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Adipose tissue-derived mesenchymal stem cells ameliorate bone marrow aplasia related with graft-versus-host disease in experimental murine models

Yukiko Nishi[a,1], Akikazu Murakami[b,1], Yuko Murayama[a], Narutoshi Tsukahara[b], Shiki Okamoto[a], Sawako Nakachi[a], Kazuho Morichika[a], Keita Tamaki[a], Hirofumi Noguchi[c], Masayuki Matsushita[d], Ken-nosuke Karube[e], Takuya Fukushima[f], Satoko Morishima[a], Hidehiro Kishimoto[b], Hiroaki Masuzaki[a,*]

[a] Division of Endocrinology, Diabetes and Metabolism, Hematology, Rheumatology (Second Department of Internal Medicine), Graduate School of Medicine, University of the Ryukyus, Okinawa, Japan
[b] Department of Parasitology & Immunopathoetiology, Graduate School of Medicine, University of the Ryukyus, Okinawa, Japan
[c] Department of Regenerative Medicine, Graduate School of Medicine, University of the Ryukyus, Okinawa, Japan
[d] Department of Molecular and Cellular Physiology, Graduate School of Medicine, University of the Ryukyus, Okinawa, Japan
[e] Department of Pathology and Cell Biology, Graduate School of Medicine, University of the Ryukyus, Okinawa, Japan
[f] Laboratory of Hematoimmunology, School of Health Sciences, Faculty of Medicine, University of the Ryukyus, Okinawa, Japan

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ABSTRACT

Graft-versus-host disease (GVHD) constitutes the most frequent complications after the allogeneic hematopoietic stem cell transplantation for a variety of hematological malignancies. In the present study, we explored the prophylactic potential of adipose tissue-derived mesenchymal stem cells (AD-MSCs) in controlling GVHD in murine models with a special focus on bone marrow aplasia related with acute GVHD. The CB6F1 mice were induced GVHD by the injection intravenously of C57BL/6 (B6-Ly-5.1) splenocytes without conditioning irradiation or chemotherapy. AD-MSCs from C3H mice were injected intravenously via tail veins. GVHD was assessed using flowcytometry analysis of peripheral blood cells and histopathologic analysis of target organs. Histopathological analyses revealed that AD-MSCs markedly suppressed the infiltration of lymphocytes into liver as well as the aplasia in bone marrow. This study is the first to clarify the effectiveness of AD-MSCs against bone marrow aplasia in GVHD, supporting a rationale of AD-MSCs for ameliorating bone marrow suppression and infectivity after allo-HSCT in human clinics.

1. Introduction

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is a curative therapy with proven efficacy in the management of hematologic malignance. In some cases, however, it is complicated by acute and chronic graft-versus-host disease (GVHD) [1–3]. Outside of primary disease relapse following allo-HSCT, arguably the most obstacle to success following this therapy is the onset of GVHD [4]. Although corticosteroids are used as a first-line therapy [5], only 60–70% of patients with acute GVHD respond to standard corticosteroid therapy, and those who failed initial therapy showed only a 10–30% chance of long-term survival [4,6,7]. A wide variety of second-line treatments for acute GVHD are available including tacrolimus, mycophenolate mofetil (MMF), sirolimus, anti-thymocyte globulin (ATG), monoclonal antibodies against IL-2 receptor, TNFα, CD52, CD147, and CD3 [8–12]. However, the prognosis of patients under these treatments remains dismal because of higher risk of infectious complications, immunosuppression-mediated toxicity, and incomplete remission of GVHD [8–12]. Therefore, better strategies for the prophylaxis and treatment of GVHD are critical to improve the outcome of HSCT.

It is well known that the principal target organs of acute GVHD are the skin, liver and gastrointestinal tract. Moreover, GVHD is often accompanied by cytopenia and bone marrow suppression [13]. In a murine model of GVHD, Shono et al. reported the destruction of bone marrow hematopoietic niches, especially osteoblasts by donor T cells, resulting in bone marrow suppression [13], an example of bone marrow

* Corresponding author at: Division of Endocrinology, Diabetes and Metabolism, Hematology, Rheumatology (Second Department of Internal Medicine), Graduate School of Medicine, University of the Ryukyus, 207 Uehara, Nishihara, Okinawa 903-0215, Japan.
E-mail address: hiroaki@med.u-ryukyu.ac.jp (H. Masuzaki).
[1] These authors contributed equally to this work.

