Adoptive Transfer of Treg Cells Combined with Mesenchymal Stem Cells Facilitates Repopulation of Endogenous Treg Cells in a Murine Acute GVHD Model

Eun-Sol Lee1,2, Jung-Yeon Lim1,2, Keon-II Im1,2, Nayoun Kim1,2, Young-Sun Nam1,2, Young-Woo Jeon1,2,3, Seok-Goo Cho1,2,3*

1 Institute for Translational Research and Molecular Imaging, The Catholic University of Korea College of Medicine, Seoul, Korea, 2 Laboratory of Immune Regulation, Convergent Research Consortium for Immunologic Disease, The Catholic University of Korea College of Medicine, Seoul, Korea, 3 Department of Hematology, Catholic Blood and Marrow Transplantation Center, Seoul St. Mary’s Hospital, The Catholic University of Korea College of Medicine, Seoul, Korea

* chosg@catholic.ac.kr

Abstract

Therapeutic effects of combined cell therapy with mesenchymal stem cells (MSCs) and regulatory T cells (Treg cells) have recently been studied in acute graft-versus-host-disease (aGVHD) models. However, the underlying, seemingly synergistic mechanism behind combined cell therapy has not been determined. We investigated the origin of Foxp3+ Treg cells and interleukin 17 (IL-17+) cells in recipients following allogeneic bone marrow transplantation (allo-BMT) to identify the immunological effects of combined cell therapy. Treg cells were generated from eGFP-expressing C57BL/6 mice (Tregegfp cells) to distinguish the transferred Treg cells; recipients were then examined at different time points after BMT. Systemic infusion of MSCs and Treg cells improved survival and GVHD scores, effectively downregulating pro-inflammatory Thx and Th17 cells. These therapeutic effects of combined cell therapy resulted in an increased Foxp3+ Treg cell population. Compared to single cell therapy, adoptively transferred Tregegfp cells only showed prolonged survival in the combined cell therapy group on day 21 after allogeneic BMT. In addition, Foxp3+ Treg cells, generated endogenously from recipients, significantly increased. Significantly higher levels of Tregegfp cells were also detected in aGVHD target organs in the combined cell therapy group compared to the Treg cells group. Thus, our data indicate that MSCs may induce the long-term survival of transferred Treg cells, particularly in aGVHD target organs, and may increase the repopulation of endogenous Treg cells in recipients after BMT. Together, these results support the potential of combined cell therapy using MSCs and Treg cells for preventing aGVHD.
Introduction

Recent studies have demonstrated that therapeutic approaches based on various cells, such as mesenchymal stem cells (MSCs), natural killer cells (NK cells), natural killer T cells (NKT cells), and regulatory T cells (Treg cells), can be efficacious in improving acute graft-versus-host disease (aGVHD) complications and survival rates after allo-HSCT [1-6]. In particular, MSCs have been widely studied in clinical HSCT to suppress the proliferation of allo-reactive T cells that are involved in aGVHD [5,7,8]. In addition, regulatory T cells (Treg cells) that are CD4+ CD25+ Foxp3+ have immunosuppressive abilities that decrease effector T cell activities [9–11]. However, current treatment using MSCs do not play a significant role in modulating or preventing aGVHD [12].

Several studies have demonstrated that the infusion of MSCs can relatively control Th1-mediated responses, but does not inhibit Th17-mediated conditions, such as autoimmune arthritis [13,14]. Treg cells are also unstable, with the potential to convert to inflammatory Th17 cells in Th1 responses in autoimmune conditions [15–17]. However, it has recently been demonstrated that interactions with MSC can induce Treg cells in various in vitro and in vivo models [18–20]. MSC-induced Treg cell formation involves several factors, including transforming growth factor beta 1 (TGF-β) and prostaglandin E2 (PGE2). In addition, co-cultures of peripheral blood mononuclear cells (PBMCs) with MSCs generated powerful regulatory CD4+ and/or CD8+ lymphocytes [19–22]. These reports suggest that MSCs may be helpful in generating and maintaining Treg cells stably in aGVHD models. Furthermore, combined cell therapy using MSCs and Treg cells may be helpful in alleviating aGVHD.

Given this background, we previously demonstrated that combined cell therapy with MSCs and Treg cells induced long-term survival in a aGVHD model and regulated Th1/Th17 cells, and Foxp3+ Treg cells, reciprocally in recipients [23]. In addition, we identified various therapeutic effects in mixed chimerism and skin allograft transplantation [24,25]. However, the underlying immunological mechanisms that occur in recipients have not been fully explained.

Satisfactory therapeutic outcomes in adoptive cell therapy depend on whether the adoptively transferred cells remain in recipients over a long period of time without conversion to other cell types. Thus, we demonstrated combined cell therapy using *ex vivo*-expanded Treg cells from eGFP-expressing C57BL/6 mice (Treg eGFP cells). We distinguished the adoptively transferred Treg eGFP cells and repopulating endogenous Treg cells following co-administration of Treg eGFP cells with MSCs in recipients after BMT. We demonstrate the functional properties of MSCs to maintain and generate Treg cells *in vivo*, and also provide a potential strategy for treating aGVHD through combined cell therapy in aGVHD.

