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# Mesenchymal stromal cell exosome–enhanced regulatory T-cell production through an antigen-presenting cell–mediated pathway

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**Abstract**

*Background aims.* The immunomodulatory property of mesenchymal stromal cell (MSC) exosomes is well documented. On the basis of our previous report that MSC exosomes increased regulatory T-cell (Treg) production in mice with allogenic skin graft but not in ungrafted mice, we hypothesize that an activated immune system is key to exosome-mediated Treg production. *Methods.* To test our hypothesis, MSC exosomes were incubated with mouse spleen $CD4^+$ T cells that were activated with either anti-CD3/CD28 mAbs or allogenic antigen-presenting cell (APC)-enriched spleen $CD11c^+$ cells to determine whether production of mouse $CD4^+CD25^+$ T cells or $CD4^+CD25^+Foxp3^+$ Tregs could be induced. MSC exosomes were also administered to the lethal chimeric human-SCID mouse model of graft-versus-host disease (GVHD) in which human peripheral blood mononuclear cells were infused into irradiated NSG mice to induce GVHD. *Results.* We report here that MSC exosome–induced production of $CD4^+CD25^+$ T cells or $CD4^+CD25^+Foxp3^+$ Tregs from $CD4^+$ T cells activated by allogeneic APC-enriched $CD11C^+$ cells but not those activated by anti-CD3/CD28 mAbs. This induction was exosome- and APC dose–dependent. In the mouse GVHD model in which GVHD was induced by transplanted human APC-stimulated human anti-mouse $CD4^+$ T cell effectors, MSC exosome alleviated GVHD symptoms and increased survival. Surviving exosome-treated mice had a significantly higher level of human $CD4^+CD25^+CD127^{low/-}$ Tregs than surviving mice treated with Etanercept, a tumor necrosis factor inhibitor. *Conclusions.* MSC exosome enhanced Treg production *in vitro* and *in vivo* through an APC-mediated pathway.

**Key Words:** *antigen-presenting cell, exosome, mesenchymal stromal cell, graft-versus-host disease, regulatory T cells*

## Introduction

Mesenchymal stromal cells (MSCs) are multipotent fibroblast-like cells that could be readily isolated from a wide variety of tissues. They also have a large capacity for *ex vivo* expansion and are reported to have the potential to differentiate into all the three lineages: ectoderm, mesoderm and endoderm (reviewed in Ullah *et al.*) [1]. MSCs are also well known for their immunomodulatory potency [2]. MSCs are currently in clinical testing for many immune diseases [3], such as steroid-resistant graft-versus-host disease (GVHD) [4,5], Crohn disease [6,7] and multiple sclerosis [8]. In a landmark multicenter nonrandomized trial led by Katarina Le Blanc and colleagues from the European Group for Blood and Marrow Transplantation Mesenchymal Stem Cell Expansion Consortium, MSC transplantation induced complete responses in 55% of 55 patients with acute GVHD grade 2–4, and this response was independent of whether the MSCs were from third-party mismatched donors, human leukocyte antigen–identical siblings or haplo-identical family members [9].

Although MSC transplantation is effective in ameliorating GVHD, it does not impair graft-versus-leukemia (GVL) reactions [10,11], and this phenomenon has been attributed in part to MSC-mediated induction of regulatory T cells (Tregs) [12]. Tregs are a subpopulation of T cells known to attenuate immune activity and abrogate autoimmune diseases and as such have been implicated as a key immune cell type in the modulation of GVHD [13]. Tregs are progressively lost during GVHD in experimental murine models and patients [14–17]. *Ex vivo* infusion of Tregs suppressed GVHD but not GVL responses [18], whereas depletion of Tregs increased GVHD [19,20]. The hypothesis that MSCs' efficacy against GVHD is due to MSC-mediated induction of

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Tregs is supported by numerous lines of evidence. MSC infusion in mice after heart, islet or kidney transplant was reported to increase Tregs and increase survival of the transplanted tissues [21–23]. MSCs also induce Tregs in mouse models of experimental colitis, allergen-driven airway inflammation and autoimmune uveitis [24–26]. In a mouse model of collagen-induced arthritis, allogenic MSCs induce antigen-specific Tregs and prevent tissue damage [27]. Significantly, MSC infusion was found to be safe and enhance Treg level in a clinical study of two kidney transplant patients [28]. More recently, a phase II multicenter, randomized, double-blind study has reported that MSC infusions in chronic GVHD (cGVHD) patients increase memory B lymphocytes and Tregs and Th1:Th2 ratio [29].

The induction of Tregs by MSCs has been proposed to be mediated by MSC secretion as co-incubation of MSCs with $CD4^+$ T lymphocytes in a transwell system was sufficient in inducing generation of Tregs [24,30]. Consistent with this, MSCs have been shown to secrete many factors known to be important in the induction of Tregs, such as interleukin (IL)-10, transforming growth factor (TGF)-β and prostaglandin $(PGE)_2$. However, as previously discussed in our review, we have rationalized that in these factors secreted by MSC, exosomes represent the most plausible secreted candidate in mediating the induction of Tregs [31].