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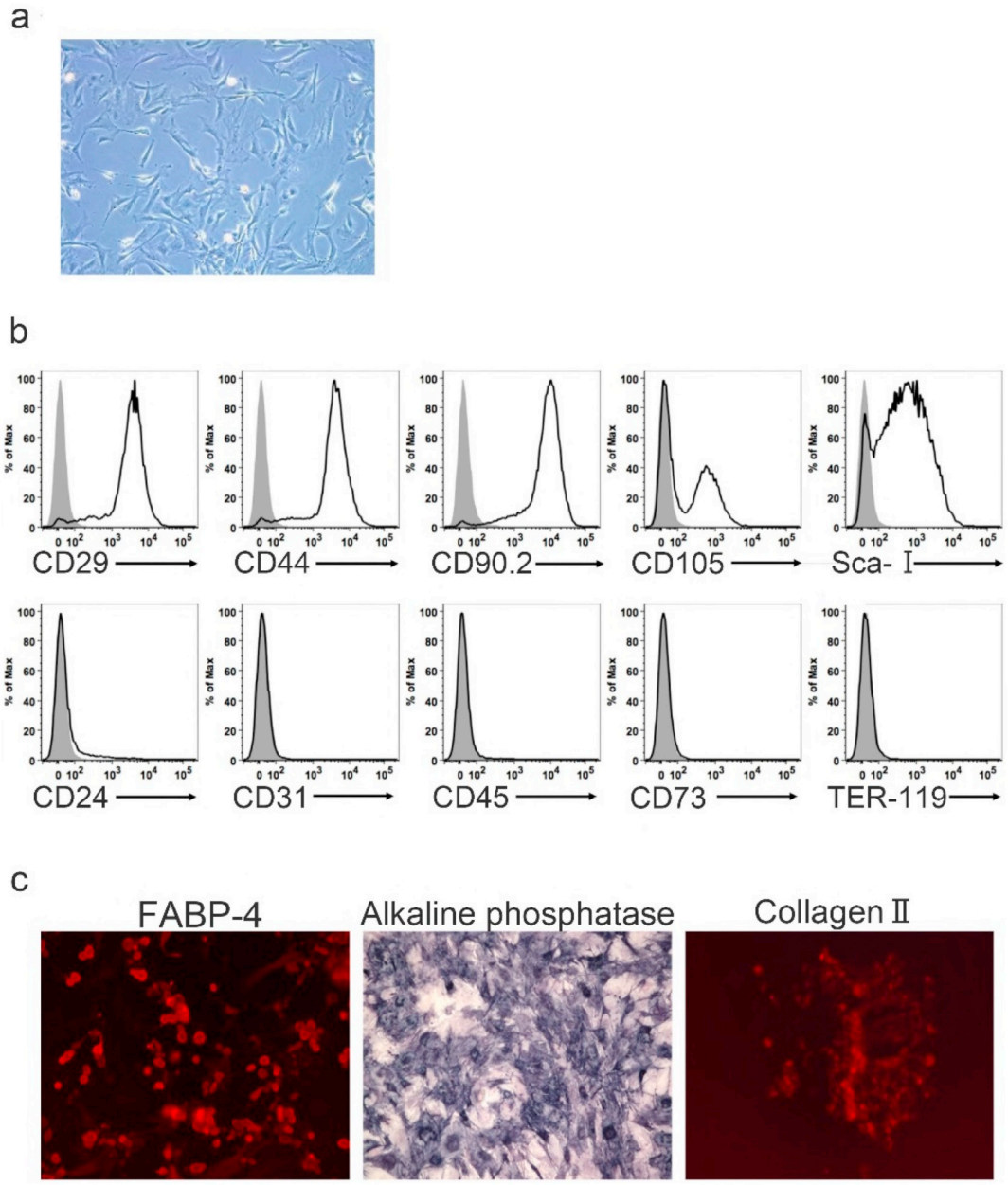

Fig. 1. Characteristics of Ad-MSCs.
(a) The morphology of Ad-MSCs (passage 3) monitored by phase contrast microscopy. (b) Flow cytometry analysis of surface markers of Ad-MSCs. Ad-MSCs were stained with phycoerythrin-conjugated anti-mouse CD29, CD44, CD90.2, CD105, Sca−I, CD24, CD31, CD45, CD73 and TER-119, respectively. Filled histograms represent cells stained by the corresponding isotype control antibody. Representative results are demonstrated. (c) Differentiation potential of Ad-MSCs. Ad-MSCs were cultured in each differentiation medium. After 2 weeks (adipogenic differentiation and osteogenic differentiation) or 3 weeks (chondrogenic differentiation), cells were stained with anti-mFABP-4, alkaline phosphatase (ALP) and anti-mCollagen, and visualized by phase contrast microscopy or fluorescence microscopy.

GVHD. Because bone marrow GVHD may cause life-threatening infection after the allo-HSCT [14], bone marrow destruction caused by acute GVHD should be prevented in clinics.

Mesenchymal stromal cells (MSCs) are a form of multipotent adult stem cells characterized by their plastic-adherent growth and expression of specific surface antigen [15]. MSCs possess the capacity to suppress immunological responses, support hematopoiesis and repair tissues [16–19]. Based on a variety of immunoregulatory properties, MSCs (bone marrow derived MSCs) have been studied as a promising platform for cell-based therapies to prevent or treat GVHD [20–22]. MSCs are present in various tissues including bone marrow, peripheral bloods, umbilical cord blood and adipose tissue. It has been highlighted that adipose tissue derived MSCs (AD-MSCs) might serve as promising therapeutic tools for GVHD with a line of unique advantage [23]. In particular, large amount of adipose tissues can be obtained in a minimally invasive manner through lipectomy or liposuction. Furthermore, previous in vitro studies showed that AD-MSCs represent equipotent or higher immunoregulatory capacity compared to bone marrow derived MSCs [24].

Although many studies reported that MSCs ameliorated GVHD, to our knowledge, there have been no reports focusing on the impact of MSCs on bone marrow aplasia related with GVHD. There are a large number of murine acute GVHD models, and most of them use lethally irradiation regimen prior to transplantation. On the other hand, in a MHC-mismatched murine model, parent-to-F1 transplants have been studied using no irradiation conditioning [25,26]. As pretreating

