



# Third-party regulatory T cells prevent murine acute graft-versus-host disease

Jung-Yeon Lim<sup>1,2</sup>, Keon-Il Im<sup>1,2</sup>, Yunejin Song<sup>1,2</sup>, Nayoun Kim<sup>1,2</sup>, Young-Sun Nam<sup>1,2</sup>, Young-Woo Jeon<sup>1,2,3</sup>, and Seok-Goo Cho<sup>1,2,3</sup>

<sup>1</sup>Institute for Translational

Research and Molecular Imaging,

<sup>2</sup>Laboratory of Immune Regulation,

Convergent Research Consortium

for Immunologic Disease, College

of Medicine, The Catholic University

of Korea, Seoul; <sup>3</sup>Department of

Hematology, Catholic Blood and

Marrow Transplantation Center,

College of Medicine, Seoul St. Mary's

Hospital, The Catholic University of

Korea, Seoul, Korea

**Background/Aims:** Adoptive therapy with regulatory T (Treg) cells to prevent graft-versus-host disease (GVHD) would benefit from a strategy to improve homing to the sites of inflammation following hematopoietic stem cell transplantation (HSCT). Although donor-derived Treg cells have mainly been used in these models, third-party-derived Treg cells are a promising alternative for cell-based immunotherapy, as they can be screened for pathogens and cell activity, and banked for GVHD prevention. In this study, we explored major histocompatibility complex (MHC) disparities between Treg cells and conventional T cells in HSCT to evaluate the impact of these different cell populations on the prevention of acute GVHD, as well as survival after allogeneic transplantation.

**Methods:** To induce acute GVHD, lethally irradiated BALB/c (H-2d) mice were transplanted with  $5 \times 10^5$  T cell-depleted bone marrow cells and  $5 \times 10^5$  CD4+CD25<sup>-</sup> splenic T cells from C57BL/6 (H-2b) mice. Recipients were injected with  $5 \times 10^5$  cultured donor-, host-, or third-party-derived CD4+CD25+CD62L<sup>+</sup> Treg cells (bone marrow transplantation + day 1).

**Results:** Systemic infusion of three groups of Treg cell improved clinicopathological manifestations and survival in an acute GVHD model. Although donor-derived Treg cells were immunologically the most effective, the third-party-derived Treg cell therapy group displayed equal regulation of expansion of CD4+CD25+Foxp3<sup>+</sup> Treg cells and suppressive CD4+IL-17<sup>+</sup> T-helper (Th17) cells in *ex vivo* assays compared with the donor- and host-derived groups.

**Conclusions:** Our findings demonstrate that the use of third-party Treg cells is a viable alternative to donor-derived Treg cellular therapy in clinical settings, in which human leukocyte antigen-matched donors are not always readily available.

**Keywords:** T-Lymphocytes, regulatory; Acute graft-versus host disease; Hematopoietic stem cell transplantation

Received: October 5, 2016

Revised : December 2, 2016

Accepted: December 30, 2016

Correspondence to  
Seok-Goo Cho, M.D.

Department of Hematology,

Catholic Blood and Marrow

Transplantation Center,

College of Medicine,

Seoul St. Mary's Hospital,

The Catholic University of Korea,

222 Banpo-daero, Seocho-gu,

Seoul 06591, Korea.

Tel: +82-2-2258-6052

Fax: +82-2-599-3589

E-mail: chosg@catholic.ac.kr

## INTRODUCTION

Graft-versus-host disease (GVHD) is the most severe complication of allogeneic hematopoietic stem cell transplantation (HSCT). GVHD is characterized by an imbalance between the effector and regulatory arms of

the immune system, which results in the overproduction of inflammatory cytokines. Several studies have demonstrated that CD4+CD25<sup>-</sup> conventional T cells (Tcons) are critical for the development of GVHD, since depletion of T cells from donor bone marrow (BM) effectively prevents acute GVHD; thus, increasing animal

survival rates in murine studies [1,2]. Recently, these promising results have been translated into clinical applications; CD4+CD25+Foxp3+ regulatory T (Treg)-based cellular therapy is considered as a powerful approach for both acute and chronic GVHD prevention.

Treg cells are not a homogenous cell population, but can be divided into at least two subpopulations according to CD62L expression. CD62L (L-selectin) is a member of the selection adhesion molecule family, and is an important T cell homing receptor, as well as a marker for T cell development [3]. The CD62L+ subset of cells is a more potent suppressor than the CD62L- population or unfractionated CD4+CD25+ Treg cells; in addition, it can be expanded far more easily in culture, and is more responsive to chemokine-driven migration to secondary lymphoid organs [4].

Despite these promising results, there are several factors that limit the clinical applications of GVHD therapy. Several groups are investigating the clinical utility of *ex vivo* expansion of donor-derived Treg cells, to increase their number, because Treg cells are a rare cell population; others are improving culturing strategies to enhance Treg cell function. Moreover, in terms of actual clinical performance, it is difficult to request another donation of an unrelated donor's blood following HSCT for the purpose of generating Treg cells. Brunstein et al. [5] recently demonstrated the safety and clinical efficacy of administration of third-party cord blood-derived Treg cells after a primary cord blood transplantation. Therefore, third-party-derived Treg cells are particularly suitable for such studies, as they can be prepared in advance and then banked for further use.

Several studies have demonstrated that Treg cells from different sources, such as a donor, recipient, or third-party, have been tested separately in preclinical and clinical transplantation studies, but no comparison among these three types of Treg sources has been systematically reported simultaneously. In the present study, we used a mouse model to test the efficacy of donor, host, or third-party-derived Treg cells.

## METHODS

### Mice

C57BL/6 (H-2b), BALB/c (H-2d), and DBA1J (H-2q) mice,

8 to 10 weeks old, were purchased from Orient (Seongnam, Korea). Mice were maintained under specific pathogen-free conditions in an animal facility with controlled humidity (55% ± 5%), light (12/12-hour light/dark), and temperature (22°C ± 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*. The protocols used in this study were approved by the Animal Care and Use Committee of The Catholic University of Korea (2010-0204-02).

