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Original Article

Effects of regulatory dendritic cells on graft-versus-host disease and graft-versus-leukemia in mice after allogeneic bone marrow transplantation

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Abstract: We aimed to investigate the effects of regulatory dendritic cells (DCreg) on acute graft-versus-host disease (GVHD) and graft-versus-leukemia (GVL) in leukemia mouse model after allogeneic bone marrow transplantation (allo-BMT). DCreg with donor bone marrow cells and splenocytes were subsequently transplanted into myeloablatively irradiated mice. The mice were divided into the TBI (Total body irradiation), leukemia, allo-BMT (bone marrow and spleen cells from donors were injected into recipients after TBI), allo-BMT with immature DC (imDC), and allo-BMT with DCreg (DCreg) groups. DCreg were cultured for longer time than imDC and stimulated by LPS for 1 d. Survival time in the DCreg and imDC groups was significantly prolonged compared with that of allo-BMT group ($P < 0.05$). Moreover, mild histological changes of GVHD or leukemia were observed in mice from DCreg group, with significantly decreased clinical GVHD scores compared with those in allo-BMT and imDC groups. Serum interferon-γ level was decreased in imDC and DCreg groups compared with that in allo-BMT group with even more significantly reduced in DCreg ($P < 0.01$). Additionally, Serum interleukin-10 level was gradually increased in imDC and DCreg groups ($P < 0.01$) but decreased in allo-BMT group. In conclusion, DCreg cell infusion reduced the incidence and ameliorated the severity of GVHD in allo-BMT mouse model, while preserved the effect of GVL.

Keywords: Regulatory dendritic cells, allograft, transplantation, immunotolerance, antileukemia

Introduction

Allogeneic bone marrow transplantation (allo-BMT) is a therapeutic approach for the treatment of hematological malignancies, including leukemia, lymphoma, and myelodysplastic syndrome. However, graft-versus-host disease (GVHD) and leukemia recurrence are critical factors affecting the survival rate and prognosis after transplantation [1, 2]. Immunosuppressive agents and T cell depletion are currently being used in clinic to prevent GVHD. Nevertheless, the efficacy is limited by severe infections and leukemia recurrence. To alleviate the complications and improve the therapeutic outcome for patients, it is urgent to develop new strategies and methods which can effectively prevent GVHD but also preserve or even enhance GVL effects simultaneously [3-5].

Previous studies reported that regulatory dendritic cells (DCreg), a subgroup of immature DC could negatively regulate immune functions via several low co-stimulatory molecules, such as CD80, CD86, or MHC-II [6, 7]. Additionally, DCreg regulates immune response by influencing inhibitors secretion (such as IL-10) and regulatory T cells (Treg) function [6, 7]. Currently, several studies reported that $CD11c^{low}CD45RB^{high}$DC, a distinct subset of DCreg which express specifically the CD45RB marker and secrete IL-10, have effects on the regulation of negative immune function and are being used for clinical treatment for several diseases [8, 9]. Our previous studies showed that mouse bone marrow-derived $CD11c^{low}CD45RB^{high}$DC had the capacity to resist lipopolysaccharide (LPS) stimulation with difficulty in converting to mature DC. Furthermore, $CD11c^{low}CD45RB^{high}$DC was associated with preservation of immune

tolerance and proliferation potential, and could induce T cell resistance against specific antigens, consistent with the findings of Wakkach *et al.* [10]. Additionally, $CD11c^{low}CD45RB^{high}$DC could inhibit mixed lymphocyte reactions (MLR) and, more importantly, this tolerance could be transferred to other individuals.

In mice, adoptive infusions of $CD11c^{low}CD45RB^{high}$DC induced OVA-specific immunological unresponsiveness in T cells and mediated antigen-specific Tr1 production [6, 11]. Meanwhile, the survival time of mice receiving allo-BMT was increased significantly and the inflammatory response was inhibited after infusion of $CD11c^{low}CD45RB^{high}$DC [11, 12]. According to Chorny *et al.* [13], vasoactive intestinal peptides cultured DCreg not only ameliorated GVHD in mice, but also maintained the GVL effects. Consistent with this, our previous finding also showed that infusion of immature DC (imDC) and gene modified imDC into mice could effectively prevent acute GVHD development [14]. However, whether the persistent tolerance of DCreg could improve the transplant graft tolerance and prevent GVHD still remains unknown. In this study, we assessed the effects of $CD11c^{low}CD45RB^{high}$DC on GVHD and GVL in leukemia mouse model after allo-BMT.

Materials and methods

Materials

RPMI 1640 culture medium, fetal bovine serum, and IMDM culture medium were purchased from Gibco (Shanghai, China). Tris, boric acid, osmic acid, L-glutamine, glutaraldehyde, and HEPES were provided by Sigma (St. Louis, MO, USA). mouse GM-CSF, IL-4, IL-10, and TGF-β_1 were bought from PeproTech (Rocky Hill, NJ, USA). FITC labeled mouse CD11c antibody and FITC-I-A/I-E antibody, PE labeled CD80, CD86, and CD45RB antibodies, and isotype control antibody was purchased from eBioscience (San Diego, CA, USA). Anti-mouse H-$2K^b$-FITC and anti-mouse H-$2K^d$-PE were obtained from BD Biosciences (San Jose, CA, USA). FACS Calibur flow cytometer was offered by BD Biosciences.