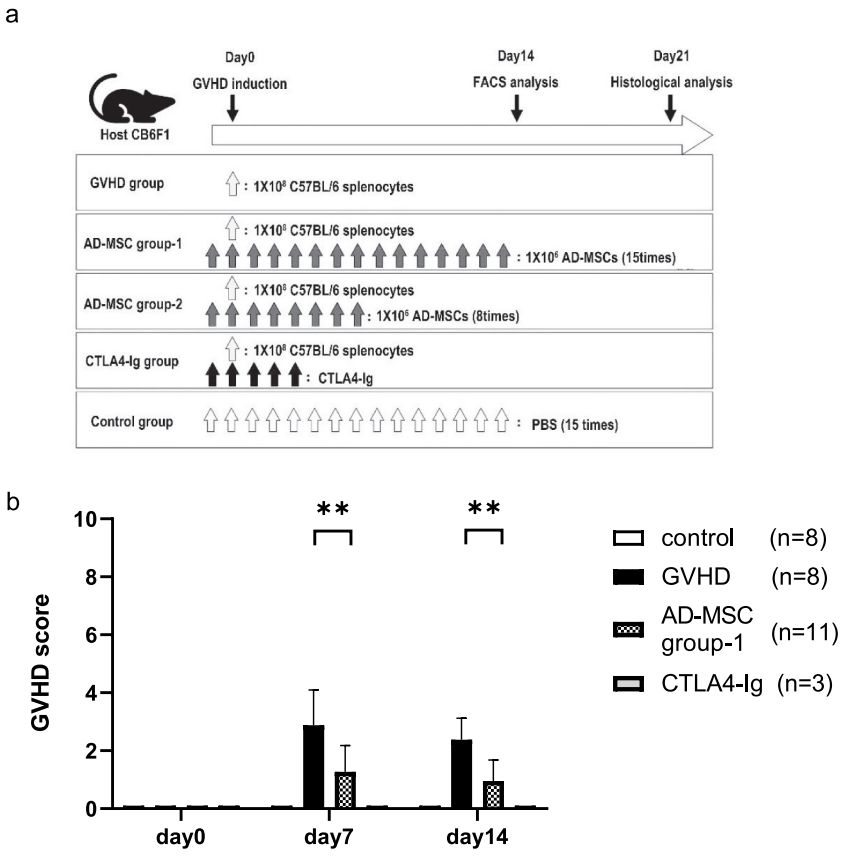

Fig. 2. Schematic outline of the experimental protocol.
(a) To induce GVHD, single cell suspension splenocytes (1×10^8) from C57BL/6 (B6-Ly-5.1) donor mice in PBS were injected intravenously via the tail vein into unirradiated CB6F1 host mice on day 0. AD-MSCs group-1 was received intravenous injections of 1×10^6 AD-MSCs from day -1 to day 15, and AD-MSCs group-2 was received 1×10^6 AD-MSCs from day -1 to day 8. GVHD amelioration murine model (CTLA4-Ig group) was prepared by administration of CTLA 4-Ig i.v. on day 0 and day1, and i.p. on day 2, 3 and 4. Control group was received PBS. PBMCs from each group of mice were analyzed by FACS on day 14 and histopathologic analysis of organs were performed on day 21. (b) Clinical GVHD score of mice. **: $P < 0.001$.

chemotherapy and irradiation are responsible for bone marrow destruction [14], it has been difficult to analyze bone marrow per se in preexisting GVHD murine models. The model without irradiation is advantageous for assessing the impact of GVHD itself on bone marrow. In this context, we employed murine GVHD model of MHC homozygous-donor to heterozygous-recipient without pretreating chemotherapy or irradiation.

2. Material and methods

2.1. Animals

C57BL/6 J(H-2^b), (BALB/c × C57BL/6) F1 (CB6F1) (H-2^{dXb}) and C3H/He(*H*-2^k) female mice of 6–8 weeks of age were purchased from Japan SLC, Inc. (Shizuoka, Japan). This study was conducted in compliance with the Animal Welfare Act, the implementing Animal Welfare Regulations and in accordance with the principles of the Guide for the Care and Use of Laboratory Animals. Study protocol was reviewed and approved by the Animal Experiment Ethics Committee of the University of the Ryukyus (A2016029).

2.2. Isolation, culture, and in vitro differentiation of AD-MSCs

To isolate AD-MSCs, adipose tissues were obtained from the inguinal region of C3H/He mice. The adipose tissues were washed with PBS and minced with scissors and blades in culture dish, then placed on the hydroxyapatite matrix (BMK, Tokyo, Japan) and cultured in Completed StemXVivo® MSC Expansion Media (R&D systems, MN, USA) for five days at 37 °C under 5% CO_2. Expanded AD-MSCs on the hydroxyapatite matrix were dissociated by trypsinization and maintained. When the cells reached 90% confluency, they were sub-cultured and expanded for two or three passage, at which point they were used for experiments.

Multilineage potential was assessed using Mouse mesenchymal stem cell functional identification kit (R&D systems) and TRACP & ALP double-stain kit (TAKARA, Shiga, Japan). Briefly, cells were incubated under specific conditions to induce differentiation into adipocytes, osteoblasts, and chondrocytes. After differentiation, multilineage potential was evaluated. The mature phenotypes of adipocytes and osteocytes was defined with anti-mFABP4, anti-mCollagen II, respectively. Osteoblasts formation was determined by staining of intracellular alkaline phosphatase (ALP). Control cells were cultured only with MSC expansion medium.

2.3. Flow cytometry analysis

Third passaged AD-MSCs were detached to single cell suspension using Trypsin-EDTA (Thermo Fisher Scientific). AD-MSCs aliquots (1×10^6) were washed and resuspended in FACS buffer (PBS containing 2% FBS) and stained with phycoerythrin-conjugated anti-mouse CD24, CD29, CD31, CD44, CD45, CD73, CD90.2, CD105, Sca−I, TER-119 and CD4 (BioLegend, CA, USA). Peripheral blood cells were resuspended in FACS buffer and stained with antibodies specific for CD4 (FITC), CD8 (PE), CD45R/B220 (pacific blue), CD45.1 (PerCP-Cy5.5), CD44 (Alexa Fluor 647), CD62L (PE-Cy7) (BioLegend, CA, USA). Appropriate isotype controls were used for gating purpose. Fluorescence- activated cell sorting (FACS) analysis was performed on an FACS Verse (BD Biosciences). The data were analyzed by FlowJo software (TreeStar Inc., Ashland OR).