### Bone marrow transplantation and acute GVHD induction

Recipient mice (BALB/c, H-2d) were irradiated with 800 cGy and injected intravenously (IV) with  $5 \times 10^6$  T cell-depleted bone marrow cells (TCD-BM) and  $5 \times 10^6$  CD4+CD25- splenic T cells from donor mice (C57BL/6, H-2b). Control groups were comprised of irradiated mice receiving only  $5 \times 10^6$  TCD-BM cells (which did not induce GVHD). Survival after bone marrow transplantation (BMT) was monitored daily, and the degree of clinical GVHD was assessed weekly using a system that scored changes in five clinical parameters: weight loss, posture, activity, fur texture, and skin integrity.

### Treg cell generation

To obtain Treg cells, isolated CD4+ T cells from donors (C57BL/6), recipients (BALB/c) and third parties (DBA1J) were cultured with anti-CD3 (1 µg/mL), anti-CD28 (1 µg/mL), human recombinant transforming growth factor (5 ng/mL) and retinoic acid (100 µM) for 3 days. The expanded induced Treg cells were then sorted by flow cytometry to obtain a ~90% pure CD4+CD25+CD62L+ population [6].

### Treg cell therapy

Mice were injected IV with  $5 \times 10^5$  Treg cells derived from one of a donor, host or third-party, after BMT (BMT + day 1). Control mice received IV injections of an equal volume of phosphate-buffered saline (PBS) (Gibco, Carlsbad, CA, USA) at the same time points. Donor Treg, host Treg, and third-party Treg refer to donor mice-derived Treg cell, host mice-derived Treg cell, and third party mice derived Treg cell, respectively.

### Histopathological analysis of acute GVHD

Survival after BMT was monitored daily, and the degree of clinical GVHD was assessed weekly using a scoring system that sums changes in five clinical parameters: weight loss, posture, activity, fur texture, and skin integrity. Mice were killed at day 15 after BMT for blinded histopathological analysis of GVHD targets (skin, liver, and small and large intestine) [7]. Organs were harvested, cryo-embedded, and subsequently sectioned. Tissue sections were fixed in 10% buffered formalin and stained with hematoxylin and eosin for histological examination.

### Flow cytometry

Mononuclear cells were immunostained with various combinations of the following fluorescence-conjugated antibodies: intercellular adhesion molecule 1 (ICAM-1), cytotoxic T lymphocyte antigen-4 (CTLA-4), programmed death-1 (PD-1), inducible costimulator (ICOS), CD103, CD25, CD4, Foxp3, interleukin (IL)-17, interferon- $\gamma$  (IFN- $\gamma$ ), IL-4, IL-10, and IL-6. These cells were also intracellularly stained with the following antibodies: IL-4, IL-6 (BD Biosciences, Franklin Lakes, NJ, USA), IL-10 (BioLegend), IL-17, and Foxp3 (eBioscience, San Diego, CA, USA). Before intracellular staining, the cells were restimulated for 4 hours with 25 ng/mL PMA and 250 ng/mL ionomycin in the presence of GolgiStop (BD Biosciences). Intracellular staining was conducted using an intracellular staining kit (eBioscience) according to the manufacturer's protocol. Flow cytometric analysis was performed on a FACS LSRFortessa (BD Biosciences).

### ELISA assay for cytokine levels

Concentrations of IL-4, IL-21, IL-10, IFN- $\gamma$ , IL-6, and IL-17 were measured using a sandwich ELISA as follows. Anti-mouse IL-4, IL-21, IL-10, IFN- $\gamma$ , IL-6, or IL-17 monoclonal antibodies (R&D Systems, Minneapolis, MN, USA) were added to a 96-well plate (Nunc, Roskilde, Denmark) and incubated overnight at 4°C. Wells were blocked with blocking solution (PBS containing 1% bovine serum albumin and 0.05% Tween 20) for 2 hours at room temperature. Test samples and standard recombinant mouse IL-4, IL-21, IL-10, IFN- $\gamma$ , IL-6, and IL-17 (R&D Systems) were added to separate wells and the plate was incubated at room temperature for 2 hours, after which it was washed. Biotinylated IL-4, IL-21, IL-

10, IFN- $\gamma$ , IL-6, and IL-17 polyclonal antibodies (R&D Systems) were added, and the reaction was allowed to proceed for 2 hours at room temperature. The plate was washed, ExtrAvidin-alkaline phosphatase (1:2,000 dilution; Sigma-Aldrich, St. Louis, MO, USA) was added, and the reaction was allowed to proceed for an additional 2 hours. The plate was washed and 50  $\mu$ L p-nitrophenyl phosphate disodium salt (Pierce Chemical Co., Rockford, IL, USA) (diluted to 1 mg/mL in diethanolamine buffer) was applied. Experiments were performed according to the manufacturer's instructions.

### Statistical analysis

Statistical significance was determined using Student two-tailed *t* test and one-way analysis of variance with Bonferroni correction applied for multiple comparisons. In all analyses, *p* values less than 0.05 were considered to indicate statistical significance.

## RESULTS

### Phenotypes of *ex vivo* expanded Treg cells according to Treg source

Recent studies revealed the importance of diverse patterns of homing receptors for the appropriate tissue distribution and function of Treg cells. Three groups of retinoic acid-induced CD4+CD25+CD62L+ Treg cells, analyzed by flow cytometry, showed > 96% purity and positive surface staining for several phenotypic Treg markers, including CTLA-4 (Fig. 1A), ICOS (Fig. 1B), ICAM-1 (Fig. 1C), CD103 (Fig. 1D), and PD-1 (Fig. 1E). However, DBA1J (third-party)-derived Treg cells showed weakly positive surface staining for ICOS, CTLA-4 and PD-1 as compared with C57BL/6 (donor) [8] and BALB/c (host)-derived Treg cells [9].