Animals

C57BL/6 (H-2^b) mice (SPF grade) were used as the donors, and BALB/c (H-2^d) mice (SPF grade) were used as the recipients. All mice were between 8-12 weeks old and purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. [SCXK (Hu) 2012-0002]. The mice were kept in the animal center (SPF grade) of Xuzhou Medical University. The cages were sterilized by immersing in 1:10000 of Gaolvjing and were changed every day. Food, water, and padding were all sterilized. Erythromycin (250 mg/L) and gentamicin (320 mg/L) were added into the drinking water a week before transplantation. All procedures performed in the experiments were in accordance with animal ethical standards.

SHI-1 cell thawing and passage

A highly invasive monocytic leukemic cell line SHI-1, with specific t (6; 11) (q27; q23) chromosomal abnormality and MLL/AF6 fusion transcript [15], was kindly provided by the Institute of Hematology of Jiangsu Province. The frozen SHI-1 cells were taken from liquid nitrogen containers and put into 37°C water bath to thaw the cells within 1 min. The SHI-1 cell suspension was transferred into a sterilized 15 ml centrifuge tube and washed with basal IMDM culture medium twice. Then, the cells were cultured in culture flasks with 10 ml of 10% IMDM complete culture medium at 37°C with 5% CO_2. When the culture medium became yellow and the cell number increased distinctly, the cell suspension was collected and centrifuged at 800 × g, the supernatant was discarded, 2 ml of IMDM complete culture medium was added, and the cells were passaged (1:3). The third cell generation was used for the following experiments.

Separation of bone marrow cells and spleen cells

C57BL/6 mice were sacrificed by cervical dislocation, and bone marrow was obtained from the thigh and tibia bones under aseptic conditions. The suspension of bone marrow mononuclear cells (MNC) was prepared and the cell density was adjusted to 5×10^7/ml using RPMI culture medium. The spleens from C57BL/6 mice were obtained and used for preparation of a suspension of spleen cells. Lymphocyte separation medium was used to separate MNC, and the cell density was adjusted to 5×10^7/ml. Trypan blue cell staining showed that cell viability was above 95%.

Acquisition and identification of DC

Bone marrow-derived MNC was obtained from C57BL/6 mice under aseptic conditions and seeded into a 6-well culture plate with RPMI 1640 complete culture medium. Different cytokines were added into corresponding groups to culture different DC types. For the imDC induction, we used RPMI 1640 culture medium containing rmGM-CSF (20 ng/mL) and rmIL-4 (10 ng/mL) to culture the cells for 5 days. To induce mature DC, we used RPMI 1640 culture medium containing rmGM-CSF (20 ng/mL) and rmIL-4 (10 ng/mL) to culture the cells for 6 days, and then stimulated the cells by LPS for 24 h to allow them to mature. To induce DCreg, we used RPMI 1640 culture medium containing rmGM-CSF (20 ng/mL), rmIL-10 (20 ng/mL) and transforming growth factor-β_1 (TGF-β_1, 20 ng/mL) to culture the cells for 6 days, and then stimulated the cells by LPS for 24 h to allow them to mature. The DC morphology was observed with optical microscope, cell ultrastructure was observed with electronic microscope, and cell immunophenotype was measured by flow cytometry.

The ability of DCs in stimulating T lymphocyte proliferation was measured with a CCK-8 method, and *in vitro* chemotactic cell capabilities were measured with a transwell system. In brief, DCs were collected from different groups followed by addition of mitomycin (25 μg/ml) and incubated for 45 min at 37°C, 5% CO_2. After washing two times, DCs (1×10^5/well) were co-cultured with T lymphocytes in 1640 medium in 96-well plate with triplicates for each group for 120 h at 37°C, 5% CO_2. At the last 16 h, 10 ml CCK-8 reagent was added into each well and incubated for 4 h followed by measuring the absorbance at 450 nm using a microplate reader. Only T lymphocytes were added for the control group. Proliferation rate was calculated as the OD value of experimental group divided by the OD value from the control group × 100%.

Meanwhile, DC cells migration was measured by Transwell system. Briefly, cultured DCs were collected from each group and added into the upper layer of the transwell (150 μl at 5×10^5/ml). At the meantime, 450 μl 10% FBS 1640 medium was added into the lower layer. Then the transwell system was placed at an incubator (37°C, 5% CO_2) for 72 h followed by collecting the cells from lower layer and counted the number of cells. The migration rate was calculated as number of the cells migrated into the lower layer divided by the original number of cells × 100%.