MSC exosomes are 100- to 200-ηm endosome-derived vesicles [32] with a protein- [33] and RNA-rich [34] cargo and were first reported when they were shown to reduce reperfusion injury in a mouse of acute myocardial ischemia [35–37]. Our group has demonstrated through pulse chase studies that our $CD9^+$, $CD81^+$ extracellular vesicles are endosome-derived [32] and are therefore bona fide exosomes. Since then, we have reported that MSC exosomes are also immunologically active [38] and could induce Tregs *in vitro* and *in vivo* [38]. They do not express MHC class I or II or co-stimulatory molecules such as CD40, CD80 and CD86 and can polarize THP-1 cells and primary mouse or human monocytes toward an M2 macrophage-like phenotype with elevated expression of anti-inflammatory IL-10 and an attenuated expression of pro-inflammatory (e.g., IL-1β, IL-6, tumor necrosis factor [TNF]-α, IL-12p40) genes. When pretreated with exosomes, THP-1 cells can induce Treg differentiation. In mice with allogeneic skin grafts, infusion of MSC exosomes increased Tregs and enhanced survival of allogenic skin graft. However, in nongrafted animals, infusion of MSC exosomes have no effect on Treg production. We hypothesize that MSC exosomes are immunologically modulatory only in the context of an activated immune response and not in a homeostatic immune system.

In this report, we tested this hypothesis by evaluating the capacity of MSC exosomes to induce Tregs *in vitro* using naive $CD4^+$ T cells that had been activated by anti-CD3 and CD28 antibody. MSC exosomes had minimal effects on the polarization of the activated $CD4^+$ T cells into $CD4^+CD25^+$ $Foxp3^-$ T cells. However, in the presence of allogenic $CD11C^+$ APCs, MSC exosomes induced significant polarization of naïve $CD4^+$ T cells to $CD4^+CD25^+Foxp3^+$ Treg in a dose-dependent manner. Infusion of MSC exosomes led to a significant improvement in mouse model of xenogeneic GVHD symptoms and reduction in mortality with an increase of human $CD4^+CD25^+CD127^{low/-}$ Tregs in a mouse model of GVHD induced by human peripheral blood mononuclear cells (PBMCs).

## Methods

### *Mice*

Six- to 8-week-old female BALB/c ($H\text{-}2^d$) and C57BL/6 ($H\text{-}2^b$) mice were purchased from InVivos Pte Ltd (Singapore) and housed in a specific pathogen-free facility. The 6- to 8-week-old female NSG mice were provided from Jackson Laboratory, as an outsourced service of an *in vivo* experiment to assess the effect of a novel therapeutic of MSC exosomes on survival in a model of human PBMC-induced GVHD (with irradiation). All manipulations were performed according to the protocols approved by the Institutional Animal Care and Use Committee.

### *Preparation of MSC exosomes*

MSC exosome was prepared as previously described [35,36,39]. Briefly, immortalized human embryonic stem cell–derived MSCs were grown in a chemically defined medium for 3 days and the conditioned medium was harvested and 0.22-mm filtered. The conditioned medium was concentrated 100 × for exosomes by tangential flow filtration (Sartorius; MWCO 100 kDa) and stored in −20°C freezer until use. The exosomes were assayed for protein concentration using Coomassie Plus (Bradford) Assay (Thermo Fisher Scientific), as per manufacturer's instruction, and characterized for particle size distribution and concentration by Zetaview (Particle Metrix) according to the manufacturer's protocol. The mean protein and particle contents of the exosome preparations were 1.9 mg/mL and $1.9 \times 10^{11}$ particles/mL.

### *Incubation of MSC exosomes with $CD4^+$ T cells activated by anti-cd3cd28 mAb*

$CD4^+$ T cells were isolated from C57BL/6 mouse spleens as previously described [38]. Briefly, the spleens

Table I. GVHD clinical scoring system.

| Criteria | Grade 0 | Grade 1 | Grade 2 |
|---|---|---|---|
| Weight loss | <10% | **>10% to <20%** | >20% |
| Posture | Normal | Hunching noted at rest | Severe hunching impairs movement |
| Activity | Normal | Mild to moderately decreased | Stationary unless stimulated |
| Fur texture | Normal | Mild to moderate ruffling | Severe ruffling/poor grooming |
| Skin integrity | Normal | Scaling of paws/tail | Obvious areas of denuded skin |
| Paleness | Normal | Slightly pale | Severe paleness |

were first homogenized to splenocytes using gentleMAC Dissociator (Miltenyi Biotec) and naive $CD4^+$ T cells were then isolated using EasySep mouse $CD4^+$ T cell Negative Selection Kit (Stem Cell Technologies). The T cells were plated at $1 \times 10^6$ per well in 24-well plates that were pre-coated with 5 μg/mL anti-CD3 mAb (145-2C11, eBioscience) with 5 μg/mL soluble anti-CD28 mAb (37.51, eBioscience) in the absence or presence of either 10 or 30 μg/mL MSC exosomes. In the positive control, the T cells were incubated with 10 ηg/mL rhTGF-β1 (eBioscience). The cells were grown in RPMI-1640 medium (Life Technologies/Gibco) supplemented with 10% FCS, 100 U/mL penicillin, 100 mg/mL streptomycin, 2 mmol/L L-glutamine, 1 mmol/L sodium pyruvate, 10 mmol/L HEPES buffer (Life Technologies/Gibco) and 10 μmol/L 2-mercaptoethanol (Sigma-Aldrich) at 37°C, 5% $CO_2$. After 6 days, the cells were harvested and stained for fluorescein isothiocyanate–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 according to the manufacturer's instructions (eBioscience). fluorescence-activated cell sorting (FACS) analysis of the cells was performed on a B LSR II flow cytometer (BD Biosciences).