### Regardless of Treg source, CD4+CD25+CD62L+ Treg cells are protective for lethal acute GVHD

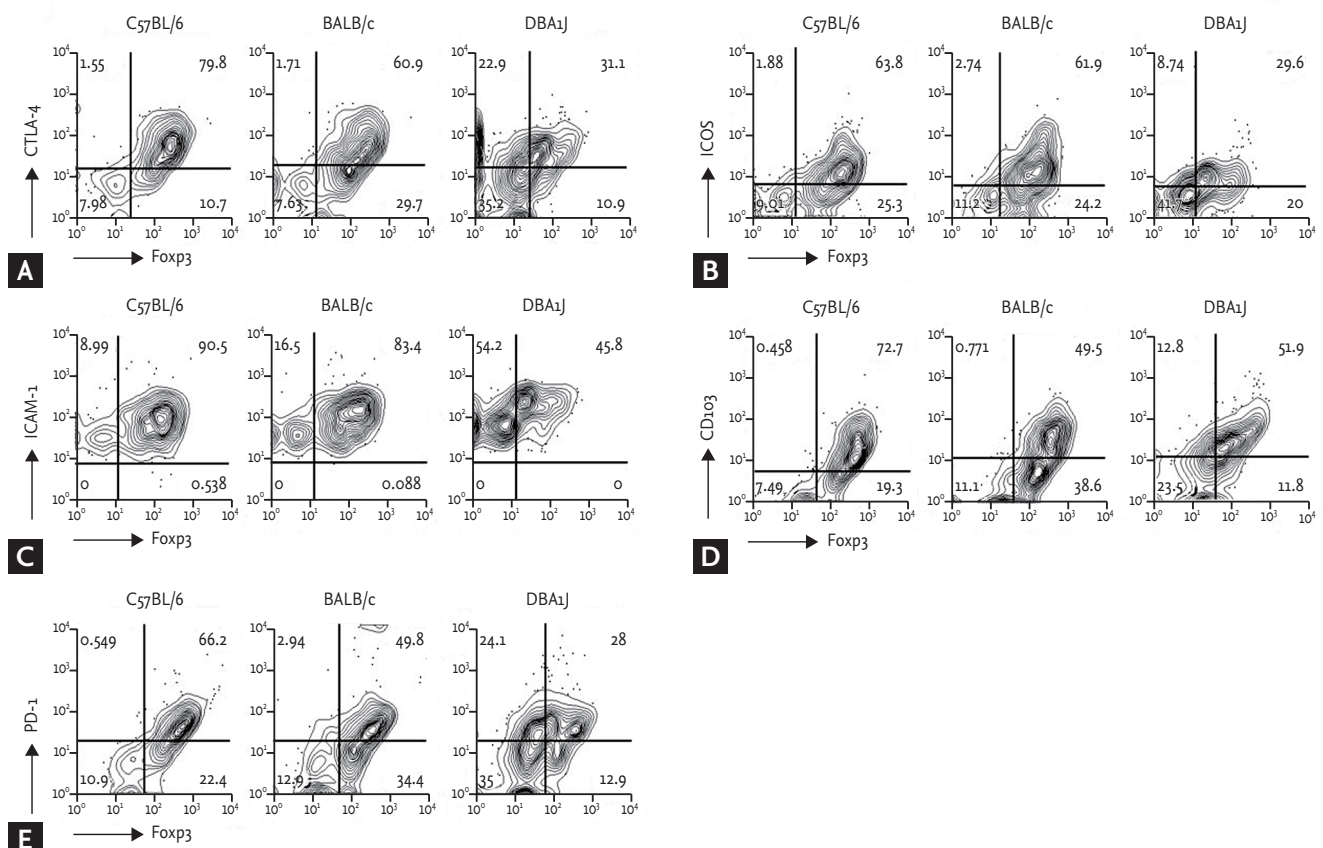
We established a model of acute GVHD following complete major histocompatibility complex (MHC)-mismatched HSCT, to assess the effects of *ex vivo* expanded Treg cells of various origin on GVHD, engraftment, and immune reconstitution. In this model,  $5 \times 10^6$  TCD-BM cells from C57BL/6 (H-2b) mice were injected into lethally irradiated (total body irradiation; 800 cGy) B/c (H-

2d) recipient mice to establish hematopoiesis. Co-transfer of  $5 \times 10^5$  donor CD4+CD25- Tcons resulted in lethal GVHD with a median survival of 10 to 20 days. Infusion of CD4+CD25+CD62L+ Treg cells at a 1:1 ratio to Tcons protected animals from GVHD-related mortality. In these experiments, irradiated BALB/c (H-2d) mice were divided into five groups: mice in group 1 received TCD-BM cells from C57BL/6 mice; in group 2, in addition to TCD-BM cells, mice also received donor Tcons to induce GVHD; mice in groups 3 to 5 were transplanted as in group 2 and also received IV infusions with donor-, host-, or third-party-derived CD4+CD25+CD62L+ T cells on day 1 post-transplantation. Recipient mice were monitored for clinical scores of GVHD, weight, and survival. As expected, none of the animals from group 1 died after transplantation, whereas all the animals from group

2 died within 20 days; no significant differences in survival rates were observed among groups 3 to 5 (Fig. 2) and GVHD clinical scores indicated that donor-derived Treg cell-treated mice did not develop clinical signs of GVHD for > 100 days as compared with host-derived Treg cell-treated and third-party-derived Treg cell-treated mice (Fig. 3A). Also, mice receiving an infusion of donor-derived Treg cells gained a significant amount of weight (Fig. 3B). Notably, the improved GVHD scores of mice in group 3 were consistent with a continued health status until at least day 100 after HSCT.

### Regardless of Treg source, CD4+CD25+CD62L+ Treg cell therapy reduces the histopathological manifestations of acute GVHD

Histological analysis of skin from the group of mice with



**Figure 1.** Immunophenotypes of *ex vivo* expanded retinoic acid-induced regulatory T (Treg) cells according to Treg source. Retinoic acid-induced CD4+CD25+ Treg cells showed > 96% purity using flow cytometry. Treg cells induced from C57BL/6 (donor, H-2b), BALB/c (host, H-2d), or DBA1j (third-party, H-2q) splenic CD4+ T cells were characterized by positive expression of intracellular Foxp3, (A) cytotoxic T lymphocyte antigen-4 (CTLA-4), and surface expression of the indicated markers (B) inducible costimulator (ICOS), (C) intercellular adhesion molecule 1 (ICAM-1), (D) CD103, (E) programmed death-1 (PD-1) in the gated CD4 T-cell populations. The reported percentages are those of double-positive cells. Results are representative of two independent experiments.