Establishing the mouse leukemia model and grouping

Total body irradiation of ^{60}Co γ-ray (total dose: 7.5 Gy, dose rate: 0.66 Gy/min, irradiation time: 682 s) was performed for the BABL/c recipient mice on the day of transplantation for 4 hours, and then bone marrow transplantation was performed. The mice were randomly divided into 5 groups (TBI, leukemia, allo-BMT, imDC, and DCreg) using a random number table with 15 mice in each group (not including the mice that were sacrificed for chimera analysis or cytokine measurement). For the mice in the TBI group, 0.3 ml of sterilized normal saline was injected through the caudal vein; for the mice in the leukemia group, syngeneic bone marrow cells (5×10^6), spleen cells (5×10^5), and SHI-1 cells (1.2×10^7) were injected through the caudal vein into each mouse after TBI; for the mice in the allo-BMT group, bone marrow cells (5×10^6) and spleen cells (5×10^5) from the donors were injected through the caudal vein into each recipient after TBI; for the mice in the imDC group, bone marrow cells (5×10^6) and spleen cells (5×10^5) from the donors, SHI-1 cells (1.2×10^7), and imDC (5×10^6) were injected through the caudal vein into each recipient after TBI; and for the mice in the DCreg group, bone marrow cells (5×10^6) and spleen cells (5×10^5) from the donors, SHI-1 cells (1.2×10^7), and DCreg (5×10^6) were injected through the caudal vein into each recipient after TBI.

Observation index after transplantation

1) Mice survival: recipient food intake and mental status were observed every day after transplantation; mice survival time was also recorded to calculate survival rate. 2) Hematopoiesis reconstruction: peripheral blood cell count and blood smear examinations were performed on days 7, 14, and 21 after transplantation to evaluate hematopoiesis reconstruction. A white blood cell count of $< 0.5 \times 10^9$/L represented transplantation failure, whereas acount of $> 1.0 \times 10^9$/L represented hematopoietic recovery. 3) Diagnosis of acute GVHD: WBC $> 1 \times 10^9$/L, and with symptoms including tireness,

Table 1. Analysis of GVHD severity by a scoring system

Criteria	Grade 0	Grade 1	Grade 2
Weight loss	~10%	> 10% to < 25%	> 25%
Posture	Normal	Hunching noted only at rest	Severe hunching impairs movement
Activity	Normal	Mild to moderately decreased	Stationary unless stimulated
Fur texture	Normal	Mild to moderate ruffling	Severe ruffling poor grooming
Skin integrity	Normal	Scaling of pawskail denuded	Obvious areas of skin

poor appetite, poor mobility, reduced body weight, roachback, ruffled fur, and diarrhea. GVHD severity was evaluated by a scoring system (**Table 1**) reported by Cooke *et al.* [16], and scores were evaluated from 5 aspects including weight loss, posture, activity, fur texture, and skin integrity with the highest score of 10. 4) Development of leukemia: WBC > 20 × 10^9/L, hepatomegaly, splenomegaly, and a large amount of leukemia cells in peripheral blood; and histopathology showed a large number of acute monocytic leukemia cell infiltration in liver, spleen, lung, and other tissues. 5) Transplantation-related death: deaths caused by bleeding or infection induced by hematopoietic inhibition within 2 weeks after transplantation (except deaths caused by leukemia or GVHD) were defined as transplantation-related death.

Pathological examination

The liver, small intestine, lung, and spleen were collected from the moribund recipients with GVHD or recipients sacrificed on +30 d in each group. The tissues were then sliced, fixed with 100 g/L of formaldehyde, paraffin-embedded, HE stained, and observed with optical microscope to detect pathological GVHD changes and leukemia cell infiltration. A pathological scoring system developed by Blazar and Kaplan *et al.* [17, 18] was used to evaluate the pathological changes of the target organs in mice with acute GVHD.

Detection of bone marrow cell chimeras

Bone marrow cells of 1 × 10^6 were randomly selected from recipients at +18 d or from the ones that survived more than 30 d. After cells were treated with FITC-anti-H-2K^b and PE-anti-H-2K^d, flow cytometer was used to evaluate the percentage of lymphocytes that expressed donor-derived H-2K^b.

Detection MLL-AF6 fusion gene with nested RT-PCR

Total RNA was extracted from liver and spleen tissues from the mice using TRIzol one-step method, then reverse RNA transcription was performed using M-MLV (2 μg) reverse transcriptase to obtain cDNA. PCR was performed according to the methods reported in previous studies [19]. Agarose gel electrophoresis (1.5%) was undertaken to analyze the PCR products. A gel imaging system was used to observe and photograph the images under ultraviolet.

ELISA detection of the peripheral blood cytokine

Peripheral blood from the recipient mice (1.5 mL) was collected via the fossa orbitalis vein on days 0, 5, 10, 15, 20, and 30 post-BMT and centrifuged to obtain serum, which was preserved at -80°C until use. The levels of IFN-γ and IL-10 in peripheral blood serum were detected with ELISA kits according to the instructions by the manufacturer.