Materials and Methods

Mice

BALB/c (H-2d) mice (8–10 weeks old) were purchased from OrientBio (Sungnam, Korea). C57BL/6-tg(CAG-EGFP; H-2b) mice were purchased from Japan SLC (Shizuoka, Japan). The mice were maintained under specific pathogen-free conditions in an animal facility with controlled humidity (55±5%), light (12/12 h light/dark), and temperature (22±1°C). The air in the facility was passed through a HEPA filter system, designed to exclude bacteria and viruses. Animals were fed mouse chow and tap water *ad libitum*. For blood collection, mice were anesthetized with 2.5% isoflurane in oxygen, and sacrificed by exposure to CO2. The animal care and euthanasia protocols used in this study were approved by the Animal Care and Use Committees at Korea University and the Catholic University of Korea, College of Medicine (Permit number: 2013-0073-01).
Treg generation

To obtain Treg cells, isolated CD4+ T cells from C57BL/6-tg(CAG-EGFP) mice were cultured with plate-bound anti-CD3 (1 μg/mL; BD PharMingen, CA, USA), soluble anti-CD28 (1 μg/mL; Biolegend, San Diego, CA, USA), human recombinant transforming growth factor beta (TGF-β; 5 ng/mL; PeproTech, London, UK), and RA (100 nM; Sigma-Aldrich, St. Louis, MO, USA) for 3 days. The expanded induced Treg cells were sorted by flow cytometry to obtain a >96% pure CD4+ CD25+ Foxp3+ population.

Isolation and culture of MSCs

C57BL/6 BM cells were collected by flushing femurs and tibias with Dulbecco’s Modified Eagle’s Medium (Gibco, Carlsbad, CA, USA) containing 2 mM L-glutamine (Gibco), 1% anti-biotics (penicillin [10 U/mL]-streptomycin [10 g/mL]; Gibco), and 15% heat-inactivated fetal bovine serum (FBS) with endotoxin levels ≤5 EU/mL and hemoglobin levels ≤10 mg/dL (Gibco). Cell immunophenotypes were persistently positive for Sca-1, CD44, and CD29, but were negative for c-Kit, CD11b, and CD34 after more than 15 passages using the antibodies described below, consistent with previous reports [26].

Bone Marrow Transplantation and aGVHD Induction

Recipient (BALB/c, H-2d) mice were exposed to a 800 cGy dose of radiation from a Mevatron MXE-2 instrument (Siemens, New York, NY, USA), with a focus on skin distance of 100 cm and a rate of 70 cGy/min. Recipient mice were then injected intravenously (IV) with 5×10^6 BMCs and 5×10^6 spleen cells from donor mice (C57BL/6, H-2b). Survival after bone marrow transplantation (BMT) was monitored daily, and the degree of clinical aGVHD was assessed weekly using a scoring system that summed changes in five clinical parameters: weight loss, posture, activity, fur texture, and skin integrity. Animals with scores >7 were considered moribund and were euthanized 50 days after transplantation.

Combined cell therapy of MSCs and Treg cells controlled aGVHD

Mice were injected i.p. with 1×10^6 MSCs, and i.v. with 2×10^6 Treg cells or 1×10^6 MSCs plus 2×10^6 Treg cells twice weekly after BMT (BMT + day 0, 4). Control mice received i.v. injections of an equal volume of phosphate-buffered saline (PBS; Gibco) at the same time points.

Flow cytometric analysis

Mononuclear cells were immunostained with various combinations of the following fluorescence-conjugated antibodies: CD25-APC (eBioscience, San Diego, CA, USA), CD4-Pacific blue (eBioscience), Foxp3-PE (eBioscience), H-2D^k-perc5.5 (eBioscience), IL-17-PE (eBioscience), Foxp3-APC (eBioscience), INF-γ-APC (eBioscience), IL-4-PE (BD PharMingen), IL-17-FITC (eBioscience), H-2K^d-FITC (eBioscience), IL-6-PE (Biomek), and TNF-α-APC (BD). Before intracellular cytokine staining, cells were stimulated in culture medium containing phorbol myristate acetate (25 ng/mL; Sigma-Aldrich), ionomycin (250 ng/mL; Sigma-Aldrich), or monensin (GolgiStop, 1 μL/mL; BD PharMingen) in a 5% CO2, 37°C incubator for 4 h. Intracellular staining was performed using an intracellular staining kit (eBioscience) according to the manufacturer’s protocol. Flow cytometry was performed on a FACSCalibur flow cytometer (BD PharMingen) with the FlowJo software (TreeStar, Ashland, OR, USA).
Western blotting
Spleen tissues were collected from recipients at 10 days after allo-BMT. Protein samples were separated by SDS gel electrophoresis, and transferred to a nitrocellulose membrane (Amer- sham Pharmacia Biotech, Buckinghamshire, UK). Membranes were stained with primary antibo- dies against signal transducer and activator of transcription (STAT) 1, STAT3 (all from Cell Signaling, Danvers, MA, USA), and β-actin. Then, horseradish peroxidase (HRP)-conjugated secondary antibody was added. After washing with Tris-buffered saline plus Tween-20 (TTBS), the protein bands were detected using an enhanced chemiluminescence (ECL) detection kit and Hyperfilm-ECL reagents (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