2.4. Splenocyte transplantation and administration of AD-MSCs

To induce GVHD, single cell suspension spleen cells (1×10^8) from C57BL/6 (B6-Ly-5.1, CD45.1) mice in PBS were injected intravenously via the tail vein into unirradiated CB6F1 (CD45.2) recipient mice. Donor derived peripheral blood cells (CD45.1) were detected by

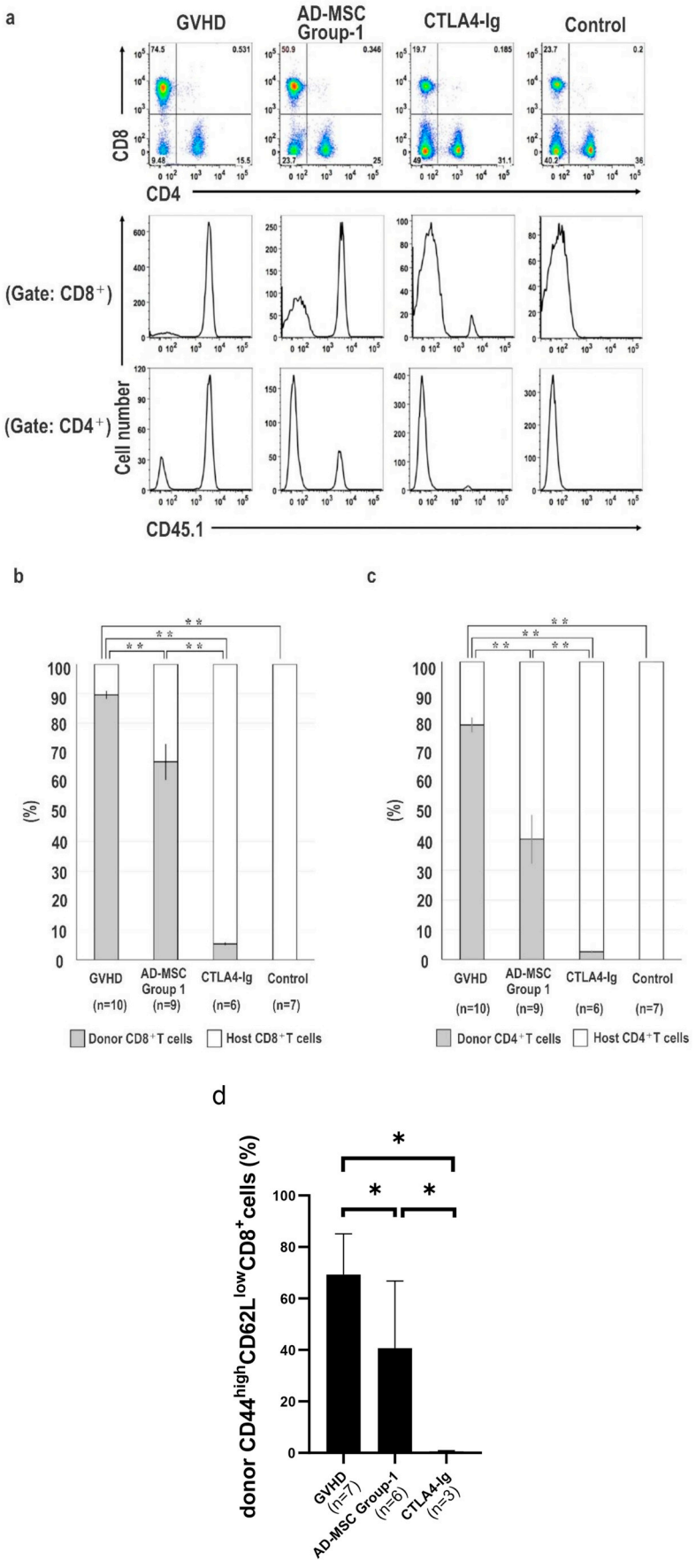

Fig. 3. Flow cytometry analysis of Ad-MSCs administrated or un-administrated GVHD induced mice T cells.
(a)Representative plots and histograms of the flow cytometric profiles of donor ($CD45.1^+$) and host ($CD45.1^-$) $CD4^+$and $CD8^+$ T cells on day 14. (b) Relative numbers of host and donor $CD8^+$ T cells are shown. (c) Relative numbers of host and donor $CD4^+$ T cells are shown. Data are shown as the mean ± SEM and are the cumulative results from four independent experiments. (d) The percentage of cells with $CD44^{high}$ and $CD62L^{low}$ donor $CD8^+$T cells in total $CD8^+$T cells. The value in GVHD group was 69.3% and 40.7% in AD-MSCs group, respectively. *: $P < 0.01$, **: $P < 0.001$.

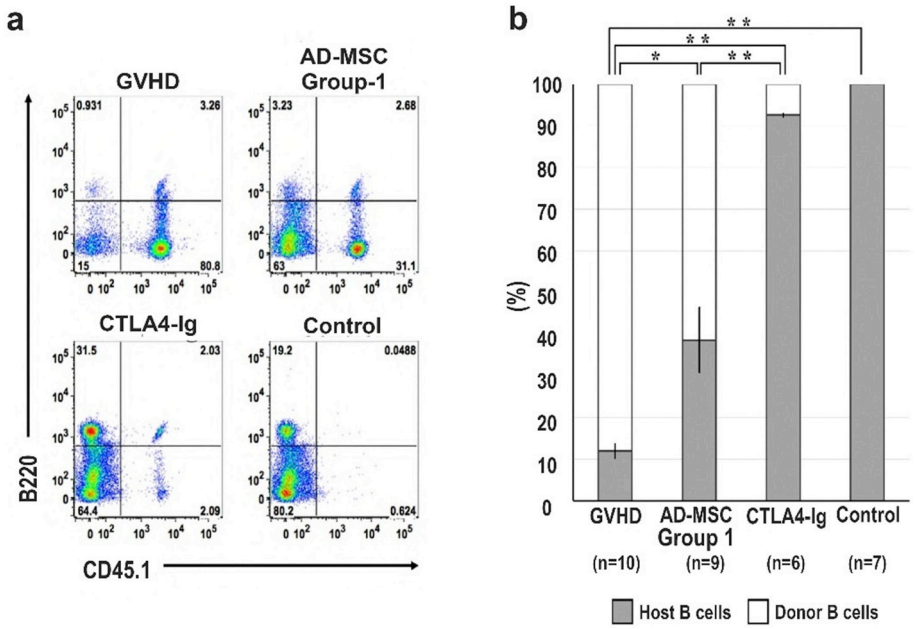

Fig. 4. Flow cytometry analysis of Ad-MSCs administrated or un-administrated GVHD induced mice B cells.
(a) Representative plots of the flow cytometric profiles of donor ($CD45.1^+$) and host ($CD45.1^-$) B cells on day 14. (b) Relative numbers of host and donor B cells are shown.

staining with PerCP-Cy5.5-conjugated CD45.1 antibody. Control group consisted of age- and sex-matched F1 mice, and received PBS alone without splenocyte transplantation. On the other hand, as a positive control of stronger immunosuppression, mice were prepared by administration of 200 μg of CTLA 4-Ig (Bristol-Myers Squibb, Tokyo, Japan) i.v. at the day of GVHD induction (day 0) and day 1, and 100 μg i.p. on day 2, 3 and 4. A single cell suspension of AD-MSCs was prepared, and 1×10^6 AD-MSCs were injected intravenously via tail veins on day -1 to day 15 (AD-MSCs group-1) or day -1 to day 8 (AD-MSCs group-2).