Statistical analysis

SPSS 16.0 software (SPSS Inc, Chicago, IL, USA) was used for statistical analysis. Data were described as means ± standard divisions (SD), and comparison of the differences between different groups was assessed by one-way analysis of variance (one-way ANOVA). *q*-test was used for the pairwise comparison, and the Kaplan-Meier test was applied for survival rate comparison. $P < 0.05$ was considered statistically significant.

Results

Mature DC and DCreg enhanced proliferation of allogeneic lymphocytes

DC morphology and immunophenotype after *in vitro* induction were published in our previous

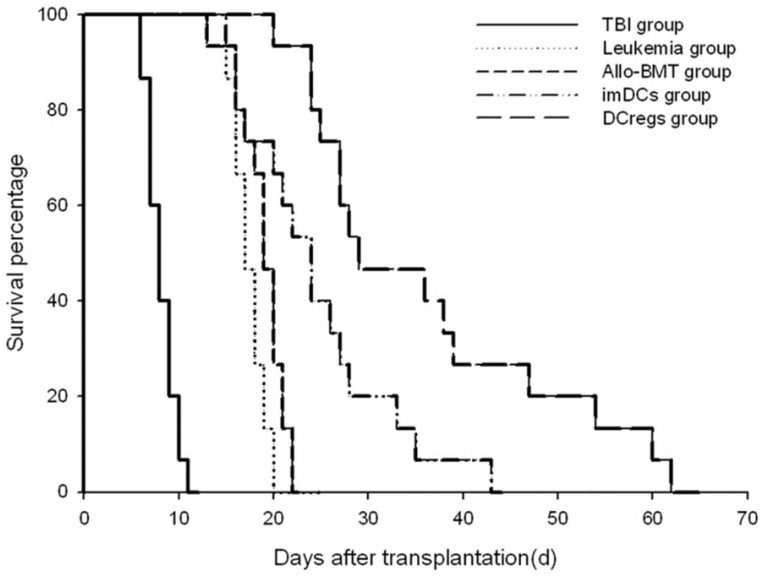

Figure 1. Post-BMT survival curves of mice in different. transplantation groups. imDC group versus allo-BMT group, $P < 0.05$; DCreg group versus imDC and allo-BMT group, $P < 0.05$.

study [12]. The regulatory DC subgroup was featured as $CD11c^{low}CD45RB^{high}$ DCreg. MLR results showed that imDC only mildly stimulated T lymphocyte proliferation (cell proliferation rate: 270.76 ± 12.25%), while mature DC effectively stimulated T lymphocyte proliferation (cell proliferation rate: 348.94 ± 14.45%, $P < 0.01$). Conversely, the T lymphocyte proliferation was significantly lower after stimulation with DCreg (cell proliferation rate: 237.05 ± 7.60%) compared with imDC ($P < 0.01$) and mature DC stimulation ($P < 0.01$).

The mature DC, imDC and DCreg possessed strong capability of migration

To understand the DC capability of migration, we investigated the migration of three DC groups by chemotactic assay. The migration rate of mature DC, imDC, and DCreg were 8.13 ± 0.97%, 15.94 ± 0.87% and 26.81 ± 0.43%, respectively, suggesting that the migration capability of DCreg was the strongest in the three DC group.

The imDC and DCreg potently prolonged mice survival after transplantation

In order to investigate the effect of different DC on mice survival, mice survival time was compared. In the TBI group the first death was found on +6 d, and then the mice gradually died (average survival time (AST) of 8.13 ± 1.51 d). For mice in leukemia group, the first death was found on +9 d, and all mice died within +24 d, with an AST of 17.27 ± 3.41 d. Using Blood smear and pathological organ examinations, we found that leukemia cells were infiltrated in the leukemia group mice. The first death in the allo-BMT group was found on +10 d, and all mice died within 25 d, with an AST of 18.93 ± 3.33 d. However, in the imDC group, there were still 20% mice alive on 30 d, with an AST of 24.47 ± 7.91 d, which was significantly longer than that in the allo-BMT group ($P < 0.05$). Surprisingly, in the DCreg-treated mice, the first death was found on +19 d, with 46.7% mice alive on +30 d, and 13.3% alive on +60 d, with an AST of 36.00 ± 13.76 d. Taken together, these results indicated that DCreg significantly prolonged the survival time in allo-BMT model mice ($P < 0.05$) (**Figure 1**).

DCreg infusion significantly prevented the development of GVHD

To identify the effect of Dcreg on GVHD, we evaluated clinical acute GVHD phenotypes including tiredness, poor mobility, reduced body weight, roachback, ruffled fur, hair loss, diarrhea, and even bloody stool in allo-BMT, imDC, and DCreg groups. The most severe symptoms were found in allo-BMT group, while the least severe symptoms were found in DCreg group. The GVHD clinical score was 7.60 ± 0.99, 4.93 ± 0.96, and 3.8 ± 0.68 in the allo-BMT, imDC, and DCreg groups, respectively. This result showed that DCreg infusion significantly prevented the development of GVHD compared with other two groups ($P < 0.05$).