### *Incubation of MSC exosomes with $CD4^+$ T cells activated by allogeneic $CD11c^+$ cells*

$CD11c^+$ spleen cells were isolated from homogenized BALB/c mouse spleens using mouse $CD11c^+$ MACS MicroBeads UltraPure (Miltenyi Biotec), and naive $CD4^+$ T cells were isolated from homogenized C57BL/6 mouse spleens as described earlier. The $CD4^+$ T cells were plated at $1 \times 10^6$ per well in 24-well plates, and $CD11c^+$ cells were added such that the ratio of $CD11c^+$ cells to $CD4^+$ T cells is 1:2, 1:5, 1:10 or 1:20. The mixed cell cultures were then incubated in the absence or presence of MSC exosomes at 1, 5, 15, 30 or 60 μg/mL. After 6 days, the cells were harvested and analyzed by FACS for the presence of $CD4^+CD25^+Foxp3^+$ Treg cells as described earlier.

### *Human PBMC-induced mouse GVHD model*

Efficacy testing of MSC exosomes in a mouse model of GVHD was conducted by In Vivo Services at Jackson Laboratory. Briefly, on day 0, mice were grouped per body weight and irradiated with 100 cGy per mouse. Four hours post-irradiation, mice received $1–2 \times 10^7$ human PBMC injected via tail vein. 1 or 10 μg MSC exosomes per mouse (n = 20), phosphate-buffered saline (as vehicle control, n = 20) and 100 μg Enbrel per mouse (as drug-positive control, n = 12) were intraperitoneally injected into each mouse beginning on day 1. Subsequent doses were administered every 3 days thereafter until death of animal or termination of study at day 34, whichever is earlier. Cage side observations were made daily throughout the study. Clinical observations, body weights and disease activity index (DAI) measurements were performed before GVHD induction and three times a week thereafter. Clinical observations included weight loss, posture, activity, appearance of hair coat and skin based on the grading scale described in Table I to monitor the progression of GVHD. Clinical observations switched to daily and survival testing began when the earliest possible clear clinical signs were observed. Clinical signs included ≤10% weight loss from the starting weight, cold to touch, lethargic, hunched posture and scruffy coat. On day 21, approximately 100 μL of whole blood per mouse was collected for FACS analysis of the respective percentage of human/mouse $CD45^+$ live cell subpopulation and $CD3^+$, $CD3^+CD4^+$ and $CD3^+CD8^+$ cell subpopulation out of the total human $CD45^+$ live cells. On day 34, mice were euthanized by $CO_2$ asphyxiation, terminal body weights were recorded, terminal whole blood was collected and spleens were harvested for FACS analysis of the respective percentage of $CD45^+$, $CD3^+$, $CD3^+CD4^+$, $CD3^+CD8^+$ and $CD4^+CD25^+CD127^{low/-}$ cell subpopulation.

### *Statistical analysis*

Data are shown as mean ± SD values. Mean values for each group were compared using Student's *t* test. $P < .05$ was considered statistically significant.

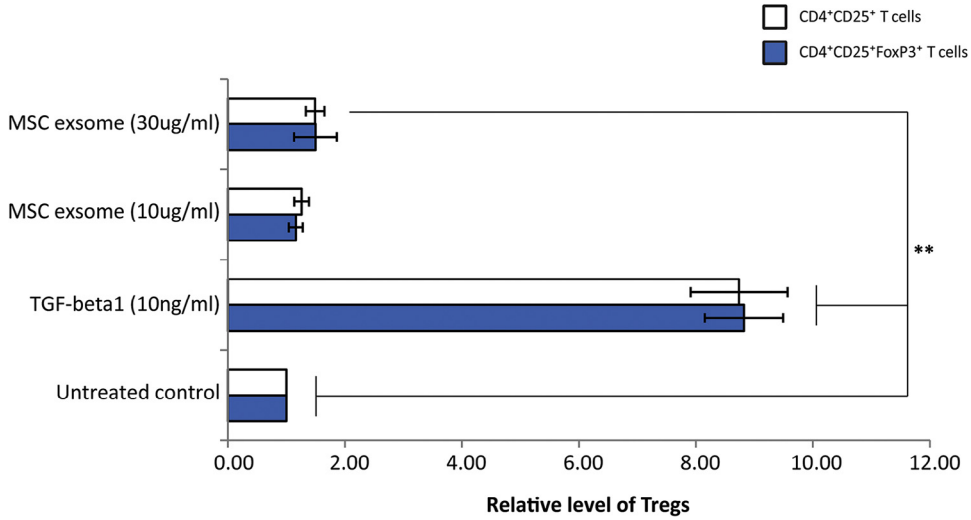



Figure 1. Anti-CD3/CD28 mAb activation of $CD4^+$ T cells and Treg induction by MSC exosome. $CD4^+$ T cells were purified from C57BL/6 mouse spleens and incubated with or without (w/wo) MSC exosomes (10 or 30 ug/mL) or TGF-β1 (10 ηg/mL) for $CD4^+CD25^+Foxp3^+$ Treg cells in the presence of anti-CD3 mAb (5 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 (A) $CD4^+CD25^+$ cells or (B) $CD4^+CD25^+Foxp3^+$ Treg cells. The percentage of $CD4^+CD25^+$ cells or $CD4^+CD25^+Foxp3^+$ Treg cells was normalized to that of $CD4^+$ T cells exposed to only anti-CD3 mAb (5 μg/mL) and anti-CD28 mAb (5 μg/mL) (untreated control). Each bar represents the mean (±SD) of three independent assays performed in duplicate or triplicate. $^{**}P < 0.001$.