2.5. Assessment of GVHD and histopathologic analysis of GVHD target organs

The severity of GVHD was assessed by the percentage of weight loss, posture, activity, fur texture, and skin integrity. Individual mice from coded cages received a score of 0 to 2 for each criteria (maximum score of 10) [27].

The liver and bone marrow isolated from mice were fixed in 10% formalin, embedded in paraffin, sectioned at 6 μm thickness, deparaffinized using xylene, dehydrated through an alcohol gradient and stained with hematoxylin and eosin. The number of cells of bone marrow was counted using ImageJ software (National Institutes of Health Image, USA) [28].

2.6. Carboxyfluorescein succinimidyl ester (CFSE) labeled AD-MSCs assay

AD-MSCs were suspended in phosphate buffer saline (PBS) at 2–3 × 10^6 cells/ml, and incubated with 5 mM carboxyfluorescein succinimidyl ester (CFSE) (eBioscience Inc., CA, USA) for 10 min at room temperature. Cells were re-suspended in RPMI Medium with 10% FBS for 5 min to stabilize the CFSE staining, and washed 3 times with the medium. Cells were re-suspended in PBS after a final wash step, and were injected into mice according to the protocol as AD-MSC group-1 (material and methods 2.4). Twenty days after the injection, bone marrow cells were analyzed by flow cytometry. A portion of CFSE-labeled AD-MSCs were cultured in medium (StemXVivo® MSC Expansion Media, R&D systems, MN, USA) for 20 days, and subsequently analyzed by flow cytometry.

2.7. Hematopoietic colony forming cell assay

Ability of hematopoietic colony forming was assessed using Hematopoietic Colony Forming Cell Assay kit (CELL BIOLABS, INC., CA, USA). Bone marrow cells were seeded at 3000 cells/well of a 96-well tissue culture plate, and cultured for 7 days in the presence of 50 ng/mL mouse SCF (PEPROTECH, NJ, USA), 10 ng/mL mouse IL-3 (PEPROTECH), 10 ng/mL mouse GM-CSF (R&D system). Colony quantitation was assessed according to the assay protocol.

2.8. Statistical analysis

One-way and two-way analysis of variance (ANOVA) was used for multiple comparison among more than three groups. A value of $P < 0.05$ denoted statistical significance.

3. Results

3.1. Characterization of isolated AD-MSCs

AD-MSCs were isolated from adipose tissues obtained from the inguinal region of C3H/He mice. The cultured AD-MSCs exhibited a fibroblast-like, spindle-shaped morphology (Fig. 1A). Flow cytometry analysis was used to assess whether the AD-MSCs showed typical profile of MSC surface marker. The AD-MSCs highly expressed a variety of markers for authentic mesenchymal stem cells including Sca−I, CD29, CD90 and CD105, while they were negative for hematopoietic and endothelial markers such as CD31 and CD45 (Fig. 1B). Differentiation assay showed that capability of multi-potential differentiation into adipocytes, osteocytes, and chondrocytes (Fig. 1C).

3.2. AD-MSCs effectively protect mice against GVHD

The prophylactic effect of AD-MSCs on GVHD was investigated in mouse GVHD models. Weight-matched CB6F1 mice were used as recipient mice, and they were divided into five groups, as described in