Analysis of gene expression using real-time quantitative PCR
Total RNA was extracted using the TRIzol reagent (Invitrogen). Total RNA (2 μg) was reverse transcribed at 50°C for 2 min, followed by 60°C for 30 min. Quantitative PCR was performed using a FastStart DNA Master SYBR Green I kit and a LightCycler 480 detection system (Roche, Meylan, France), as specified by the manufacturer. The crossing point (Cp) was defined as the maximum of the second derivative of the fluorescence curve. Negative controls contained all elements of the reaction mixture, except the template DNA. For quantification, the relative mRNA expression of specific genes was obtained by the ΔΔCt method, using β-actin for normalization. The following gene-specific primers (5’→3’) were used: β-actin (forward, GAA ATC GTG CGT GAC ATC AAA G and reverse, TGT AGT TTC ATG GAT GCC ACA G), Foxp3 (forward, GGC CCT TCT CCA GGA CAG A and reverse GGC GCT GAT CAT TGG GTT GT), eGFP (forward, TGA ACC GCA TCG AGC TGA AGG G and reverse, TCC AGC AGG ATG TGA TCG C), RORγt (forward, TGT CCT GGG CAT TGG GTT GT), and SOCS3 (forward, CGC CTC AAG ACC TTC AGC TC and reverse, CTG ATC CAG GAA CTC CCG AA).

Histopathology of aGVHD
Mice were sacrificed on day 21 after BMT for blinded histopathological analysis of aGVHD targets (liver and small intestine). Organs were harvested, cryoembedded, and then sectioned. Tissue sections were fixed in 10% buffered formalin (Sigma-Aldrich) and stained with hematoxylin (Sigma-Aldrich) and eosin Y 1% solution (Muto Pure Chemical Co., Ltd., Tokyo, Japan) for histological examination. The scoring system for each parameter was 0 for normal, 0.5 for focal and rare, 1 for focal and mild, 2 for diffuse and mild, 3 for diffuse and moderate, and 4 for diffuse and severe, in accordance with previously reported aGVHD histology [27].

Immunohistochemistry of aGVHD target organs
Paraffin sections (5 μm thick) were deparaffinized in xylene followed by hydration through gradedethanols. Antigen retrieval was performed by heating specimens at 92–95°C for 10 min with Universal Antigen Retrieval Agent (CTS015; R&D Systems, Minneapolis, MN). Endoge- nous peroxidase was blocked by incubating sections with 0.3% hydrogen peroxide for 15 min, and the non-specific binding sites were blocked in PBS with 1% horse serum for 30 min. Sec- tions were covered with primary antibody, and the slides were incubated in a moist chamber overnight at 4°C (Invitrogen anti-GFP rabbit IgG, A11122). After three washes in PBS, sections were incubated with secondary antibody (FITC-conjugated goat anti-rabbit IgG #115922) at 1:200 in incubating solution (1% BSA, 1% normal donkey serum, 0.3% Triton X-100 and 0.01% sodium azide in PBS) for 1 h at room temperature. After three washes in PBS, sections were incubated with DAPI for 10 min at room temperature, before rinsing once with PBS, and
mounting in fluorescence mounting medium (Dako #S3023). Fluorescence images were acquired using an LSM 510 confocal microscope (Carl Zeiss, Jena, Germany).

Statistical Analysis
Statistical analyses were performed using Graphpad Prism (ver. 5.01). Comparisons between groups were analyzed statistically using the Kruskal-Wallis test. Pair-wise group comparisons used the Mann-Whitney U-test, and p values were adjusted for multiple comparisons using Bonferroni’s method to determine the statistical significance of these comparisons. A p value < 0.05 was considered statistically significant.

Results

Immunophenotypes of culture-expanded MSCs and Treg cells
To characterize culture-expanded MSCs and Treg cells from C57BL/6 mice, surface protein expression of MSCs was examined using flow cytometry at the 10th–15th passage. MSCs showed a typical fibroblast-like morphology, and were uniformly positive for Sca-1, CD44, and CD29, but were negative for c-Kit, CD11b, CD34, CD106, CD45, and CD31 (Fig 1A) [26]. CD4+CD25+Foxp3+ Tregs showed >96% purity on flow cytometry and positive surface staining for several phenotypic Treg markers, including CD44, glucocorticoid-induced tumor necrosis factor receptor (GITR), intercellular adhesion molecule-1 (ICAM-1), inducible costimulator (ICOS), and programmed death-1 (PD-1). They also showed weak positive surface staining for CD62L and CD103 (Fig 1B).

Donor-derived MSCs and Treg cells improve clinical outcomes in a murine aGVHD model
aGVHD is caused by donor-derived T cells that attack recipient tissues after allo-HSCT from a major histocompatibility complex (MHC)-unrelated donor. Recipients (BALB/c, H-2d) were lethally irradiated and received BM cells with spleen cells from donor mice (C57BL/6, H-2d). At days 0 and 4 after BMT, recipients were administered MSCs (1×10^6) and Treg-gfp cells (2×10^6) from donor-derived mice (H-2b). Mice were evaluated at different time points after BMT. The mice were monitored for survival, body weight, and clinical aGVHD scores. Survival was 80% in the Treg cells group and 100% in the combined cell therapy group (Fig 2A). Body weights were not significantly different compared to the Treg cell group (Fig 2B), and aGVHD scores were improved in the combined cell therapy group compared to the single-therapy groups (Fig 2C). Interestingly, MSCs alone had lower survival and clinical scores. Histological analyses of aGVHD target organs also showed improvements in the combined cell therapy group (Fig 2D). These data suggest that combined cell therapy with donor-derived MSCs and Treg cells was effective for aGVHD.