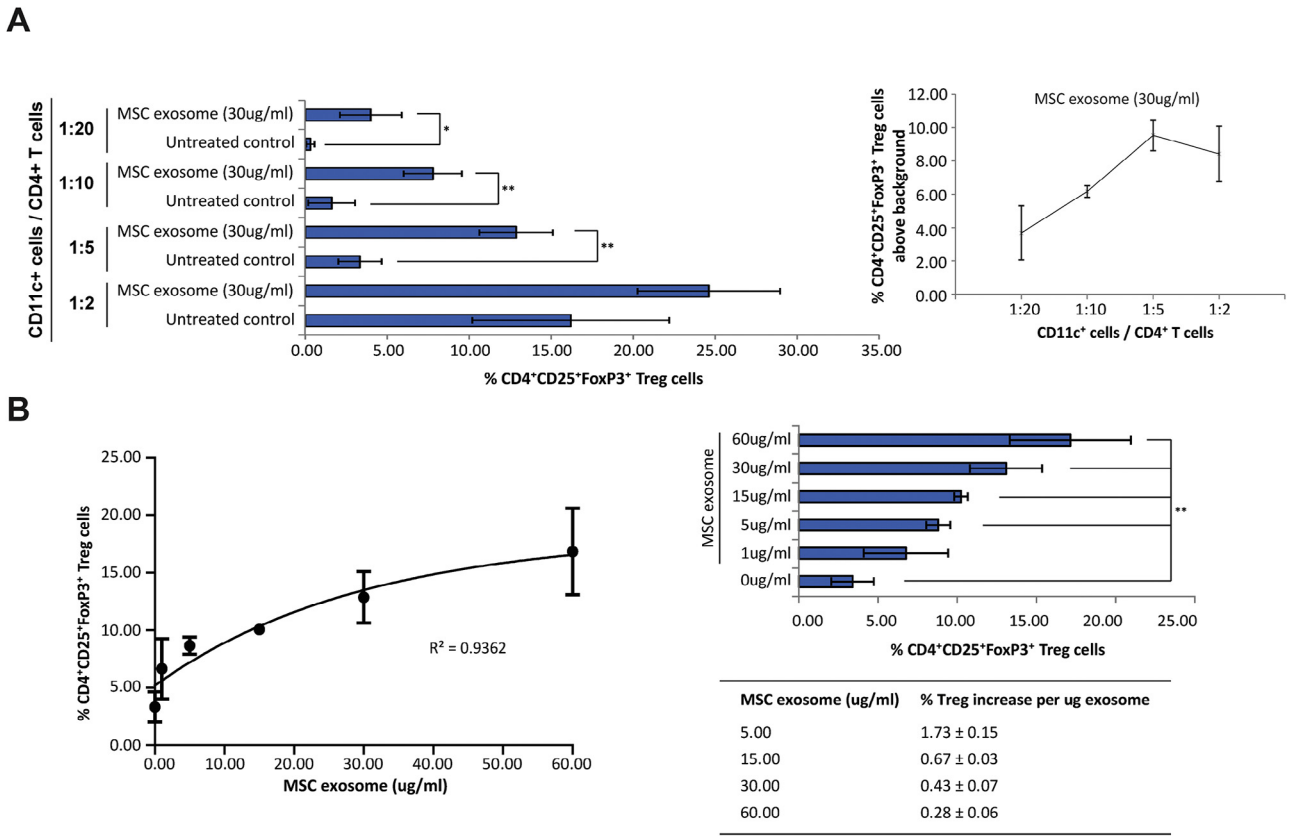
## Results

### *MSC exosomes had minimal effect on the polarization of CD3/CD28-costimulated $CD4^+$ T cells to $CD4^+CD25^+$ $FoxP3^+$ tregs*

We had previously shown that infusion of MSC exosomes increased Tregs in mice grafted with allogenic skin but not in ungrafted mice [38]. Therefore, we hypothesized that MSC exosomes enhance differentiation of Treg only when T cells are activated. To test this, $CD4^+$ T cells were purified from C57BL/6 mouse spleen and activated with anti-CD3 mAb and anti-CD28 mAb in the presence of either MSC exosomes (10 or 30 μg/mL) or rhTGF-β1 (10 ηg/mL). The rhTGF-β1 treatment served as positive control as it was previously shown to a potent stimulator of Treg differentiation in vitro [40,41]. Six days later, the cells were analyzed by FACS for the presence of $CD4^+CD25^+$ Treg cells or $CD4^+CD25^+FoxP3^+$ Treg cells. The percentage of Treg cells was normalized to that of $CD4^+$ T cells exposed to only anti-CD3 mAb (5 μg/mL) and anti-CD28 mAb (5 μg/mL) but not exosomes (untreated control) (Figure 1). Induction of $CD4^+CD25^+$ or $CD4^+CD25^+$ $FoxP3^+$ Treg cells was not significantly different in the exosome-treated group. The increase in either $CD4^+CD25^+$ cells or $CD4^+CD25^+$ $FoxP3^+$ Treg cells over that in the untreated control was small and not likely to have biological consequences. In contrast, rhTGF-β1 induced a ninefold increase in $CD4^+CD25^+$ in $CD4^+CD25^+$ $FoxP3^+$ Treg cells (Figure 1). Therefore, MSC exosome has minimal effect on production of $CD4^+CD25^+FoxP3^+$ Tregs from anti-CD3/CD28 co-stimulated $CD4^+$ T cells.

