS-treated group were performed. The mean rejection score over time was determined. Each data point represented the mean with standard error of the mean. P value was determined by analysis of variance, $*P < 0.001$. **(B)** Representative skin allograft in PBS or MSC exosome-treated mice at days 9, 11, and 15 after grafting. **(C)** Tregs in spleens of PBS or MSC exosome-treated mice. Fifteen days after grafting, splenocytes were purified from PBS or MSC exosome-treated mice, and stained for CD4, CD25, and Foxp3. The Treg levels were normalized to that of PBS vehicle control and presented as mean (\pm SD) of triplicate samples, $*P > 0.5$; $**P < 0.0005$. **(D)** MSC exosomes were injected directly into the spleen at a dosage of 0.3 μ g/mouse, and PBS as a vehicle control. After 3, 6, and 9 days, the spleens were isolated from PBS ($n = 10$) or MSC exosome ($n = 10$)-treated mice and analyzed for CD4⁺CD25⁺Foxp3⁺ Treg by flow cytometry. Data were normalized to the untreated control and presented as a mean (\pm SD) of triplicate samples, $*P > 0.5$.

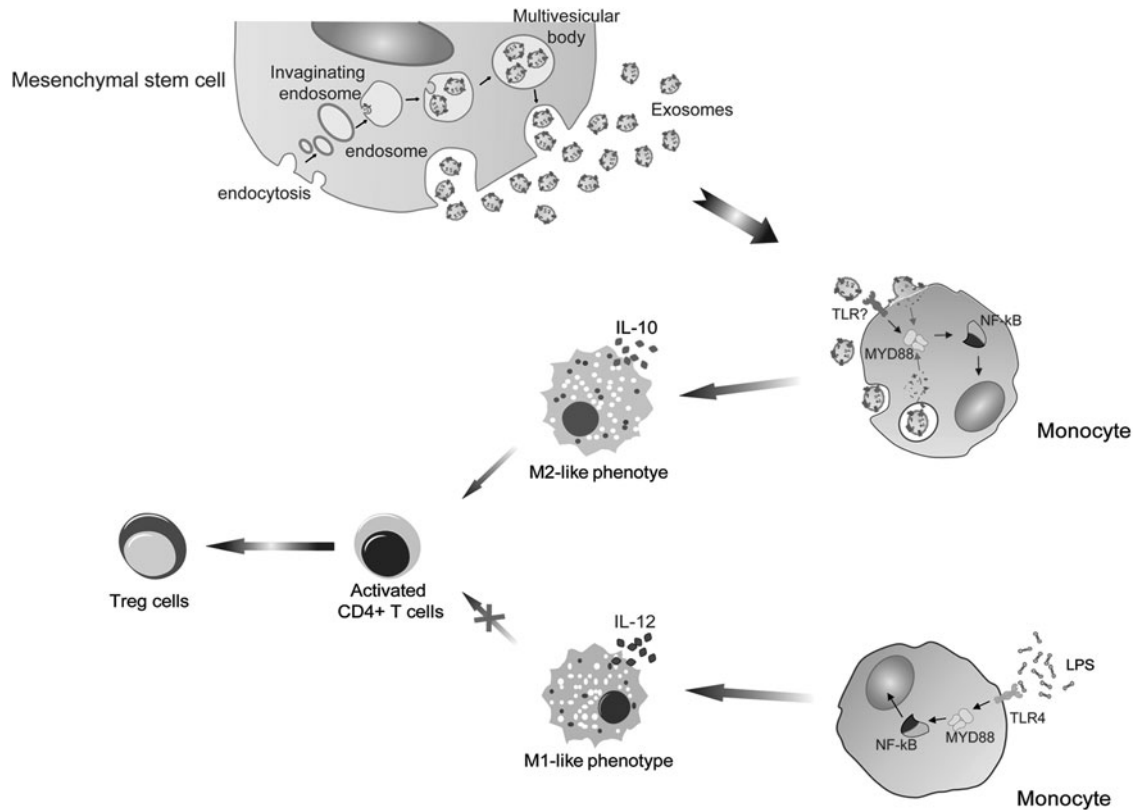


FIG. 5. A proposed model for the immunomodulatory activity of MSC. We propose that MSC secretes exosomes to mediate their immunomodulatory effects. These exosomes induce an anti-inflammatory $IL-12^{lo}IL-10^{hi}$ M2-like phenotype in monocytes via a TLR-dependent signaling pathway. The identity and subcellular location (ie, membrane or cytoplasm) of the TLR/s remain to be elucidated. This monocyte with M2-like phenotype, in turn, induces Treg cell expansion.

IL10 expression. This profile is reminiscent of M2 macrophages that are known to promote tissue repair and limit injury [44,45]. Despite eliciting a different cytokine response, the 3-h cytokine expression in both LPS- and MSC exosome-treated THP-1 cells was abrogated by MYD88 deficiency, demonstrating that despite their differences, LPS and MSC exosome similarly induced the 3-h cytokine expression through a TLR-signaling pathway.

The M2-like macrophage phenotype of MSC exosome-treated THP-1 cells suggests that similar to M2 macrophages, the exosome-treated THP-1 cells may induce Tregs [46]. Indeed, exposure to 100 ng/mL exosomes for 24 or more hours enabled THP-1 cells to induce Treg polarization when exosome-treated THP-1 cell were incubated with activated $CD4^{+}$ T cells at a ratio of 1:1,000. This observation suggested that MSC exosomes could be immunosuppressive *in vivo* by inducing anti-inflammatory IL-10 and Tregs to attenuate immune activity. To test this, we assessed the effects of exosomes on allogeneic skin graft rejection in mice. We observed a delay of 2 days in graft rejection for exosome-treated animals, and this delay is comparable to that reported for mice treated with 30 mg/kg cyclosporine A [30]. This exosome-mediated delay in graft rejection was concomitant with an increase in Tregs. However, it should be noted that the MSC exosome did not increase Tregs in mice that did not receive skin grafts (Fig. 4B), suggesting that MSC exosomes induce Treg polarization only in an activated immune system. If true, this mitigates the risk of

compromised immune surveillance in MSC exosome-based therapy. Consistent with this, clinical observations thus far have indicated that while MSCs could induce solid organ transplantation tolerance [47], MSC therapy did not attenuate GVL responses or increase infections in patients [2]. Most importantly, the capacity of MSC exosomes to attenuate an activated immune system provides a rationale for its use in attenuating a hyperactive immune system such as GVHD. A preliminary clinical study demonstrated that MSC exosomes dramatically alleviated the symptoms of a treatment-resistant grade IV acute GVHD patient and the patient remained stable for 5 months [48]. Therefore, our study demonstrated that exosomes could mediate the widely reported immunosuppressive activity of MSCs by activating MYD88-dependent signaling in monocytes to induce a M2-like phenotype (Fig. 5). These activated monocytes, in turn, polarized activated $CD4^{+}$ T cells to Tregs, which then attenuated an activated immune system.

In conclusion, MSC exosomes are immunologically active, and they have the potential to attenuate an activated immune system through the induction of anti-inflammatory cytokines and Tregs. This feature provides a rationale for the use of exosomes in treating immune diseases.

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Author Disclosure Statement

No competing financial interests exist.

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