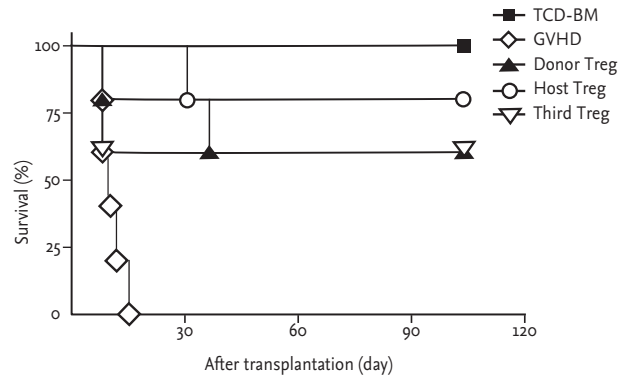


acute GVHD showed scattered eosinophilic degradation (apoptosis) and slight vacuolation of individual epidermal cells. These corresponded to of a score of 8 according to the criteria used [10]. The other Treg cell-treated groups (donor, host, and third-party Treg cells) showed reduced histologic scores, but there were no significant differences among them (Fig. 4A). Histological grading of GVHD of the small intestine showed effacement and blunting of the villous architecture, mucous cell depletion and sloughing of epithelial cells with patchy mucosal ulceration, yielding an acute GVHD histologic score of 2. These outcomes were improved with the addition of donor Treg cells in the group of mice with GVHD. Histological analysis of the liver showed a slight infiltration of lymphoid cells in the portal space and remarkable changes in the bile duct epithelium. Therefore, the total histological scores of each host, third-party, and donor Treg group were lower than that of GVHD group, yet the total score of donor Treg was the lowest. (Fig 4B).

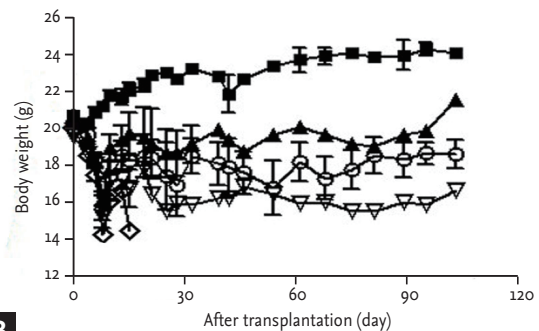
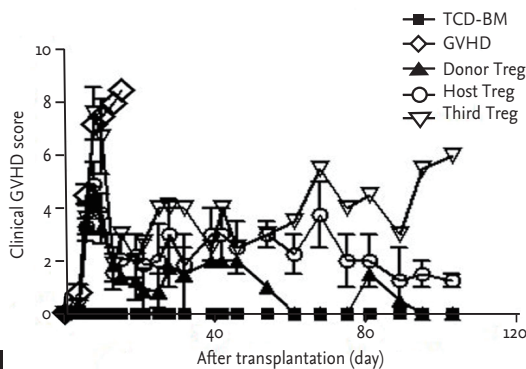
**Third-party-derived CD4+CD25+CD62L+ Treg cells did not modulate the balance of Th1 and Th2 cells in lethal acute GVHD induced by CD4+CD25- T cells**

To confirm the immunoregulatory function of donor Treg, host Treg, and third-party Treg cell therapy groups relative to T-helper 1 (Th1) and Th2 cells, flow cytometry was used to measure cytokine expression. Mice of the host, third-party Treg cell and GVHD control groups produced similar or slightly higher levels of IFN-γ, whereas those of donor Treg cell-treated GVHD mice

showed markedly decreased production of inflammatory cytokines. However, three groups of Treg cell-treated GVHD mice displayed increased IL-10 expression in the spleen compared with GVHD control mice (Fig. 5A). Differences in IL-4 levels between the groups was not statistically significant (Fig. 5B). Analyses performed



**Figure 2.** Improved survival in groups of mice treated with three types of Treg cell after bone marrow transplantation (BMT). Recipient mice (BALB/c, H-2d) were divided into five groups and irradiated with 800 cGy followed by intravenous injection with  $5 \times 10^6$  T-cell depleted bone marrow (TCD-BM) cells as a control. Four of the groups were also infused with  $5 \times 10^5$  CD4+CD25- T cells from donor spleen cells of C57BL/6 background, to induce graft-versus-host disease (GVHD). Three of these four groups were then designated as treatment groups:  $5 \times 10^5$  donor-, host-, or third-party-derived Treg cells were infused into mice after BMT. Survival rates in an acute GVHD mouse model with adoptive transfer of three types of Treg cells are compared to those in a GVHD control group following allogeneic BMT.