### *MSC exosomes increased polarization of $CD4^+$ T cells to $CD4^+CD25^+FoxP3^+$ tregs in the presence of allogeneic $CD11c^+$ cells*

Because APCs are critical in both activating T cell and inducing tolerance (as reviewed elsewhere) [42–44], we next determined whether MSC exosomes could render a more efficient polarization of T cells to Tregs in the presence of APC-enriched allogeneic $CD11c^+$ cells. $CD4^+$ T cells were isolated from spleen of C57BL/6 mice and co-cultured for 6 days with allogenic APC-enriched $CD11c^+$ cells from BALB/c mouse spleen at ratio of 1:2, 1:5, 1:10 or 1:20 in presence or absence of 30 μg/mL MSC exosomes. Allogenic APC-enriched $CD11c^+$ cells alone have the potential to polarise $CD4^+$ T cells to Tregs in a dose-dependent manner. At a high ratio of one $CD11c^+$ cell to two $CD4^+$ T cells, Treg induction was 51 ± 23 times higher than the induction level at the ratio of 1:20. This induction potential was enhanced by MSC exosomes and was inversely proportional to the cell ratio. The rate of enhancement was 13 ± 6 at 1:20 ratio of $CD11c^+$ cells to $CD4^+$ T cells and this decreased to 2 ± 0.3 at the 1:2 ratio. However, the largest exosome-enhanced increase in Treg production above background was at the ratio of 1:5 (Figure 2A). This enhancement was also exosome dose–dependent.




| MSC exosome (ug/ml) | % Treg increase per ug exosome |
|---|---|
| 5.00 | 1.73 ± 0.15 |
| 15.00 | 0.67 ± 0.03 |
| 30.00 | 0.43 ± 0.07 |
| 60.00 | 0.28 ± 0.06 |

Figure 2. (A) $CD4^+$ T-cell activation by allogeneic APC-enriched $CD11c^+$ cells and Treg induction by MSC exosome. Allogeneic $CD11c^+$ cells from BALB/c mouse spleens were incubated with $CD4^+$ T cells from C57BL/6 mouse spleens with or without (w/wo) 30 μg/mL MSC exosomes at a different cell ratio ($CD11c^+$ cells/$CD4^+$ T cells = 1:2, 1:5, 1:10 or 1:20). After 6 days, the cells were harvested and analyzed by FACS for the presence of $CD4^+CD25^+Foxp3^+$ Treg cells. Each bar represents the mean (±SD) of three independent assays performed in triplicate. Background was determined by untreated control. $^*P < 0.05$, $^{**}P < 0.001$. (B) Effect of exosome concentration on Treg induction. Allogeneic $CD11c^+$ cells from BALB/c mouse spleens were incubated with $CD4^+$ T cells from C57BL/six mouse spleens w/wo a different dose titration of MSC exosomes (1, 5, 15, 30 or 60 μg/mL) at an optimized cell ratio of 1:5 ($CD11c^+$ cells/$CD4^+$ T cells = 1:5; $CD11c^+$ cells: $1 \times 10^5$ cells/well, $CD4^+$ T cells: $5 \times 10^5$ cells/well). After 6 days, the cells were harvested and analyzed by FACS for the presence of $CD4^+CD25^+Foxp3^+$ Treg cells. Each bar represents the mean (±SD) of three independent assays performed in triplicate. $^*P < 0.05$, $^{**}P < 0.001$.

The most efficient dose was 5 μg/mL MSC exosome, which had enhancement of 1.7 ± 0.2 per μg exosome (Figure 2B). Together, these observations demonstrated that MSC exosomes enhanced $CD4^+CD25^+FoxP3^+$ Treg differentiation through an allogeneic APC-enriched $CD11c^+$ cell–mediated process.

### *MSC exosomes alleviated GVHD and improved survival of mice with an increase in human $CD4^+CD25^+CD127^{low/-}$ tregs*

Because MSC exosomes can enhance murine Treg production from $CD4^+$ T cells in the presence of allogeneic APC-enriched $CD11c^+$ cells *in vitro*, we next determined whether the MSC exosomes could also enhance human Treg production in the well-established chimeric human-SCID mouse model. It was reported that in this mouse model, human APCs play a requisite role in stimulating the human anti-mouse $CD4^+$ T-cell effectors to induce GVHD [45]. Briefly, NSG mice were irradiated with 100 cGy, injected with $1–2 \times 10^7$ human PBMC via tail vein 4 h later, followed by intraperitoneal injections of 1 or 10 μg MSC exosomes, phosphate-buffered saline vehicle control and 100 μg Enbrel positive control on day 1 and then every 3 days thereafter. Cage side observations were made daily, and GVHD was evaluated using an assessment of combined disease activity index (DAI) score. MSC exosome administration alleviated GVHD severity and improved survival ($P < 0.05$; Figure 3A,B). Surprisingly, both exosome doses of 1 and 10 μg had similar efficacy in alleviating GVHD severity and improving survival. The efficiency of xenogeneic graft engraftment in human PBMC-induced mouse GVHD model was assessed by determining the relative presence of human cells in the blood of the grafted mice by flow cytometry. Human $CD45^+$ cells in the vehicle- and exosome-treated animals were present at similar levels