The imDC and DCreg reduced the pathological GVHD score compared with allo-BMT

In order to assess the effect of imDC, DCreg, and allo-BMT on GVHD score, the pathological changes of liver, small intestine, and lung were investigated in accordance with the clinical

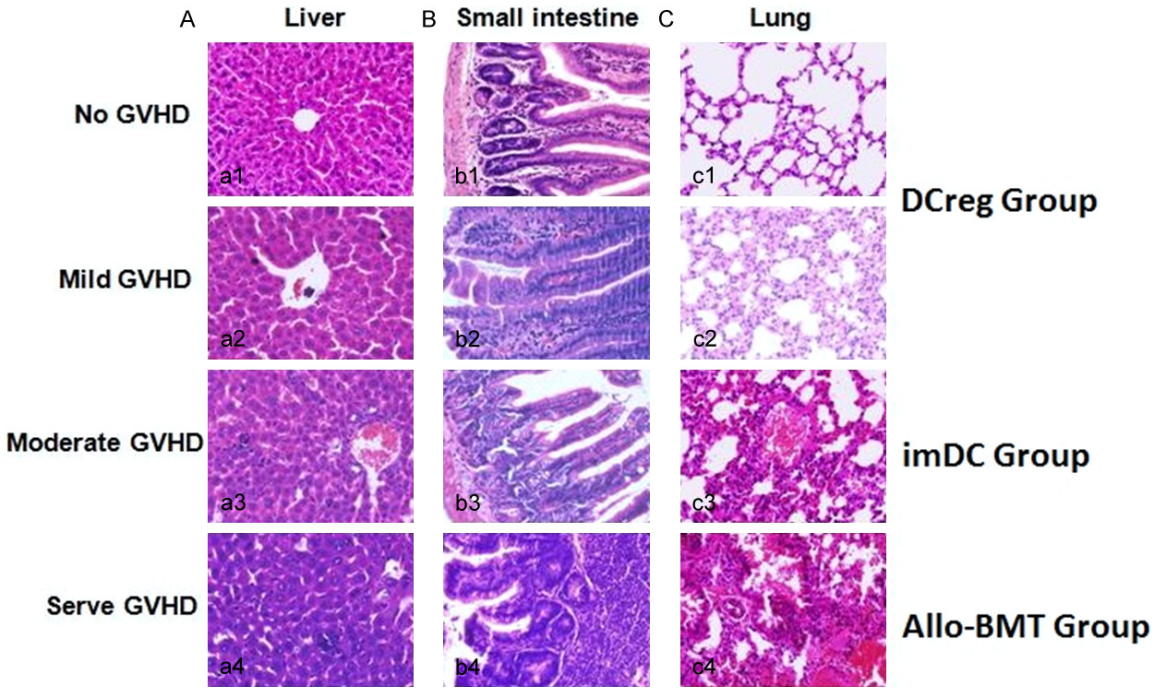

Figure 2. Histopathological changes of liver, small intestine, and lung in mice (HE, × 400). A: Liver; B: Small intestine; C: Lung; a1, b1, c1: Liver, small intestine, and lung in mice which showed no evidence of GVHD; a2, b2, c2: Liver, small intestine, and lung in mice with mild GVHD; a3, b3, c3: Liver, small intestine, and lung in mice with moderate GVHD; a4, b4, c4: Liver, small intestine, and lung in mice with severe GVHD. A pathological scoring system developed by Blazar and Kaplan *et al.* [17, 18] was used as the criteria for mild, moderate and severe GVHD.

Table 2. GVHD pathologic scoring of liver, small intestine, and lung in mice after transplantation (n = 3, mean ± SD)

Groups	Liver	Small intestine	Lung
Allo-BMT	6.33 ± 0.58	7.00 ± 1.00	6.67 ± 0.58
imDC	4.67 ± 0.58*	4.67 ± 0.58*	2.33 ± 1.16*
DCreg	3.00 ± 1.00*,#	2.67 ± 0.58*,#	2.00 ± 1.00*,#

*$P < 0.05$ *vs.* allo-BMT group; #$P < 0.05$ *vs.* imDC group.

phenotypes in each group after transplantation. In allo-BMT and imDC groups, there were obvious derangement of liver cells, focal necrosis, lymphocyte infiltration in the periportal area, destruction and collapse of the small bile duct in liver tissues, intestinal epithelial cells necrosis and partial defect excalation with significant inflammatory cells infiltration in the underlayer, intestinal villous atrophy degeneration, and even severe structural damage to the small intestine. Furthermore, inflammatory cells infiltration and lung structure damages were observed in 25% pulmonary vessels. Meanwhile, inflammatory cells were infiltrated into 2-3 parenchymal cell layers. However, in DCreg group, we found only mild or even no GVHD presentations in the target organs (**Figure 2**). The pathological GVHD score was significantly lower in imDC and DCreg groups than that in allo-BMT group ($P < 0.05$). Also, the pathological GVHD score was also significantly lower in DCreg group than that in imDC group ($P < 0.05$) (**Table 2**).