Co-administration of donor-derived MSCs and Treg cells downregulate Th1 responses in an aGVHD model
Th1 cells play an important role in aGVHD pathogenesis, whereas Th2 cells reduce aGVHD [28,29]. We examined the improvement in aGVHD using donor-derived MSCs and Treg cells, and performed an ex vivo immunological analysis. We observed the percentage of Th1 and Th2 cells in recipient spleen at 12 days after BMT. The percentages of IFN-γ and TNF-α secreted by Th1 cells decreased in the combined cell therapy group versus MSCs or Treg cells alone. IL-4, which is secreted by Th2 cells, showed no significant difference among the groups (Fig 3A). In addition, the expression of phosphorylated-(p) signal transducers and activators of
transcription 1 (STAT1) was significantly downregulated in the combined cell therapy group (Fig 3B). The mRNA expression of T-box transcription factor TBX21 (T-bet) and trans-acting T-cell-specific transcription factor GATA-3 (GATA3), transcription factors of Th1 and Th2 cells, were measured in recipient spleen using real-time PCR. The mRNA levels of T-bet were decreased in the combined cell therapy group, whereas GATA-3 was not significantly different among groups. There were no groups had significant group differences in T-bet or GATA3 (Fig 3C). It has been suggested that Th1 expression was effectively downregulated by combined cell therapy in the aGVHD model.

Effective downregulation of Th17 cells by co-infusion of MSCs and Treg cells

The functional imbalance of Treg cells and Th17 cells is key in severe aGVHD; several reports have demonstrated instability of Treg cells that convert into Th17 cells under Th1 conditions [15–17]. We identified IL-17 and IL-6 expression in recipient spleen. IL-17 cells, within CD4⁺ T cells, were significantly reduced in the combined cell therapy group, compared to MSCs alone or the GVHD group, 12 days after BMT. While IL-17 levels in the combined cell therapy group were reduced compared to those of the Treg group, there was no significant difference. A trend toward a reduction of IL-6 levels was observed in the combined cell therapy group; however, there was only a significant difference between the GVHD and combined cell therapy
groups (Fig 4A). In addition, STAT3, which plays a role in downregulating Foxp3 in aGVHD, was decreased in the combined cell therapy group (Fig 4B). In addition, we identified that the transcription factor, RAR-related orphan receptor gamma (RORγt), which binds IL-17 in naïve CD4+ T helper cells, was significantly decreased in the combined cell therapy group compared to the Treg cell group (Fig 4C).

We identified the percentage of IL-17+ cells within CD4+ T cells in the recipient spleen and blood at three time points after BMT. We used ex vivo-expanded Treg cells from C57BL/6 eGFP+ (H-2b) background mice that had > 96% purity on flow cytometry. Recipients were administered donor-derived MSCs (1×10^6) and eGFP+ Treg cells (2×10^6) on days 0 and 4 after allo-BMT. We examined eGFP+IL-17+ cells of CD4+ T cells that represent IL-17+ cells converted from infused eGFP+ Treg cells after BMT. IL-17 expression was detected in < 0.5% of CD4+ T cells in the spleen, and eGFP+ IL-17+ cells were barely detected on day 7. IL-17+ cells increased slowly by day 21; however, eGFP+ IL-17+ cells were still a small proportion of total CD4+ T cells (Fig 4D). Moreover, the detected IL-17+ cells were completely of donor (H-2b) origin, endogenously generated in the recipient after BMT (data not shown). Similar to the spleen, IL-17+ cells were detected at a maximum percentage of 3% of CD4+ T cells in blood. In addition, IL-17+ cells were downregulated in the combined cell therapy group compared to the...
Treg cells single group (Fig 4E). These results suggest that MSCs could aid in maintaining adoptively transferred Treg cells stably in recipients.

MSCs contribute to the maintenance of adoptive transferred Treg cells, and help in the repopulation of endogenous Treg cells in recipients

We examined the origin of Foxp3+ Treg cells that increased in recipients after BMT. We used ex vivo-expanded Treg cells from C57BL/6 background eGFP+ (H-2b) mice that showed > 96% purity on flow cytometry. Recipients were administered donor-derived MSCs (1×10⁶) and Treg[eGFP] cells (2×10⁶) on days 0 and 4 after allo-BMT.

CD4⁺ CD25⁺ cells from recipients generally increased in the groups that received cells for therapy, except the GVHD group. Furthermore, Foxp3⁺ cells were increased in the combined cell therapy group, compared to the other groups (Fig 5A). In addition, we identified several transcription factors, suppressor of cytokine signaling 3 (SOCS3) and forkhead box protein 3 (Foxp3), which are important in evaluating aGVHD, and which significantly increased in the combined cell therapy group compared to the Treg cells alone group (Fig 5B and 5C).

We identified the percentage of eGFP⁺ cells in the Foxp3⁺ cells in the spleen and blood of the recipient by flow cytometry. Foxp3⁺ Treg cells were mostly of eGFP⁺ origin in both the Treg cells single group and the combined cell therapy group on day 7. On day 14, the eGFP⁺ cells in the Foxp3⁺ Treg cells showed a lower percentage than on day 7, but were still maintained in both the Treg cells single group and combined cell therapy group. However, eGFP
+ cells were still only detected at a high level in the combined cell therapy group on day 21, but not in the Treg cells single group (Fig 5D). Also notable was the fact that donor-derived (H-2b) Foxp3+ Treg cells significantly increased in the combined cell therapy group. These results suggest that combined therapy with MSCs and Treg cells increased the repopulation of Foxp3+ Treg cells from the recipient after BMT.