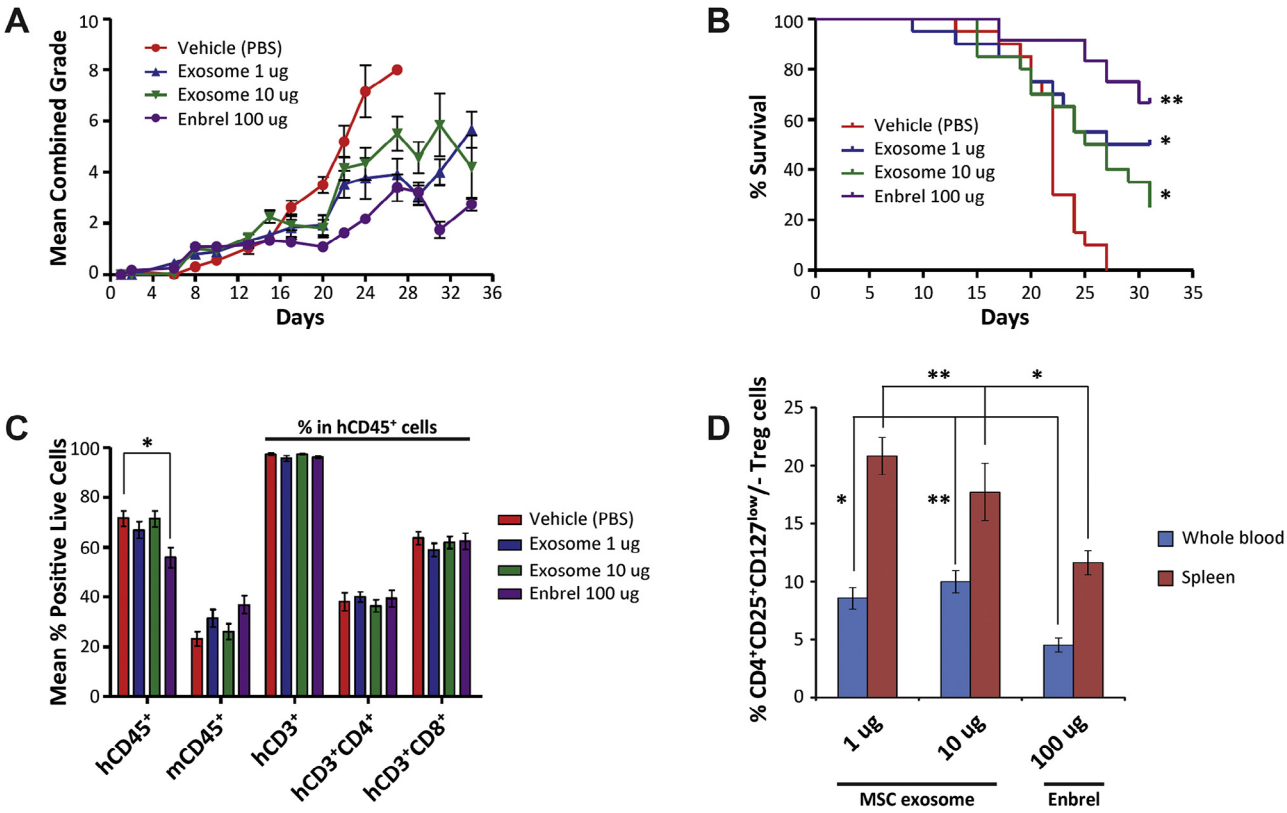



Figure 3. (A) Assessment of combined disease activity index (DAI). Onset of GVHD was assessed through a disease activity index of various clinical observations. Clinical observations included weight loss, posture, activity, appearance of hair coat, skin integrity and paleness based on the grading scale. Baseline measurements were taken of these clinical signs and measurements continued for the duration of the study. DAI scores were combined for each animal and averaged by treatment group. $*P < 0.05$, $**P < 0.001$. (B) Kaplan-Meier survival curve. The Kaplan-Meier survival plot represents the percentage of animals surviving to the study end point, which does not include the remaining live animals that were taken down on day 34 upon request to end the study. These percentages were generated using GraphPad Prism 5.0 software. Survival curve comparisons were analyzed by the Mantel-Cox and Gehan-Breslow-Wilcox tests (95% confidence interval) through GraphPad Prism software. $*P < 0.05$, $**P < 0.001$. (C) Flow cytometry analysis of mouse whole blood. Flow cytometry measurements were averaged by treatment group at day 21. Mean percent of human $CD45^+$ live cells represents subpopulation out of all live cells (mouse and human). Mean percent cells for all other markers represent the percentage of each cell population out of the total human $CD45^+$ live cells. Error bars represent standard error of the mean (SEM). (D) Flow cytometry analysis of human $CD4^+CD25^+CD127^{low/-}$ Tregs in mouse whole blood and spleen. Flow cytometry measurements of human $CD4^+CD25^+CD127^{low/-}$ Tregs were averaged by MSC exosome and Enbrel-treated groups at the terminal of *in vivo* study. Mean percent cells for $CD4^+CD25^+CD127^{low/-}$ Treg markers represent the percentage of each group cell population out of the total human $CD45^+$ live cells. Error bars represent SEM. $*P < 0.05$, $**P < 0.001$.

of 70–75% (Figure 3C), whereas that in the Enbrel-treated animals was significantly lower at 55.9% ($P < 0.05$). Because almost 100% of the human $CD45^+$ cells were $hCD3^+$ cells, the reduced level of $hCD45^+$ cells in the Enbrel-treated animals was consistent with the immunosuppressive potency of Enbrel. The distribution of $hCD3^+$ cells into $hCD3^+$ $CD4^+$ and $hCD3^+$ $CD8^+$ fractions were similar for all four treatment groups. The level of human Tregs in surviving mice was determined by measuring the relative level of $hCD4^+CD25^+CD127^{low/-}$ cells in $hCD4^+$ in whole blood and spleen on day 34 (Figure 3D). The level of Tregs in the 1-µg versus 10-µg exosome-treated mice was not significantly different for the blood and spleen. However, the level of Tregs in the blood and spleen of Enbrel-treated animals was significantly lower than the respective levels in exosome-treated mice ($P < 0.001$).