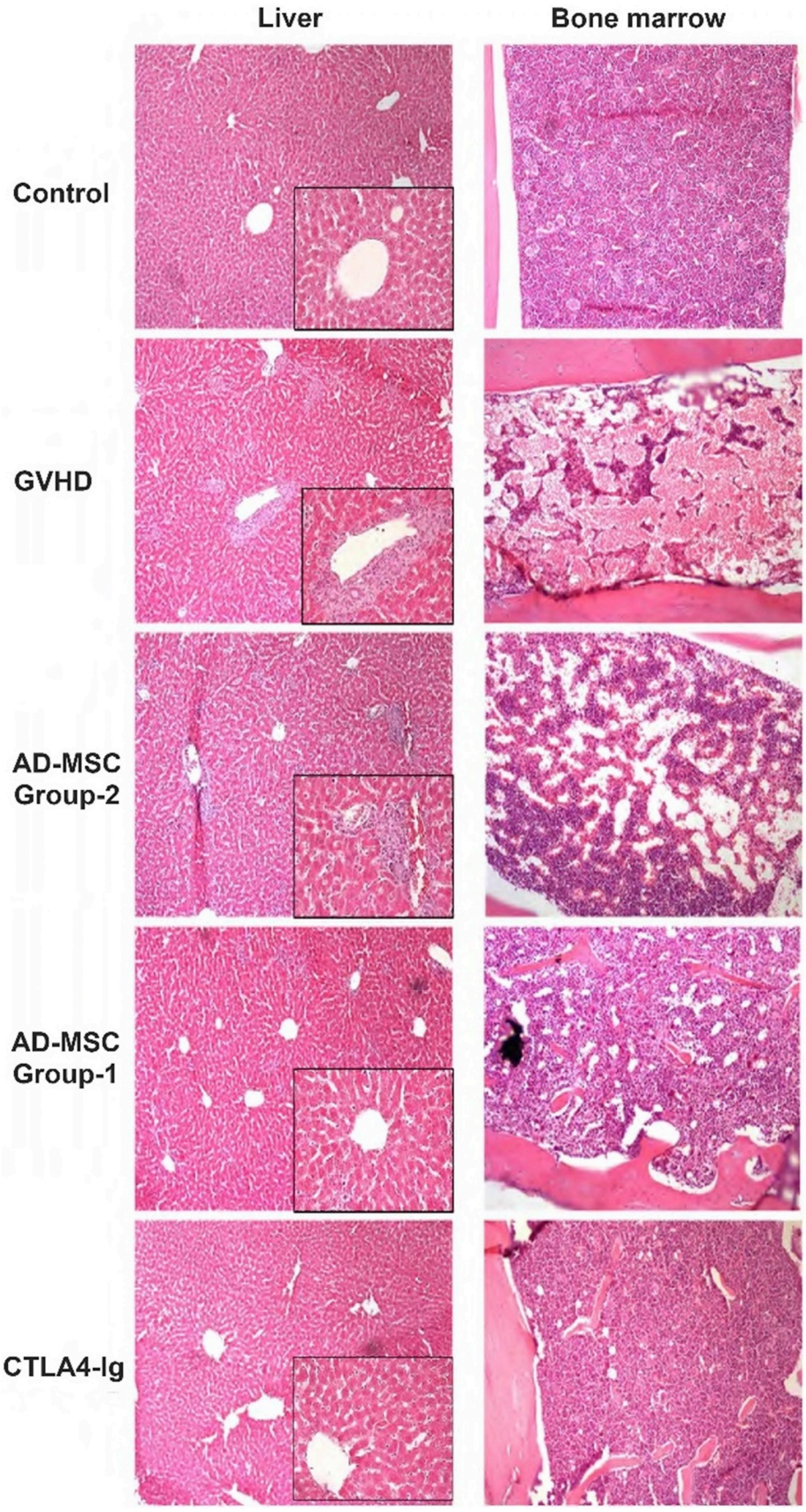

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Fig. 5. Pathological improvement in GVHD by AD-MSC administration.
(a) Induction of GVHD and administration of AD-MSCs were performed as described in Fig. 2. AD-MSCs group-1 was received intravenous injections of 1×10^6 AD-MSCs from day -1 to day 15, and AD-MSCs group-2 was received 1×10^6 AD-MSCs from days -1 to day 8. On day 21, mice from each of the 5 groups were sacrificed. Paraffin sections of the liver and bone marrow were stained by hematoxylin and eosin (magnification ×200 and ×400). AD-MSCs were apparently exempted from infiltration of lymphocyte in liver and bone marrow aplasia in a dose-dependent manner. (b) The numbers of cells in the bone marrows were shown. The number of bone marrow cells in AD-MSCs group was 133/10000 μm^2 and that in GVHD group was 81/10000μm^2, respectively. Aplasia of bone marrow related with GVHD was significantly suppressed in AD-MSCs group as compared to GVHD group. *: $P < 0.05$.

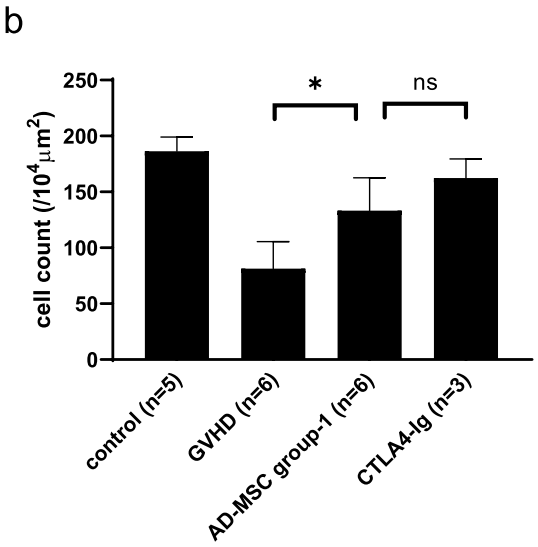

Fig. 5. (*continued*)

Fig. 2A. GVHD was induced by the intravenous injection of 1×10^8 C57BL/6 (B6-Ly-5.1) splenocytes via tail vain on day 0. AD-MSCs group-1 was received intravenous injections of 1×10^6 AD-MSCs from day -1 to day 13 post inducing GVHD. AD-MSCs group-2 was received intravenous injections of 1×10^6 Ad-MSCs from days -1 to day 8. CTLA4-Ig group which was the amelioration murine model was received intravenous injections of 200 μg CTLA4-Ig day 0 and day 1, and intraperitoneal injection of 100 μg CTLA4-Ig on days 2, 3, and 4 post inducing GVHD. Control group was received only PBS from day -1 to day 13. AD-MSC group-1 showed a significantly lower score than that of GVHD group ($P < 0.001$, Fig. 2B). The mice in control group and CTLA4-Ig group had the score of 0 during an entire experimental period.

It is well known that the major pathology of GVHD is expansion of host-received donor T cells. Thus, the levels of T cell subsets in peripheral blood on day 14 were analyzed by flow cytometry (Fig. 3). In our experiments, percentage of donor $CD4^+$and $CD8^+$T cells were 78.4% (± 2.4%) and 89.6% (± 5.9%) in GVHD mice, respectively (Fig. 3A–C). In contrast, percentage of donor $CD4^+$and $CD8^+$T cells were 40.4% (± 8.0%) and 66.9% (± 1.2%) in AD-MSCs mice, respectively. The percentage of activated T cells ($CD44^{high}$ and $CD62L^{low}$) in donor $CD8^+$T cells tended to be higher in GVHD group (80.6% ± 2.7%) than in AD-MSCs group (70.3% ± 6.0%) (Fig. 3D). These results suggested that AD-MSCs have suppressive effect on activation and expansion of donor T cells after transplantation. As previously reported [29], strong suppression of donor T cell activation and expansion was observed in CTLA4-Ig mice (Fig. 3).

A previous report demonstrated that host B cells dramatically decreased in a MHC-mismatched parant-to-F1 murine transplant model [30]. In the present study, the percentage of host B cells (B220+ and CD45.1-) in total lymphocytes was significantly higher in AD-MSC mice (5.6% (± 2.5%)) than in GVHD mice (0.46% (± 0.06%)) (Fig. 4).