Likewise, Foxp3+ Treg cells were mostly of eGFP origin in recipient blood on day 7. However, endogenous Treg cells (H-2b), generated in the recipient, were increased in both the Treg cells and combined cell therapy groups. The Treg cells were increased in the combined cell therapy group compared to the Treg cells alone group on day 21 after allo-BMT, although the difference was not statistically significant on day 14 (Fig 5E).

Thus, our data suggest that combination therapy to treat aGVHD, with co-administration of MSCs and Treg cells, was effective for not only enhancing the maintenance of adoptive
transferred Foxp3+ Treg cells, but also for increasing the amount of repopulated endogenous Treg cells in recipients.

eGFP+ cells were detected at high levels in aGVHD target organs, especially in the combined cell therapy group

We identified eGFP expression in the recipient liver and small intestine; aGVHD target organs were observed by immunofluorescence confocal microscopy. eGFP expression levels were detected in both the Treg cell group and the combined cell therapy group. However, the population of eGFP+ cells was higher in the combined cell therapy group than the Treg cells single group (Fig 6A). In addition, eGFP mRNA expression was examined in the recipient spleen and
small intestine on day 7 after BMT. eGFP expression in both the spleen (Fig 6B) and small intestine (Fig 6C) was increased in the combined cell therapy group, but the difference was not statistically significant in the spleen. Thus, these results provide evidence for the ability of combination therapy to reduce aGVHD severity.

Discussion

The population of Treg cells is an important factor in determining satisfactory therapeutic outcomes of aGVHD after allo-HSCT. For this reason, we expected that preserving the long-term survival of adoptively transferred Treg cells may provide an important strategy for preventing aGVHD without having them convert into other cells.

Several reports demonstrated that in vitro co-cultures of MSCs with T cells were able to induce Foxp3-expressing CD4+ T cells. This MSC-induced Foxp3+ Treg cell formation has also been demonstrated in several in vivo mouse models through MSC-mediated T cell suppression and the consequent induction of Treg cells [18-22,30-32]. For that reason, we considered that MSCs may be helpful in effectively maintaining and generating Treg cells in an aGVHD model via co-infusion of MSCs and Treg cells.

In contrast to our previous study, which used host-derived MSCs and Treg cells, we used donor-derived MSCs and Treg cells for treatment in this study. Our main objective was to distinguish between adoptively transferred donor-derived Treg cells and repopulating donor-derived endogenous Treg cells after BMT. The data suggest an underlying synergistic interaction between the combined cell therapy.

Numerous studies have shown that MSCs suppress GVHD in mice [33] and humans [34] by suppressing the proliferation of allo-reactive T cells [5]. However, our data show that MSCs alone had lower rates of survival and clinical scores. Recent data have shown that MSCs are influenced by environmental conditions, including inflammatory cytokines present during the
early post-transplant period [35,36]. The immunosuppressive effects exhibited by MSCs are not constitutive, but are triggered by specific immune responses, including IFN-γ and TNF-α [32]. Thus, a lack of inflammatory cytokines in the model used in this study may have hindered the immunosuppressive effects of MSCs due to early administration on days 0 and 4 after BMT. Further studies are needed to determine the effects of MSC injection at different time points during the post-transplant period.

Several reports have shown that aGVHD is a Th1-mediated response in animal models [37]. Our results indicated that IFN-γ and TNF-α were downregulated in the combined cell therapy group versus MSCs or Treg cells alone, whereas IL-4 expression was not significantly different among the groups. The mRNA levels of T-bet were slightly lower in the combined cell therapy group, while GATA-3 was not significantly different among groups. No groups had significantly different levels of T-bet or GATA3. In addition, STAT1 expression was significantly downregulated in the combination cell therapy groups. These results suggest that combined cell therapy with MSCs and Treg cells was associated with downregulation of the Th1 response in the aGVHD model. However, MSCs alone had increased levels of IFN-γ compared to the GVHD group, rather than an inhibitory effect. Previous studies showed that MSCs alone do not inhibit the development of the inflammatory cytokines involved in autoimmune diseases, including joint inflammation and autoimmune arthritis [14,38]. In fact, recent reports suggest that the administration of MSCs can aggravate inflammatory responses [39]. Thus, MSCs alone may not have been sufficient for effective downregulation of inflammatory cytokines in this study.

According to Bettelli et al. [34], conversion of Treg cells is caused by IL-6, which acts as a potent inflammatory cytokine, promoting Th17 development, and inhibiting the generation of Treg cells. We examined Th17 cells to identify the possibility of the conversion of Treg cells in recipient spleen and blood. The percentage of IL-17+ cells in CD4+ T cells was significantly decreased in the combined cell therapy group at 12 days after BMT. Although IL-6 expression in CD4+ T cells was scarcely detected in any group, there was further downregulation in the combined cell therapy group. Likewise, STAT3 and RORγ-t expression was significantly decreased in the combined cell therapy group.