## Discussion

The well-characterized immunological potency of MSCs in ameliorating immune diseases [3], such as steroid-resistant GVHD [4,5], Crohn disease [6,7] and multiple sclerosis [8], has been increasingly attributed to its paracrine secretion, in particular exosomes (reviewed elsewhere) [3,46,47]. We previously demonstrated that MSC exosomes possess many of the immunodulatory properties that have been ascribed to MSCs and have the potential to underpin the immunotherapeutic activity of MSC secretion [38]. Like their cellular source, MSC exosomes are

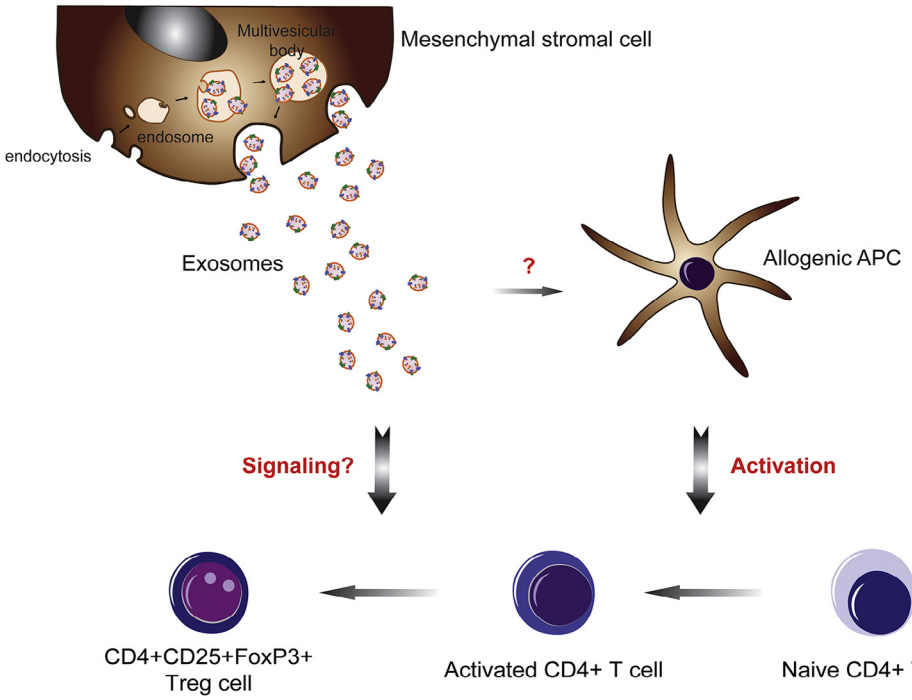



Figure 4. A proposed model for the pathways of Treg polarization by MSC exosomes. This model proposed that the induction of Tregs by MSC exosome requires activation of T cells by APCs and not by anti-CD3 and anti-CD28 mAbs. The anti-CD3/anti CD28 mAbs used to provide Signal 1 and Signal 2 in T-cell activation [53] may not be sufficient or appropriate in activating T cells for polarization to Tregs by MSC exosomes, and additional ligands present on APCs are necessary to prime the activation of $CD4^+$ T cells for polarization to Tregs by MSC exosomes. It is not clear at this time, however, whether MSC exosomes exert any effect on either or both activated $CD4^+$ T cells and APCs during Treg induction.

hypo-immunogenic. They are not only negative for major histocompatibility complex (MHC) class II but also MHC class I, B7-1 (CD80), B7-2 (CD86) and CD40 [48,49]. However, in contrast to the widely reported ability of MSCs to suppress mitogen-stimulated T-cell proliferation (reviewed in Abdi *et al.*) [50], MSC exosomes cannot suppress such T-cell proliferation except at a relatively high concentration [38,51]. Because this suppression had been shown not to be essential to patient outcome in MSC-treated GVHD patients [52], it is unlikely to be an important indicator of therapeutic potential of MSC exosomes in autoimmune diseases. Instead, we focus on the potential of MSC exosomes to induce Tregs as a possible mechanism for their immunomodulatory properties [38].

In this study, we determined that MSC exosomes could replicate the paracrine potency of MSCs in generating Tregs [24,30] through an APC-mediated pathway. We have previously reported that MSC exosomes alone failed to polarize T cells to Tregs or Th17 cells [38]. In addition, we have also observed that administration of MSC exosomes increased Tregs in mice grafted with an allogeneic skin and not in ungrafted animals [38]. Together these suggest that MSC exosome–mediated induction of Tregs requires T-cell activation. However, incubation of MSC exosome with $CD4^+$ T cells that were activated by anti-CD3/anti-CD28 mAbs did not induce Treg production. On the other hand, activation of the $CD4^+$ T cells using allogenic APC-enriched $CD11c^+$ cells enhanced Treg production (Figure 4). On the basis of these observations, we proposed that the induction of Tregs by MSC exosome requires activation of T cells by APCs, and not by anti-CD3 and anti-CD28 mAbs. The Signal 1 and Signal 2 initiated by anti-CD3/anti CD28 mAbs [53] may not be sufficient or appropriate in activating T cells for polarization to Tregs by MSC exosomes and that additional ligands present on APCs are necessary to prime the activation of $CD4^+$ T cells for polarization to Tregs by MSC exosomes. It is, however, not clear at this time whether MSC exosomes exert any effect on either or both activated $CD4^+$ T cells and APCs during Treg induction (Figure 4). Nevertheless, our proposal is consistent with previous observations that T-cell stimulation by anti-CD3/anti-CD28 mAbs only partially mimics stimulation by APCs and that additional co-stimulatory molecules and adhesion molecules such as 4-1BB, CD83, ICAM-1 (CD54) and LFA-3 (CD58) are required [54,55]. The potency of MSC exosomes in inducing Treg production via APC-stimulated T cells was corroborated by the well-established chimeric hu-SCID mouse of

GVHD [56]. In this model, GVHD is induced by human APC-mediated stimulation of human anti-mouse $CD4^+$ T cells [45]. GVHD symptoms and survival were significantly alleviated or improved by MSC exosomes. Consistent with our hypothesis that MSC exosomes enhance Treg production via APC-stimulated T cells, surviving exosome-treated mice had a higher Treg level in the blood and spleen than Enbrel-treated animals.