To verify the effect of AD-MSCs on target organs of acute GVHD, histopathologic analysis was performed. Liver and bone marrow tissues in each group on day 21 were shown in Fig. 5A. Infiltration of inflammatory cells in liver and bone marrow were reduced dramatically in mice administrated AD-MSCs. The suppression in infiltration of lymphocytes in liver and bone marrow aplasia was dependent on the number of AD-MSCs administration. We evaluated bone marrow aplasia in each group using ImageJ software. The number of bone marrow cells was significantly higher in AD-MSC group-1 (133 (± 29)/10000 μm^2) than that in GVHD group (81 (± 24)/10000 μm^2) ($P = 0.006$, Fig. 5B) In mice administrated CTLA4-Ig, no significant difference was observed in the histopathology of liver and bone marrow compared with control mice. Noticeably, histopathological analysis showed no apparent GVHD in gastrointestinal tract or lung in this model.

Because our results suggest the effectiveness of AD-MSCs against bone marrow aplasia in GVHD, we analyzed the potential effect of AD-MSC on cell counts in peripheral blood and bone marrow. Counts of white blood cell (WBC) and platelet on day 14 in the AD-MSC group-1 were significantly higher than those in the GVHD group ($P = 0.008$ and $P = 0.044$, respectively. Fig. 6A and B). Furthermore, the count of neutrophil on day14 in the AD-MSC group-1 was significantly higher than that in the GVHD group ($P = 0.011$, Supplemental Fig. 1). The colony forming assay of bone marrow were performed on day17. Colony count of AD-MSC group-1 also showed a trend to increase as compared to that in GVHD group (Fig. 6C). To see whether the infused AD-MSCs would proliferate in bone marrow, CFSE-labeled AD-MSCs were injected into mice, and bone marrow cells were analyzed by flow cytometry on day 20. Consequently, CFSE-positive cells were detected in CFSE-labelled cells which were cultured for 20 days in medium, while CFSE-positive cells were not detected in bone marrow cells obtained from mice (Supplemental Fig. 2). These results indicate that infused AD-MSCs would not proliferate in bone marrow.

4. Discussion

The present study demonstrated that bone marrow destruction induced by acute GVHD was potently protected by the administration of AD-MSCs, where inflammation or bone marrow destruction caused by preconditioning (i.e. irradiation or chemotherapy) was absent. We used murine allo-HSCT model of MHC homo-donor to hetero-recipient without preconditioning therapy, because this model is likely to correspond to transfusion-associated GVHD models in humans rather than allo-HSCT. In most cases, transfusion-associated GVHD is induced by transfused donor cells with homozygous MHC, and severe pancytopenia due to bone marrow destruction emerges in recipients [31].

Although our model is widely different from that in clinically-occurred GVHD, this would make it possible for us to simply evaluate the effect of AD-MSCs on bone marrow aplasia caused by acute GVHD. Bone marrow GVHD was initially reported in murine allo-HSCT model [13] and was subsequently characterized in human allo-HSCT [14]. In general, destruction of bone marrow associated with GVHD occurs soon after allo-HSCT, and this phenomenon is considered as one of the chief reasons for delayed immune reconstitution. A lot of studies demonstrated the efficacy of bone marrow derived MSCs in GVHD murine models with lethal preconditioning including improvement of survival rates and pathologic findings of gastro-intestinal and liver. To our knowledge, however, it has never been clarified the effectiveness of AD-MSCs against bone marrow in acute GVHD. Furthermore, there have been limited number of studies demonstrating the efficacy of AD-MSCs in GVHD murine models and clinical trials [32–35]. A recent systemic

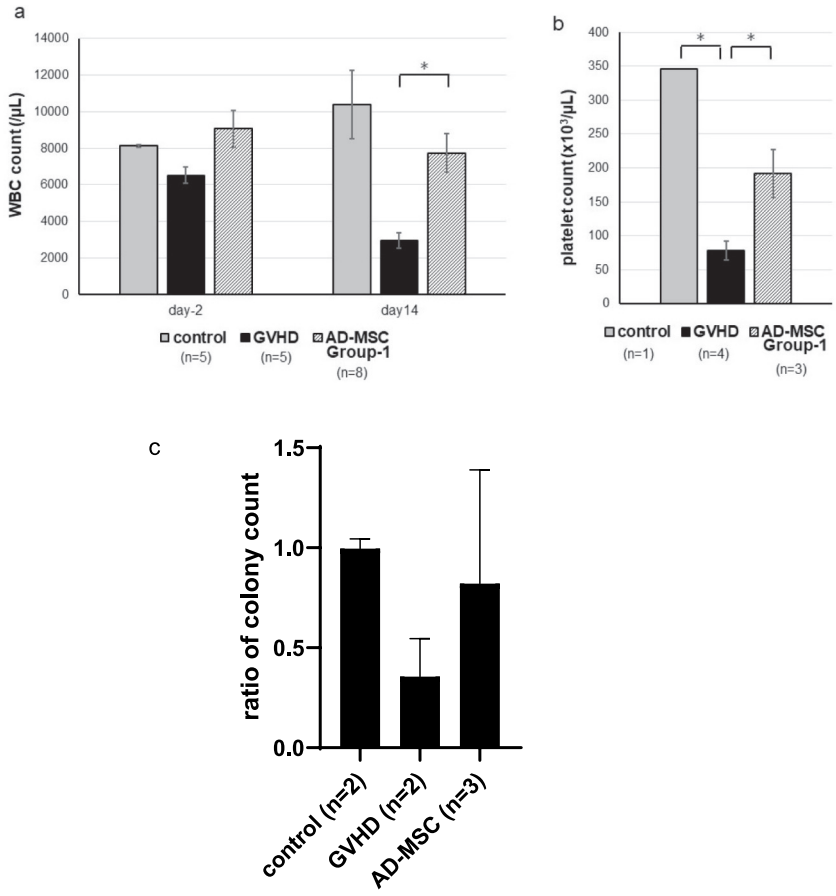

Fig. 6. Analyses of peripheral blood and bone marrow cell after transplantation.
(a) WBC count in peripheral blood on day14. (b) Platelet count in peripheral blood on day14. (c) The ratios of hematopoietic colonies of the GVHD group and the AD-MSC group-1 to the control group were shown. *: $P < 0.05$.

review of clinical studies using MSCs for the treatment or prevention of GVHD demonstrated that there was only one study on AD-MSCs among total of 30 evaluable clinical studies [36].