These data suggest that combined cell therapy can influence the downregulation of Th17 cells in aGVHD. Although we used induced Treg cells (iTreg), which are more resistant to converting into Th17 cells than natural Treg cells (nTreg) [40], according to Koenencke et al. [36], even adoptively transferred iTreg cells are unstable both in vitro and in vivo, lacking the suppressed activity to prevent fatal aGVHD. Thus, we examined the conversion of Treg cells using ex vivo-expanded Treg cells of high purity (> 96%), and co-administered with MSCs after BMT. Then, we observed IL-17 cells in CD4+ T cells to see whether adoptively transferred Treg cells were converted into Th17 cells. As a result, CD4+ IL-17+ cells were detected at < 5% of CD4+ T cells in the spleen and blood at day 21, and eGFP+ cells were detected as a small proportion of CD4+ IL-17+ cells. In addition, CD4+ IL-17+ cells were decreased in the combined cell therapy group on day 21 versus the Treg cells group. Thus, few of our adoptively transferred Treg cells were converted into Th17 cells in the aGVHD model. Indeed, our results suggest that combined cell therapy using MSCs and Treg cells was effective at reducing the conversion of CD4+ T cell into Th17 cells.

Our previous studies suggested that co-treatment with host-derived MSCs and Treg cells increased Foxp3+ Treg cells in aGVHD recipients [23]. In the present study, CD4+ CD25+ cells were increased in the groups that received cells for therapy, except the GVHD group. However, Foxp3+ cells were only effectively increased in the combined cell therapy group. SOCS3 and Foxp3 expression at the transcriptional level were also increased greatly in the combined cell therapy group. Thus, co-administration of MSCs and Treg cells could offer benefits for the
treatment of aGVHD [41]. Following these results, we focused on the increase in Foxp3+ Treg cells in recipients using ex vivo-expanded Treg cells to distinguish between adoptively transferred Treg cells and endogenously generated Treg cells. As a result, adoptively transferred Treg cells were detected at high levels in the spleen and blood in both the Treg cell group and the combined cell therapy group in the early transplant period. Treg cells were similarly decreased in both groups by day 14. However, Treg cells were only significantly preserved in the combined cell therapy group, compared to the Treg cells single group, on day 21. Furthermore, the Treg cells (H-2b) generated endogenously from recipients were significantly increased in the combined cell therapy group on day 21. Together, these results indicate that MSCs not only influenced the long-term survival of transferred Treg cells, but also induced the repopulation of endogenous Treg cells in the recipient after BMT.

aGVHD is a major complication of allogeneic hematopoietic stem cell transplantation (allo-HSCT), which leads to tissue damage, including the gut, skin, liver, and lungs [42,43]. We identified improvements in the histological examination through combined cell therapy in aGVHD target organs. We also examined eGFP expression in the liver and small intestine by immunofluorescence staining at 7 days after BMT. eGFP expression was detected more strongly in the combined cell therapy group than in the Treg cells single group. Moreover, eGFP mRNA expression levels in the spleen and small intestine were increased in the combined cell therapy group on day 7 after BMT. These results suggest that the adoptive transferred Treg cells influenced the aGVHD target organs, and that MSCs may contribute to maintaining the survival of transferred Treg cells in aGVHD target organs through co-infusion of MSCs and Treg cells after BMT.

In summary, the present study suggests that combined cell therapy with donor-derived MSCs and Treg cells improved the survival and manifestation of aGVHD, and downregulated Th1 and Th17 responses in recipients. Moreover, MSCs induced the long-term survival of transferred Treg cells, particularly in aGVHD target organs, and increased the repopulation of endogenous Treg cells in recipients after BMT. These data demonstrate a potential strategy of combined cell therapy with MSCs and Treg cells for preventing aGVHD.

Supporting Information
S1 Checklist. ARRIVE Guidelines Checklist 2014.
(DOC)

Acknowledgments
This study was supported by a Korean Health Technology R&D Project grant from the Ministry for Health & Welfare, Republic of Korea (HI14C3417)

Author Contributions
Conceived and designed the experiments: SGC. Performed the experiments: ESL JYL KII. Analyzed the data: SGC ESL JYL KII NK YSN YWJ. Contributed reagents/materials/analysis tools: SGC ESL JYL KII NK YSN. Wrote the paper: SGC ESL JYL KII NK YSN YWJ.

References
1. Asai O, Longo DL, Tian ZG, Hornung RL, Taub DD, Ruscetti FW, et al. (1998) Suppression of graft-versus-host disease and amplification of graft-versus-tumor effects by activated natural killer cells after allogeneic bone marrow transplantation. J Clin Invest 101: 1835–1842. PMID:9576746
2. Gaidot A, Landau DA, Martin GH, Bonduelle O, Grinberg-Bleyer Y, Matheoud D, et al. (2011) Immune reconstitution is preserved in hematopoietic stem cell transplantation coadministered with regulatory T
2. Le Blanc K, Frassoni F, Ball L, Locatelli F, Roelofs H, Lewis I, et al. (2008) Mesenchymal stem cells for treatment of steroid-resistant, severe, acute graft-versus-host disease: a phase II study. Lancet 371: 1579–1586. doi: 10.1016/S0140-6736(08)60690-X PMID: 18468541

3. Kim EJ, Kim N, Cho SG (2013) The potential use of mesenchymal stem cells in hematopoietic stem cell transplantation. Exp Mol Med 45: e2. doi: 10.1038/emm.2013.2 PMID: 23306700

4. Kim N, Cho SG (2013) Clinical applications of mesenchymal stem cells. Korean J Intern Med 28: 387–402. doi: 10.3904/kjim.2013.28.4.387 PMID: 2364795

5. Schneidawind D, Pierini A, Negrin RS (2013) Regulatory T cells and natural killer T cells for modulation of GVHD following allogeneic hematopoietic cell transplantation. Blood 122: 3116–3121. doi:10.1182/blood-2013-08-453126 PMID: 24068494