Our observation that MSC exosomes alleviated GVHD and improved survival in a xenogeneic mouse model of GVHD replicated a recent report describing a similar efficacy of human MSC extracellular vesicles (EVs) in an allogeneic mouse model of GVHD by Fujii *et al.* [57]. The latter also reported a higher level of Tregs in MSC EV-treated animals versus untreated control. Both Fujii *et al.* [57] and Fattore *et al.* [58] also reported that *in vitro*, MSC EVs induced Treg-associated effects on anti-CD3/CD28-stimulated PBMCs. Specifically, Fujii *et al* demonstrated that whereas human MSC EVs suppressed non-Tregs *in vitro*, they preserved naive but not effector Tregs within the Foxp3-expressing T-cell population [57]. On the other hand, Fattore *et al.* reported that human MSC-EVs significantly increased $CD4^+CD25^+CD127^{low}FoxP3^+$ Tregs in anti-CD3/CD28-stimulated PBMCs *in vitro* [58]. In contrast to these two studies, we did not observed Treg-associated effects when testing MSC exosomes on anti-CD3/CD28-stimulated mouse spleen $CD4^+$ T cells. However, it is premature at this time to draw specific inferences from these three studies because the earlier two studies used PBMCs, which are heterogenous and enriched in memory T cells, whereas our study used mouse spleen $CD4^+$ T cells enriched in naive T cells.

We attribute the increased survival of Enbrel-treated animals to the potent immunosuppression by Enbrel. Enbrel is a fusion protein consisting of the TNF receptor and immunoglobulin G1 Fc, and it acts as a decoy TNF receptor to reduce TNF signaling [59]. The immune suppression activity of Enbrel in Enbrel-treated animals was evident in the significant reduction of $hCD45^+$ cells in their peripheral blood ($P < 0.05$). On the other hand, we attribute the increased survival of exosome-treated animals to increased Treg production. This is consistent with the strong inverse correlation between the level of Tregs and the severity of GVHD in preclinical animal studies or aGVHD patients [60]. This correlation helps rationalize clinical studies on the use of adoptive Treg cell therapy to alleviate the severity of aGVHD (https://clinicaltrials.gov/ct2/results?cond=GVHD%2C+Acute&term=treg+&cntry1=&state1=&Search=Search; November 2017). Significantly, these studies suggest that human MSC exosomes could induce both human and mouse Tregs from APC-activated T cells.

We observed that the efficiency of Treg induction by MSC exosomes in vitro was inversely proportional to the relative ratio of APC-enriched $CD11c^+$ cells to $CD4^+$ T cells and exosome dose. At high ratio, the enhancement effect of MSC exosome was low. The optimal ratio of APCs to $CD4^+$ T cells for maximal MSC exosome was 1:5 and the most efficient exosome dose was 5 μg/mL at the optimal 1:5 cell ratio. We observed that the optimal therapeutic exosome dose in alleviating GVHD was 1 μg per mouse as there was no statistically significant differences between the 1- and 10-μg doses. There was also no obvious adverse outcome in either of the two doses.

The importance of APCs, particularly the DCs in regulating immune reactivity toward a tolerogenic or an immunogenic response [61,62], is well established. Failure to mount a dynamic and temporally appropriate response could result in autoimmune diseases or increased susceptibility to invading pathogens. However, the mechanism through which APCs regulate this dichotomy is not well understood [63]. The main challenge is the complexity in regulating this dichotomy between a tolerogenic or an immunogenic response. To illustrate, a computer model to understand the signaling and transcriptional regulatory networks involved in a stable state of Th1/Th2/Th17/Treg/Tfh/Th9/Th22 cell differentiation revealed the involvement of at least 20 signaling pathways, a dozen of transcription factors and about 30 cytokines, amounting to 101 components in total [64]. In addition to the signaling and transcriptional regulatory networks, our present study also revealed that the ratio of activated APCs to naive $CD4^+$ T cells is an important consideration in regulating the dichotomy between a tolerogenic and an immunogenic response, and our study revealed a role for MSC exosome in enhancing the effect of this ratio in inducing Tregs in a static experimental scenario.

In conclusion, this study demonstrated that MSC exosomes could replicate the immunological potential of MSCs in inducing Tregs *in vitro* and in alleviating GVHD symptoms with a concomitant increase in Tregs. This makes MSC exosomes a viable alternative cell-free therapeutic candidate to treat GVHD with minimal compromise in the GVL potency of allogeneic hematopoietic stem cell transplantation in patients with leukemia. MSC exosomes being nonviable cellular products provide many advantages over the use of cellular MSC products. Unlike viable cellular products, exosomes are more amenable to a strictly regulated and monitored manufacturing process. Being much smaller than cells, exosomes could be sterile filtered and are less likely to cause embolism during intravenous administration. In addition, nonviable and nonreplicating exosomes will not persist or amplify to cause abnormal tissue growth, autoimmune disease or occlusions in the distal microvasculature [65–67].

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