The AD-MSCs used in the present study are prepared through the simple method in that subcutaneous adipose tissue of mice was harvested, and then physically shredded the tissue and cultured on a hydroxyapatite matrix. Contrary to the ordinary AD-MSCs preparation [37], complicated steps such as treating tissues with collagenase are not included. It is therefore possible to easily obtain a large amount of AD-MSCs from a small amount of adipose tissue (see methods). As shown in Fig. 1, AD-MSCs used in the present study were positive for Sca-I, CD29, CD90 and CD105, and negative for CD73, CD34 and CD45. There are some previous studies showing that MSCs are CD73 positive in AD-MSCs [24], while another report demonstrated that the positive rate for CD73 and CD 105 in AD-MSCs varied depending on the number of passage [38,39], endorsing that same characteristic features of AD-MSCs as previously reported [40] was observed in the present study. In accordance with the property of plastic adherence and surface marker expression, AD-MSCs used in the present study also possessed the ability to differentiate into osteoblasts, adipocytes and chondroblasts in vitro. (Fig. 1C).

Because we aimed to investigate the clinical application of AD-MSCs for the prevention of bone marrow aplasia related with acute GVHD, AD-MSCs from third party were used. Importantly, in an acute GVHD murine model with lethal total body irradiation, bone marrow of the host mouse is completely destroyed by the pretreatment of irradiation [26]. Consequently, host's hematopoietic stem cells are depleted and bone marrow aplasia is sustained until the donor cells are stably engrafted. On the other hand, a GVHD mouse model without preconditioning of chemotherapy or radiation was used in the present study to assess direct effects of AD-MSCs on GVHD. The onset of acute GVHD can be confirmed by the proliferation of donor $CD4^+$ T cells, $CD8^+$ T cells and activated cytotoxic T lymphocytes as well as depletion of host B cells [29]. Following previous studies using bone marrow derived MSCs, we also observed that the administration of MHC-mismatched AD-MSCs clearly inhibited the proliferation of donor $CD4^+$ and $CD8^+$ T cells (Fig. 3). In the clinical allo-HSCT setting, the skin, gastrointestinal tract and liver are the major targets in acute GVHD. In our experimental mouse model, histopathological analysis showed no remarkable GVHD in gastrointestinal tract or lung (data not shown). On the other hand, massive inflammation of the portal region in the liver and destruction of bone marrow related with GVHD was clearly observed (Fig. 5A). During the clinical course of GVHD, bone marrow suppression without infection is often observed in patients undergoing HSCT, indicating that bone marrow is also a potential target of GVHD. Other group reported that donor T cells caused dysfunction of bone marrow stroma cells and loss of osteoblasts, resulting in the suppression of hematopoiesis and subsequent delay in the immune reconstitution [13]. In the present study, the cell colony count of bone marrow after the transplantation showed a trend to elevate in AD-MSC group as compared to those in GVHD group (2.3-fold), albeit without statistical significance. Furthermore, the peripheral blood counts for WBC, absolute neutrophil and platelet on day 14 in the AD-MSC group-1 were significantly higher than those in the GVHD group. These results may suggest that the bone marrow of mice in AD-MSCs group would contain more hematopoietic stem cells than those in GVHD group, and resultantly, efficient production of peripheral blood cells may be observed in AD-MSC group. Previous studies reported that infused MSCs were not

capable of long-term engraftment in the host microenvironment [41,42]. In agreement with this notion, the present study also demonstrated that CFSE labeled AD-MSCs were not detected in the bone marrow on day 21 after the transplantation, suggesting that injected AD-MSCs would also rarely exist and proliferate in bone marrow.

Several limitations in the experimental models of the present study should be noted. First, mouse models we employed did not mimic the clinical setting. As mice were transplanted without pre-conditioning, we could not address the direct effect of AD-MSCs on bone marrow aplasia observed in the clinical transplant with pre-conditioning, which may profoundly destroy the bone marrow niche and stroma. Second, we used considerably high doses of AD-MSCs as compared with those in previous reports [32–35]. Based on our preliminary evaluation in a variety of doses of AD-MSCs, 1×10^6 AD-MSCs for 15 days were required to exert an apparent protective effect against host B cell depletion after GVHD. We therefore determined the dose of AD-MSCs used in the present study. Third, our model could not exclude the possibility that beneficial effects of AD-MSCs on bone marrow aplasia are largely attributed to suppressive effects on global GVHD, rather than direct effects of AD-MSC on the bone marrow recovery, because MSCs have been shown to improve hematopoietic engraftment [43,44]. In this context, further investigations are warranted to discriminate the impact of AD-MSCs on bone marrow GVHD in clinical transplant setting.

In summary, the present study demonstrates that administration of AD-MSCs considerably protects bone marrow aplasia in models of MHC homozygous donor to heterozygous recipient without pretreating chemotherapy or irradiation. Our data support a rationale of AD-MSCs for ameliorating GVHD in human clinics. Further studies are warranted to clarify the synergistic effects between AD-MSCs and a series of drugs and to explore the prophylactic application of AD-MSCs against GVHD.

Conflict of interest

The authors report no potential conflict of interest.

Authors' contributions

Y. N. and A. M. performed the research, analyzed the data and wrote the manuscript; Y. M. performed the research; N. T. analyzed the data, S. O., S. N., H. N., M. M., T. F., K. K. and S. M. wrote the manuscript. H. K. and H. M. supervised the research, analyzed the data and wrote the manuscript.

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Appendix A. Supplementary data

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