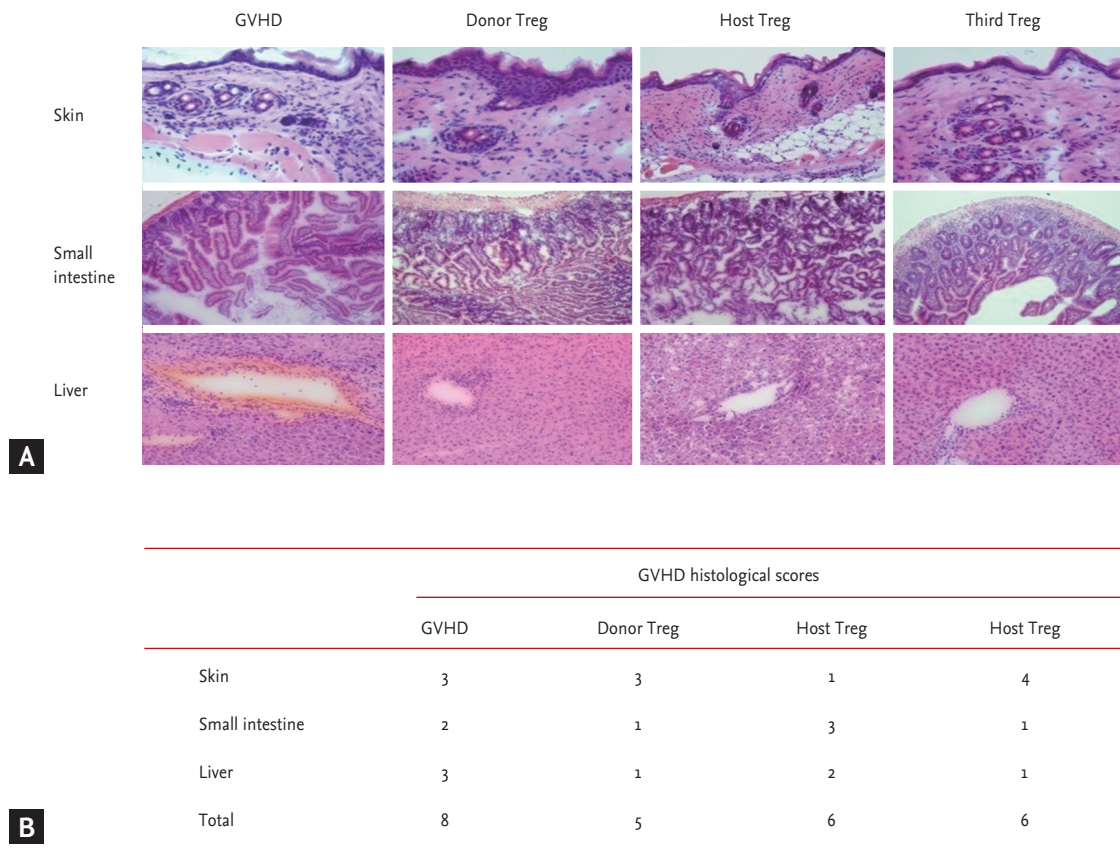
**Figure 3.** Improved clinical graft-versus-host disease (GVHD) scores in groups of mice treated with three types of Treg cell therapy after bone marrow transplantation. The degree of clinical acute GVHD (A) was assessed weekly using a scoring system that summed changes in five clinical parameters: weight loss, posture, activity, fur texture, and skin integrity. All animals were monitored for mean serial weight measurements (B) and clinical signs. Most effects were observed to be accentuated in the presence of donor-derived Treg cell therapy. TCD-BM, cell-depleted bone marrow cells; Treg, regulatory T.

on the spleens of donor Treg cell-treated GVHD mice showed that the Th1/Th2 ratio was the lowest among all of the groups (Fig. 5C). Thus, third-party-derived Treg cell therapy did not affect Th1/Th2 cytokine production. However, donor-derived Treg cell therapy markedly alters the balance between Th1 and Th2 cells in GVHD mice, resulting in an altered disease course.

### Third-party-derived CD4+CD25+CD62L+ Treg cell therapy provides an immunomodulatory effect associated with reciprocal regulation of 17 $\beta$ Th17/Treg cells

Foxp3 expression in the three groups of CD4+CD25+CD62L+ Treg cell therapy was significantly higher than in the control GVHD group, whereas expression of IL-17 and IL-6 in the three CD4+CD25+CD62L+ Treg cell

therapy groups was significantly lower than in the control group. However, among the three Treg cell therapy groups, no difference was observed in the expression of Foxp3, IL-17, or IL-6 (Fig. 6A and 6B). Th1 and Th17 cells in murine GVHD were associated with evidence of severe GVHD, and *in situ* quantification of the Treg/Th17 ratio was a specific marker for GVHD. The Treg/Th17 cell ratio was significantly higher in spleens from mice treated with the three types of CD4+CD25+CD62L+ Treg cells, as compared with the control GVHD group, in the mouse model of GVHD (Fig. 6C). Taken together, these data might explain why third-party-derived Treg cell therapy equally inhibits acute GVHD in pathogenic CD4+CD25<sup>-</sup> donor T cell-treated mice compared with donor- or host-derived Treg cell-treated groups.