6. Kim N, Im KI, Lim JY, Jeon EJ, Nam YS, Kim EJ, et al. (2013) Mesenchymal stem cells for the treatment and prevention of graft-versus-host disease: experiments and practice. Ann Hematol 92: 1295–1308. doi: 10.1007/s00277-013-1796-z PMID: 23722500

7. Klyushnenkova E, Mosca JD, Zernetkina V, Majumdar MK, Beggs KJ, Simonetti DW, et al. (2005) T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. J Biomed Sci 12: 47–57. PMID:15864738

8. Adeegbe D, Levy RB, Malek TR (2010) Allogeneic T regulatory cell-mediated transplantation tolerance in adoptive therapy depends on dominant peripheral suppression and central tolerance. Blood 115: 1932–1940. doi:10.1182/blood-2009-08-238584 PMID: 20040758

9. Joffre O, Santolaria T, Calise D, Al Saati T, Hudrisier D, Romagnoli P, et al. (2008) Prevention of acute and chronic allograft rejection with CD4+CD25+Foxp3+ regulatory T lymphocytes. Nat Med 14: 88–92. PMID: 18066074

10. Wood KJ, Sakaguchi S (2003) Regulatory T cells in transplantation tolerance. Nat Rev Immunol 3: 199–210. PMID: 12658268

11. Sudres M, Nofal F, Trenado A, Gregoire S, Charlotte F, Levrero M, et al. (2006) Bone marrow mesenchymal stem cells suppress lymphocyte proliferation in vitro but fail to prevent graft-versus-host disease in mice. J Immunol 176: 7761–7767. PMID: 16751424

12. Djouad F, Plence P, Bony C, Tropel P, Apparailly F, Sany J, et al. (2003) Immunosuppressive effect of mesenchymal stem cells favors tumor growth in allogeneic animals. Blood 102: 3837–3844. PMID: 12881305

13. Park MJ, Park HS, Cho ML, Oh HJ, Cho YG, Min SY, et al. (2011) Transforming growth factor beta-transduced mesenchymal stem cells ameliorate experimental autoimmune arthritis through reciprocal regulation of Treg/Th17 cells and osteoclastogenesis. Arthritis Rheum 63: 1668–1680. doi: 10.1002/art.30326 PMID: 21384335

14. Chen X, Oppenheim JJ (2014) Th17 cells and Tregs: unlikely allies. J Leukoc Biol.

15. Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martinez-Llordella M, Ashby M, et al. (2009) Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. Nat Immunol 10: 1774–1780. doi:10.1038/ni.1774 PMID: 19633673

16. Casiraghi F, Azzollini N, Cassis P, Imberti B, Morici M, Cugnini D, et al. (2008) Pretransplant infusion of mesenchymal stem cells prolongs the survival of a semiallogeneic heart transplant through the generation of regulatory T cells. J Immunol 181: 3933–3946. PMID: 18768848

17. Di Ianni M, Del Papa B, De Ioanni M, Moretti L, Bonfà F, Cecchini D, et al. (2008) Mesenchymal cells recruit and regulate T regulatory cells. Exp Hematol 36: 309–318. doi: 10.1016/j.exphem.2007.11.007 PMID: 18279718

18. English K, Ryan JM, Tobin L, Murphy MJ, Barry FP, Mahon BP, et al. (2009) Cell contact, prostaglandin E(2) and transforming growth factor beta 1 play non-redundant roles in human mesenchymal stem cell induction of CD4+CD25(High) forkhead box P3+ regulatory T cells. Clin Exp Immunol 156: 149–160. doi:10.1111/j.1365-2249.2009.03874.x PMID: 19210524

19. Maccario R, Pedesta M, Moretta A, Cometa A, Comoli P, Montagna D, et al. (2005) Interaction of human mesenchymal stem cells with cells involved in alloantigen-specific immune response favors the differentiation of CD4+ T-cell subsets expressing a regulatory/suppressive phenotype. Haematologica 90: 516–525. PMID: 15820948

20. Melief SM, Schrama E, Brugman MH, Tiemessen MM, Hoogduijn MJ, Fibbe WE, et al. (2013) Multipotent stromal cells induce human regulatory T cells through a novel pathway involving skewing of

21. Adoptive Transfer of Tregs Combined with MSCs in aGVHD
23. Lim JY, Park MJ, Im KI, Kim N, Jeon EJ, Kim EJ, et al. (2014) Combination cell therapy using mesenchymal stem cells and regulatory T-cells provides a synergistic immunomodulatory effect associated with reciprocal regulation of Th1/Th2 and Th17/Treg cells in a murine acute graft-versus-host disease model. Cell Transplant 23: 703–714. doi: 10.3727/096368913X664577 PMID: 23452884

24. Im KI, Park MJ, Kim N, Lim JY, Park HS, Lee SH, et al. (2014) Induction of mixed chimerism using combinatory cell-based immune modulation with mesenchymal stem cells and regulatory T cells for solid-organ transplant tolerance. Stem Cells Dev 23: 2364–2376. doi: 10.1089/scd.2013.0617 PMID: 24804993

25. Lee JH, Jeon EJ, Kim N, Nam YS, Im KI, Lim JY, et al. (2013) The synergistic immunoregulatory effects of culture-expanded mesenchymal stromal cells and CD4(+)/25(+)/Foxp3+ regulatory T cells on skin allograft rejection. PLoS One 8: e70968. doi: 10.1371/journal.pone.0070968 PMID: 23940676