The incidence of leukemia in allo-BMT, imDC and DCreg groups was similar

To study the effect of allo-BMT, imDC and DCreg on the occurrence of leukemia, mice in each group were observed. Leukemia cells infiltration in eyes was found on +18 d in mice from leukemia group, and all mice in this group died of leukemia within 24 d. Significantly enlarged liver, spleen and leukemia cell infiltrated diffused lesions were detected (**Figure 3A**). In histopathological examinations, higher numbers of leukemia cells were infiltrated in the liver and spleen (**Figure 3B**). The WBC count in the peripheral blood was 19~34 × 10^9/L, and 28%-

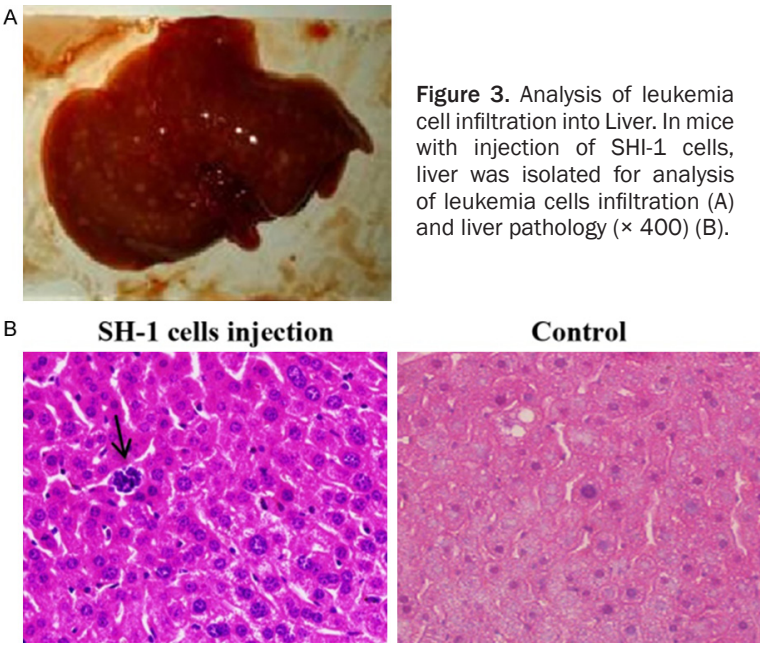

Figure 3. Analysis of leukemia cell infiltration into Liver. In mice with injection of SHI-1 cells, liver was isolated for analysis of leukemia cells infiltration (A) and liver pathology (× 400) (B).

13.3% in allo-BMT, imDC, and DCreg groups, respectively, with no statistically significant among groups ($P > 0.05$) (**Table 3**).

MLL/AF6 expression was observed in liver and spleen tissues of leukemia group

To evaluate the mRNA expression of MLL/AF6 fusion gene, liver and spleen tissues were collected from moribund leukemia mice and nested PCR was performed. The results showed that MLL/AF6 fusion gene was detected in liver and spleen tissues except control, and the earliest MLL/AF6 fusion gene was detected on day 20 (**Figure 4**).

Table 3. Incidence of Leukemia

Groups	Leukemia incidence (%)
Leukemia	100
Allo-BMT	13.3 (2/15)
imDC	20 (3/15)
DCreg	13.3 (2/15)

DCreg group vs. imDC and allo-BMT group, $P > 0.05$.

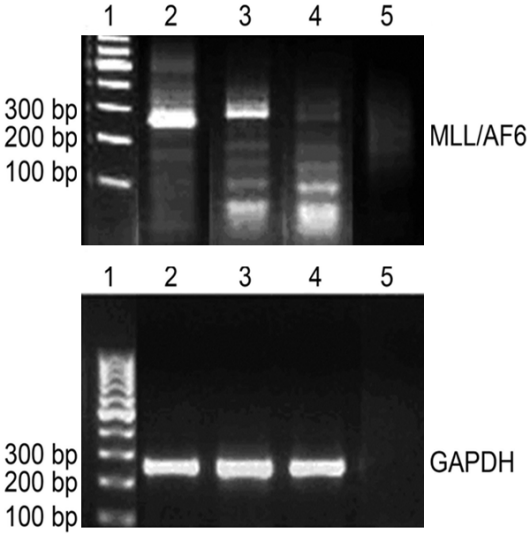

Figure 4. RT-PCR analysis of MLL/AF6 fusion gene. 1: 100 bp Marker; 2: Mouse liver; 3: Mouse spleen; 4: Healthy mouse liver; 5: ddH_2O, blank control.