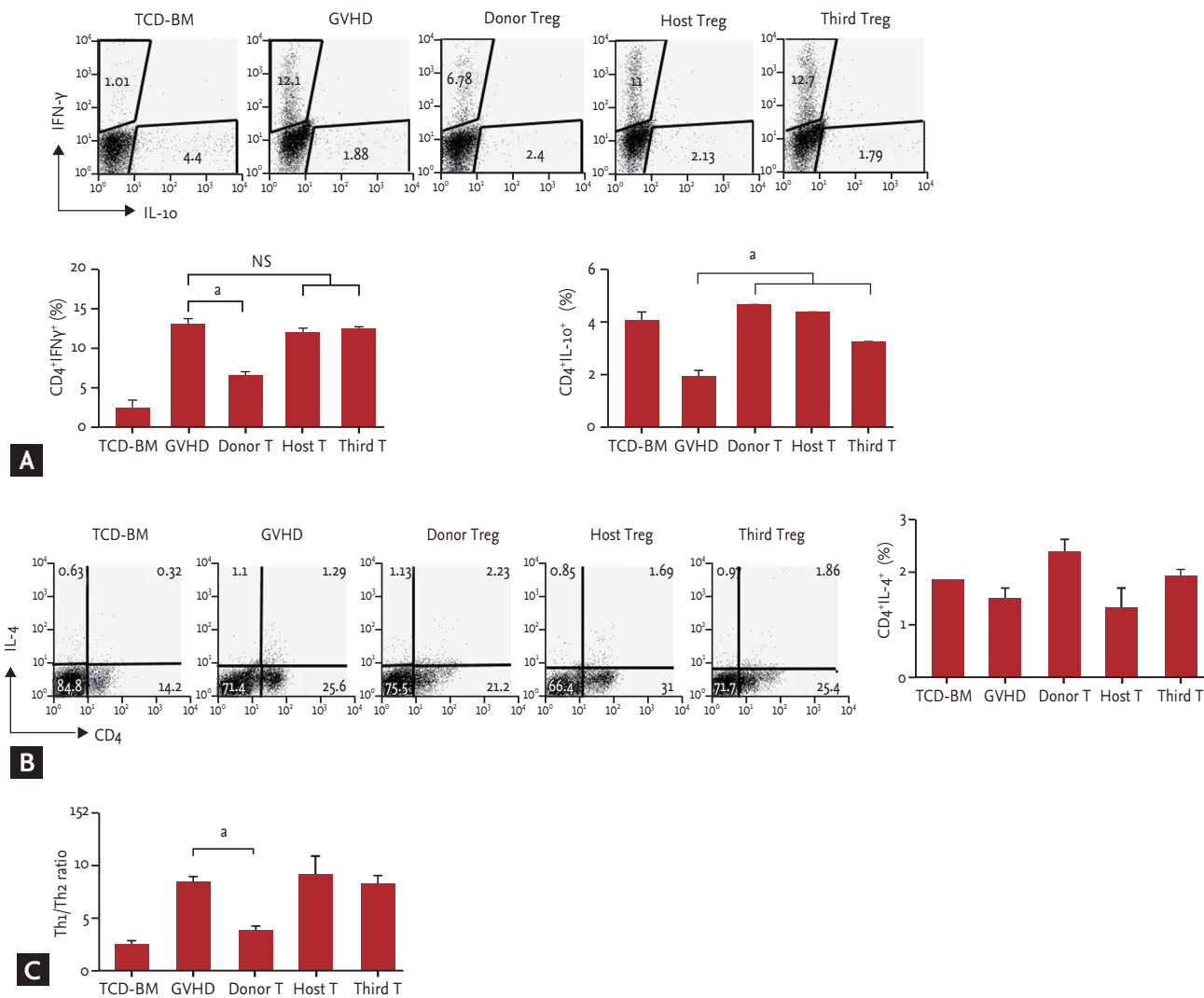
**Figure 4.** Improvement of histopathological scores in target organs of groups of mice that received adoptive transfer of regulatory T (Treg) cells after bone marrow transplantation (BMT). Histological scores were assessed weekly in target organs of acute graft-versus-host disease (GVHD): skin, small intestine, and liver. (A) Histological GVHD scores were evaluated in a mouse model of GVHD, as well as in mice infused with donor-derived Treg cells, host-derived Treg cells, or third-party Treg cells, using samples from skin ( $\times 200$ ), small intestine ( $\times 200$ ), and liver tissues ( $\times 200$ ) 15 days after BMT (with individual criteria for each specific tissue; H&E). (B) Regardless of the source of Treg cells, groups of mice treated with infusion of Treg cells demonstrated significant reductions in total GVHD histological scores compared with the GVHD control group.

DISCUSSION

Preclinical murine transplantation models have convincingly established that Treg cells have the capacity to prevent alloreactive T-cell responses in experimental models of both acute and chronic GVHD [11,12]. For example, hematopoietic stem cell grafts with a higher content of Treg cells have been correlated with less acute

GVHD [13]. Likewise, more rapid Treg reconstitution is associated with less acute GVHD, whereas patients with delayed Treg cell recovery have a higher likelihood of developing GVHD [14].

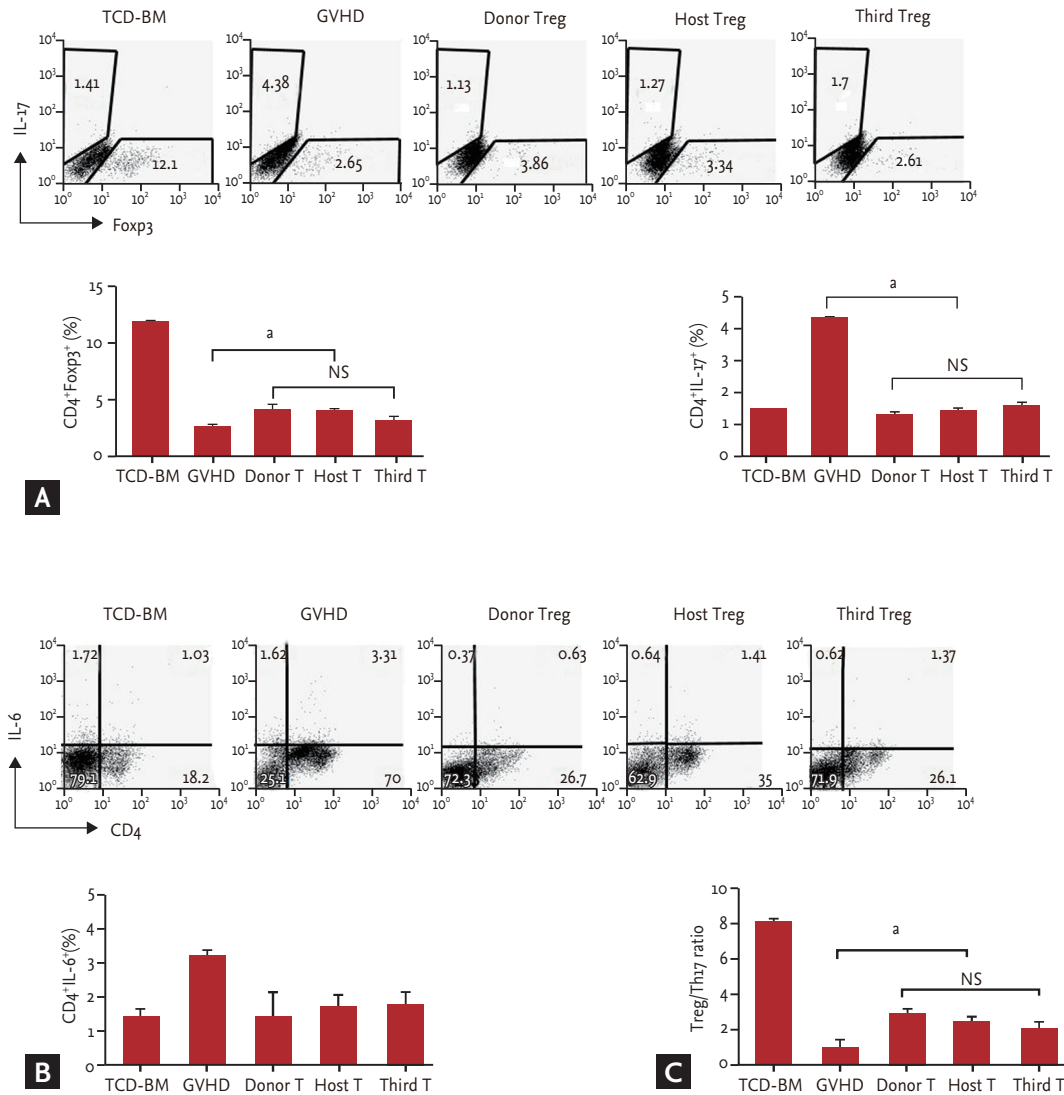
Adoptive transfer of *in vitro*-differentiated induced Treg cells, along with BM grafts containing alloreactive donor T cells, did not result in any significant protection against lethal acute GVHD [15,16], although one study did