26. Soleimani M, Nadri S (2009) A protocol for isolation and culture of mesenchymal stem cells from mouse bone marrow. Nat Protoc 4: 102–106. doi: 10.1038/nprot.2008.221 PMID: 19131962

27. Miyake T, Inaba M, Fukui J, Ueda Y, Hosaka N, Kamiyama Y, et al. (2008) Prevention of graft-versus-host disease by intrabone marrow injection of donor T cells: involvement of bone marrow stromal cells. Clin Exp Immunol 152: 153–162. doi: 10.1111/j.1365-2249.2008.03615.x PMID: 18307515

28. Krenger W, Snyder KM, Byron JC, Falzarano G, Ferrara JL (1995) Polarized type 2 alloreactive CD4+ and CD8+ donor T cells fail to induce experimental acute graft-versus-host disease. J Immunol 155: 585–593. PMID: 7608537

29. Yi T, Chen Y, Wang L, Du G, Huang D, Zhao D, et al. (2009) Reciprocal differentiation and tissue-specific pathogenesis of Th1, Th2, and Th17 cells in graft-versus-host disease. Blood 114: 3101–3112. doi: 10.1182/blood-2009-05-219402 PMID: 19602734

30. Engela AU, Baan CC, Dor FJ, Weimar W, Hoogduijn MJ (2012) On the interactions between mesenchymal stem cells and regulatory T cells for immunomodulation in transplantation. Front Immunol 3: 126. doi: 10.3389/fimmu.2012.00126 PMID: 22629256

31. Luz-Crawford P, Kurte M, Bravo-Alegria J, Contreras R, Nova-Lamperti E, Tejedor G, et al. (2013) Mesenchymal stem cells generate a CD4+CD25+Foxp3+ regulatory T cell population during the differentiation process of Th1 and Th17 cells. Stem Cell Res Ther 4: 65. doi: 10.1186/scr216 PMID: 23734780

32. Marigo I, Dazzi F (2011) The immunomodulatory properties of mesenchymal stem cells. Semin Immunopathol 33: 593–602. doi: 10.1007/s00281-011-0267-7 PMID: 21499984

33. Tobin LM, Healy ME, English K, Mahon BP (2013) Human mesenchymal stem cells suppress donor CD4+ T cell proliferation and reduce pathology in a humanized mouse model of acute graft-versus-host disease. Clin Exp Immunol 172: 333–348. doi: 10.1111/cei.12056 PMID: 23574329

34. Ball LM, Bernardo ME, Roelofs H, van Tol MJ, Contoli B, Zwaginga JJ, et al. (2013) Multiple infusions of mesenchymal stromal cells induce sustained remission in children with steroid-refractory, grade III-IV acute graft-versus-host disease. Br J Haematol 163: 501–509. doi: 10.1111/bjh.12545 PMID: 23992039

35. Polchert D, Sobinsky J, Douglas G, Kidd M, Moadanir A, Reina E, et al. (2008) IFN-gamma activation of mesenchymal stem cells for treatment and prevention of graft versus host disease. Eur J Immunol 38: 1745–1755. doi: 10.1002/eji.200738129 PMID: 18493986

36. Shi Y, Hu G, Su J, Li W, Chen Q, Shou P, et al. (2010) Mesenchymal stem cells: a new strategy for immunosuppression and tissue repair. Cell Res 20: 510–518. doi: 10.1038/cr.2010.44 PMID: 20368733

37. Szebeni J, Wang MG, Pearson DA, Szot GL, Sykes M (1994) IL-2 inhibits early increases in serum gamma interferon levels associated with graft-versus-host-disease. Transplantation 58: 1385–1393. PMID: 7809932

38. Chen B, Hu J, Liao L, Sun Z, Han Q, Song Z, et al. (2010) Fik-1+ mesenchymal stem cells aggravate collagen-induced arthritis by up-regulating interleukin-6. Clin Exp Immunol 159: 292–302. doi: 10.1111/j.1365-2249.2009.04069.x PMID: 20002448

39. Hoogduijn MJ, Roemeling-van Rhijn M, Engela AU, Korevaar SS, Mensah FK, Franquesa M, et al. (2013) Mesenchymal stem cells induce an inflammatory response after intravenous infusion. Stem Cells Dev 22: 2825–2835. doi: 10.1089/scd.2013.0193 PMID: 23767885

40. Horwitz DA, Zheng SG, Gray JD (2008) Natural and TGF-beta-induced Foxp3+/CD4+CD25+ regulatory T cells are not mirror images of each other. Trends Immunol 29: 429–435. doi: 10.1016/j.it.2008.06.005 PMID: 18676178

41. Burr SP, Dazzi F, Garden OA (2013) Mesenchymal stromal cells and regulatory T cells: the Yin and Yang of peripheral tolerance? Immunol Cell Biol 91: 12–18. doi: 10.1038/icb.2012.60 PMID: 23146942
42. Socie G, Blazar BR (2009) Acute graft-versus-host disease: from the bench to the bedside. Blood 114: 4327–4336. doi: 10.1182/blood-2009-06-204669 PMID: 19713461

43. Welniak LA, Blazar BR, Murphy WJ (2007) Immunobiology of allogeneic hematopoietic stem cell transplantation. Annu Rev Immunol 25: 139–170. PMID: 17129175