43% white blood cells were leukemia cells. Leukemia incidence was 13.3%, 20%, and

Complete bone marrow chimeras pointed to successful allo-BMT

Bone marrow cells were harvested from mice that lived more than 30 d in imDC and DCreg groups, and flow cytometer was used to detect bone marrow cell chimeras. The results showed that 95-100% of the cells were positive for $H\text{-}2K^b$, which suggested complete cell chimerism and demonstrated that the allo-BMT performed in the present study was successful (**Figure 5**). In addition, significantly increased numbers of white blood cells were observed on d 14 ($1.54 \pm 0.50 \times 10^9$/L) in mice than those on d 7 ($0.34 \pm 0.27 \times 10^9$/L) after allo-BMT ($P < 0.05$), suggesting hematopoiesis recovery after transplantation.

Serum interleukin-10 level was gradually increased in imDC and DCreg groups but decreased in allo-BMT group

The cytokine changes in mice after transplantation were shown in **Figure 6**. Plasma IFN-γ level was increased gradually in allo-BMT group and peaked on +10 d, followed by a decrease gradually. In imDC and DCreg groups, the plasma IFN-γ level was decreased gradually and reached a lowest level on +10 d, followed by an increase gradually. The decreased plasma IFN-γ level was more pronounced in DCreg group than that in imDC group ($P < 0.01$). The IL-10 level was decreased gradually in allo-BMT group but increased gradually in imDC and

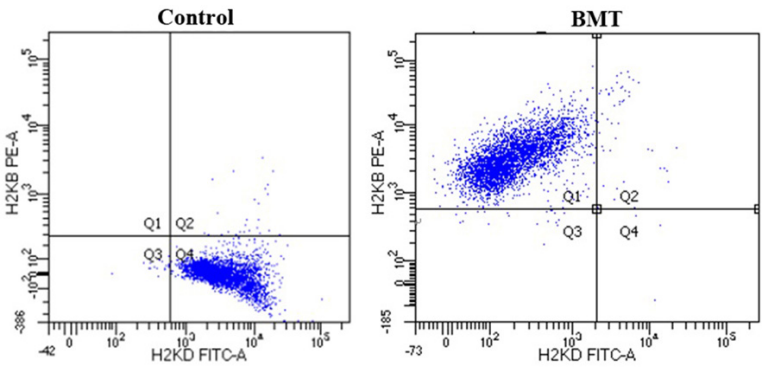

Figure 5. Chimeric rate of recipient mice who survived 30 days after transplantation in imDC and DCreg group. Flow cytometric analysis of bone marrow cells from mice surviving for > 30 d revealed that 96.8% of cells were H-2 d positive. Bone marrow cells were dualfluorescence stained with FITC-labelled anti-H-2 Kb monoclonal antibody and PE-labelled anti-H-2 Kd monoclonal antibody. FITC, fluorescein isothiocyanate; PE, phycoerythrin; Mice without BMT was served as a negative control.

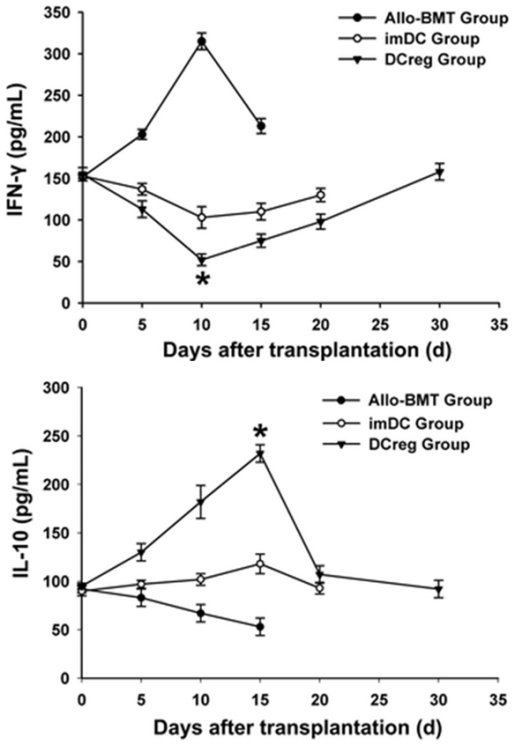

Figure 6. Post-BMT plasma interferon-γ and IL-10 levels in different transplantation groups. Peripheral blood from the recipient mice was collected at indicated time points post-BMT and centrifuged to obtain serum for analysis of the levels of IFN-γ and IL-10 by ELISA. *$P < 0.01$ vs. group allo-BMT and imDC.

DCreg groups, reaching a peak on +15 d, and then decreased gradually. The increased IL-10 level was more pronounced in DCreg group than that in imDC group ($P < 0.01$).

Discussion

The therapeutic efficacy of allo-HSCT for high-risk hematologic malignancies relies on the GVL effects to eliminate residual leukemic cells. However, GVL effects are often intertwined with GVHD, a life-threatening complication post-transplantation. GVHD and GVL are two independent and interdependent phenomenons that are overlapped but can also be separated from each other [20, 21]. Identifying the equilibrium point between GVHD and GVL's effects could keep GVHD at an acceptable degree while maintaining GVL's effects. Recent immunological findings show that acquiring graft-specific immune tolerance could be an optimal approach to resolve this problem, and inducing immune tolerance with immune competent cells has become a research hotspot [22-24].