**Figure 5.** Only donor-derived regulatory T (Treg) cells modulate the balance of T-helper 1 (Th1) and Th2 cells in lethal acute graft-versus-host disease (GVHD). Treatment of mice with donor-derived Treg cells resulted in a significant reduction in CD4+ interferon- $\gamma$  (IFN- $\gamma$ )<sup>+</sup> Th1 cells, but an enhancement of CD4+ interleukin (IL)-10<sup>+</sup> and CD4+IL-4<sup>+</sup> Th2 cells, when compared with host-derived or third-party-derived Treg cells 15 days after bone marrow transplantation. Donor-derived Treg cells led to a greater increase in (A) IL-10 and (B) IL-4 levels and a greater decrease in IFN- $\gamma$  levels versus treatment with host- or third-party-derived Treg cells in a murine model of acute GVHD. (C) Data are presented as the ratio of Th1/Th2 among CD4<sup>+</sup> T cells, which was calculated as the ratio of CD4+IL-4<sup>+</sup> Th2 cells divided by the percentage of CD4+IFN- $\gamma$ <sup>+</sup> Th1 cells. The purity of all cell subsets was > 95% as determined by flow cytometry analysis. Data are shown as mean  $\pm$  SEM. TCD-BM, cell-depleted bone marrow cells. <sup>a</sup>*p* < 0.05.

demonstrate its efficacy in a lupus-like chronic GVHD model [17]. A major reason for the lack of observed protection in the acute GVHD models was the limited *in vivo* survival of these cells, accompanied by instability of Foxp3 expression that resulted in an early loss of suppressive function post-transplantation [15,18]. However, increased numbers of CD62L+ Treg cells in grafts have been found to correlate with reduced GVHD incidence

[19], which is likely due to the ability of these cells to enter secondary lymphoid tissue, where allorecognition by donor T cells and acute GVHD initiation occur. This is consistent with reported data from murine models of acute GVHD, in which the CD62L+ Treg cell population suppresses acute GVHD to a greater extent than the corresponding CD62Llow population [20,21]. Somewhat lacking, but we showed that *ex vivo*-expanded iso-



**Figure 6.** All three groups of Treg cell therapy provide an immunomodulatory effect associated with reciprocal regulation of 17+ T-helper (Th17)/regulatory T (Treg) cells. Regardless of cell source, Treg cell therapy resulted in a significant reduction in CD4+ interleukin (IL)-17+ Th17 cells, but an enhancement in CD4+Foxp3+ Treg cells compared with graft-versus-host disease (GVHD) control at 15 days after bone marrow transplantation. (A) Treg cell therapy groups led to an increase in Foxp3 levels and a greater decrease in IL-17 levels and (B) IL-6 levels as compared with the GVHD control in a murine model of acute GVHD. However, statistical analysis revealed no significant differences. (C) Data are presented as the ratio of Treg/Th17 among CD4+ T cells, which was calculated as the ratio of CD4+Foxp3+ Treg cells divided by the percentage of CD4+IL-17+Th17 cells. The purity of all cell subsets was > 95% as determined by flow cytometry analysis. Data are shown as mean ± SEM. IFN-γ, interferon-γ; TCD-BM, cell-depleted bone marrow cells; NS, not significant. <sup>a</sup>*p* < 0.05.



lated third-party-derived CD4+CD25+CD62L+ Treg cells showed similarly potent suppressive activity to that of donor- or host-derived cells.

The present study demonstrates that *ex vivo* expanded third-party-derived CD4+CD25+CD62L+ Treg cells are a valuable and useful alternative to Treg cells derived from the same donor or host as the Tcon cells, to effectively suppress acute GVHD. Our results showed that the major limitation to the adoptive transfer of third-party-derived Treg cells was that this therapy resulted in slightly worse clinical symptoms *in vivo* in comparison with donor-derived Treg cell therapy, which were present for immunomodulatory functions during alloreactive T cells. However, both third-party-derived CD4+CD25+CD62L+ Treg cell therapy and donor-derived CD4+CD25+CD62L+ Treg cell therapy provide an immunomodulatory effect associated with reciprocal regulation of Th17 and Treg cells after allogeneic BMT.

Recently, clinical studies have been published showing that generation of third-party-derived cord blood Treg cells via the *ex vivo* expansion strategy has the potential to provide a readily available, clinically relevant, “off-the-shelf” cellular therapy to prevent GVHD and improve transplant outcomes [22]. Although the clinical trials using Treg cells to prevent acute GVHD revealed no obvious safety issues, there are still several concerns regarding this application in the clinic. One disadvantage of administration of third-party cord blood-derived Treg cells is possible relapse and increased incidence of infectious complications caused by the suppressor function of Treg cells. However, in previous clinical trial studies, no such complications were observed [22]. This report demonstrates that adoptive transfer of third-party-derived CD4+CD25+CD62L+ Treg cells is a promising alternative to donor-derived Treg-based cellular therapy, which could result in reduction of GVHD incidence and improved transplant outcomes.

## KEY MESSAGE

1. With the combination of surface markers, Treg cells are usually characterized as CD4+CD25+CD62L+ cells.
2. Third-party Treg cells provide an immunomodulatory effect associated with reciprocal regulation of Th17/Treg cells in graft-versus-host disease model.

## Conflict of interest

No potential conflict of interest relevant to this article was reported.

## Acknowledgments

This study was supported by Research Fund of Seoul St. Mary's Hospital, The Catholic University of Korea. And, supported by a grant (NRF-2016R1A2B4007282) from the Korea Healthcare Technology R&D Project, Ministry for Health, Welfare, and Family Affairs, Republic of Korea and the Basic Science Research Program through the National Research Foundation of Korea funded by the Ministry of Education, Science, and Technology.

## REFERENCES

1. Cao J, Chen C, Zeng LY, et al. Influence of donor Treg cells on GVHD and GVL effects after allogeneic bone marrow transplantation in mice. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* 2010;18:181-184.
2. Kataoka Y, Iwasaki T, Kuroiwa T, et al. The role of donor T cells for target organ injuries in acute and chronic graft-versus-host disease. *Immunology* 2001;103:310-318.
3. Gallatin WM, Weissman IL, Butcher EC. A cell-surface molecule involved in organ-specific homing of lymphocytes. *Nature* 1983;304:30-34.
4. Fu S, Yopp AC, Mao X, et al. CD4+ CD25+ CD62+ T-regulatory cell subset has optimal suppressive and proliferative potential. *Am J Transplant* 2004;4:65-78.
5. Brunstein CG, Miller JS, Cao Q, et al. Infusion of *ex vivo* expanded T regulatory cells in adults transplanted with umbilical cord blood: safety profile and detection kinetics. *Blood* 2011;117:1061-1070.
6. Jeon EJ, Yoon BY, Lim JY, et al. Adoptive transfer of all-trans-retinal-induced regulatory T cells ameliorates experimental autoimmune arthritis in an interferon-gamma knockout model. *Autoimmunity* 2012;45:460-469.
7. Fukui J, Inaba M, Ueda Y, et al. Prevention of graft-versus-host disease by intra-bone marrow injection of donor T cells. *Stem Cells* 2007;25:1595-1601.
8. Lee ES, Lim JY, Im KI, et al. adoptive transfer of treg cells combined with mesenchymal stem cells facilitates repopulation of endogenous Treg cells in a murine acute GVHD model. *PLoS One* 2015;10:e0138846.

9. Lim JY, Park MJ, Im KI, et al. 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* 2014;23:703-714.
10. Shi M, Adachi Y, Cui Y, et al. Combination of intra-bone marrow-bone marrow transplantation and subcutaneous donor splenocyte injection diminishes risk of graft-versus-host disease and enhances survival rate. *Stem Cells Dev* 2011;20:759-768.
11. Riley JL, June CH, Blazar BR. Human T regulatory cell therapy: take a billion or so and call me in the morning. *Immunity* 2009;30:656-665.
12. Fozza C, Dazzi F. Regulatory T cells in stem cell transplantation: main characters or walk-on actors? *Crit Rev Oncol Hematol* 2012;84:18-25.
13. Rezvani K, Mielke S, Ahmadzadeh M, et al. High donor FOXP3-positive regulatory T-cell (Treg) content is associated with a low risk of GVHD following HLA-matched allogeneic SCT. *Blood* 2006;108:1291-1297.
14. Li Q, Zhai Z, Xu X, et al. Decrease of CD4(+)CD25(+) regulatory T cells and TGF-beta at early immune reconstitution is associated to the onset and severity of graft-versus-host disease following allogeneic haematogenesis stem cell transplantation. *Leuk Res* 2010;34:1158-1168.
15. Koenecke C, Czeloth N, Bubke A, et al. Alloantigen-specific de novo-induced Foxp3+ Treg revert in vivo and do not protect from experimental GVHD. *Eur J Immunol* 2009;39:3091-3096.
16. Beres AJ, Haribhai D, Chadwick AC, Gonyo PJ, Williams CB, Drobyski WR. CD8+ Foxp3+ regulatory T cells are induced during graft-versus-host disease and mitigate disease severity. *J Immunol* 2012;189:464-474.
17. Zheng SG, Wang JH, Koss MN, Quismorio F Jr, Gray JD, Horwitz DA. CD4+ and CD8+ regulatory T cells generated ex vivo with IL-2 and TGF-beta suppress a stimulatory graft-versus-host disease with a lupus-like syndrome. *J Immunol* 2004;172:1531-1539.
18. Yamashita J, Koi K, Yang DY, McCauley LK. Effect of zoledronate on oral wound healing in rats. *Clin Cancer Res* 2011;17:1405-1414.
19. Lu SY, Liu KY, Liu DH, Xu LP, Huang XJ. High frequencies of CD62L+ naive regulatory T cells in allografts are associated with a low risk of acute graft-versus-host disease following unmanipulated allogeneic haematopoietic stem cell transplantation. *Clin Exp Immunol* 2011;165:264-277.
20. Taylor PA, Panoskaltsis-Mortari A, Swedin JM, et al. L-Selectin(hi) but not the L-selectin(lo) CD4+25+ T-regulatory cells are potent inhibitors of GVHD and BM graft rejection. *Blood* 2004;104:3804-3812.
21. Ermann J, Hoffmann P, Edinger M, et al. Only the CD62L+ subpopulation of CD4+CD25+ regulatory T cells protects from lethal acute GVHD. *Blood* 2005;105:2220-2226.
22. Parmar S, Liu X, Tung SS, et al. Third-party umbilical cord blood-derived regulatory T cells prevent xenogenic graft-versus-host disease. *Cytotherapy* 2014;16:90-100.