DC is known to be the most powerful APC in the body and the only one that could activate naive T lymphocytes. It could not only initiate immune tolerance but also regulate immune response. As DCreg plays an important role in initiating and maintaining immune tolerance, it has been gained increasing attention in the immune tolerance field [25-27]. During hematopoietic stem cell differentiation into DC, different cytokines could substantially influence the differentiation. In our previous studies [12], mice bone marrow cells were stimulated with cytokines including GM-CSF, IL-10, and TGF-β_1, followed by differentiation into DCreg. The high CD45RB and low CD11c, CD80, CD86, and MHC-II (IA/IE) are expressed in DCreg cells and are defined as immature DC cells. This novel DC subgroup, namely $CD11c^{low}CD45RB^{high}$DC, has high ability on chemotaxis migration, low ability on stimulating allogeneic T lymphocyte proliferation, and shows immuno-hypo-responsiveness in mixed lymphocyte reactions, offering a new method for inducing graft tolerance. In the present study, leukemia allo-BMT mouse model was induced to investigate the effects of infusing $CD11c^{low}CD45RB^{high}$DC on GVHD and GVL's effects in mice after bone marrow transplantation.

In our previous studies, SHI-1, a human monocytic leukemia cell line with high infiltration and invasion, was used to induce humanized leukemia mouse model [15, 28, 29]. In this study, more SHI-1 cells were infused and leukemia incidence increased accordingly. All mice in leukemia group developed leukemia after infusion of SHI-1 cells in the present study. Extensive leukemia cell infiltration was found in the liver, spleen, and eyes of the leukemic mice with all mice died of monocytic leukemia. Spleen tissues from moribund mice were collected to detect the MLL/AF6 fusion gene by nested RT-PCR, and the first transcripts were identified on day 20 after transplantation, in accordance with previous findings [30, 31]. We compared the anti-leukemia effects among imDC, DCreg, and allo-BMT groups, and found that only a small proportion of mice that received allogeneic transplantation developed leukemia. The survival rate and AST were significantly higher in imDC and DCreg groups than those in allo-BMT group, which were more pronounced in DCreg group than those in imDC group. Also, no leukemia-related complications were found in recipients that lived for a long time. These findings showed that infusion of imDC or DCreg into allo-BMT mice could preserve GVL's effects to a certain degree as demonstrated by significantly higher GVL effects in DCreg group than imDC group. With regard to GVHD development, GVHD severity was significantly ameliorated in imDC group than allo-BMT group, while the severity was even lower in the DCreg group, in which the survival time was significantly longer. These findings suggested that infusion of imDC could reduce GVHD severity, but these effects were limited by several factors. After infusion, antigen stimulation increased the potential of mature imDC. Costimulatory molecule expressions were up-regulated with increased capability of presenting antigens and this reduced immune tolerance gradually decreased the GVHD-alleviating effects. However, DCreg could preserve imDC features and effectively decreased GVHD severity and increased the survival rate.

GVHD is an immune response induced by the activated donor T cells recognizing the recipients' incompatible antigens. Recent studies suggested that GVHD is caused by a "cytokine storm" [32, 33]. Most recent studies suggest that Th1 cells could release pro-inflammatory cytokines including IL-2, IFN-γ, and TNF, which could aggravate GVHD. Th2 cells could release Th2-type cytokines including IL-4, IL-5, and IL-10 to antagonize the pro-inflammatory effects of Th1 cells and prevent GVHD [34, 35]. In the present study, we found that both imDC and DCreg reduced IFN-γ levels and increased IL-10 release, further confirming that imDC and DCreg could induce the donor $CD4^+$ T cells which are activated by the recipients' antigens to develop into immune tolerance cells. DCreg could also produce IL-10, promote the $CD4^+$ T cells to be differentiated into Treg cells (which could release IL-10), and induce the naïve T cells to develop into $CD4^+CD25^+Foxp3^+$ Treg cells [36, 37] and thus increase mice survival time after transplantation. However, DCreg's GVHD-alleviating effects are also limited, and most mice died of GVHD. We speculated that the followings might be involved: 1) after infusion, the *in vitro*-induced DCreg could be stimulated by exogenous antigens and inflammations and mature gradually, thus finally losing the ability to induce immune tolerance; and 2) APC in the recipients could also play a role in GVHD development. The APC from donors was selectively intervened, while APC activation in the recipients could not be sufficiently inhibited. Therefore, in future studies, we plan to optimize the experimental conditions, perform multiple infusions at different time points as well as increase the infusion dose gradually. Additionally, genetically modified DCreg could also be used to induce stable immune tolerance.

In summary, donor-derived DCreg infusion could effectively reduce GVHD incidence and severity in leukemia allo-BMT mouse model, while the GVL effects could be preserved significantly. These findings provide an effective alternative approach for applying DCreg during allo-HSCT.

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Disclosure of conflict of interest

None.

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