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Treating donor mice with rhIL-11 and rhG-CSF promotes transplant-tolerance and preserves the effects of GVL after allogeneic bone marrow transplantation

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Abstract

We investigated the impact of recombinant human interleukin-11 (rhIL-11) and granulocyte colony-stimulating factor (rhG-CSF) on bone marrow transplantation. Treatments for leukemic mice were (A) no treatment, (B) mock transplantation, and transplantation from the following donors: (C1) syngeneic, (C) controls, (D) rhG-CSF treated, (E) rhIL-11 treated, and (F) rhIL-11 and rhG-CSF treated. Graft-versus-host disease incidences were 100%, 60%, 78%, and 30% in C, D, E, and F, respectively. The 30 d leukemia-free survival improved significantly in F (70%) compared to C (0%), D (40%), and E (20%) ($P < 0.01$). Thus, treating donor mice with rhIL-11 and rhG-CSF promoted transplant-tolerance and recipient survival.

Keywords: Combination; Graft-versus-host disease; Graft-versus-leukemia effect; Recombinant human granulocyte colony-stimulating factor; Recombinant human interleukin-11; T cell

1. Introduction

Currently, allogeneic hematopoietic stem cell transplantation (Allo-HSCT) provides the highest probability of a cure for many hematological malignancies [1–3]. However, graft-versus-host disease (GVHD), comprising multiorgan damage and immunological deficiency, remains one of the most common complications following Allo-HSCT [4]. The use of peripheral blood stem cell grafts (PBSC) mobilized with recombinant human granulocyte colony-stimulating factor (rhG-CSF) has led to rapid immune and hematopoietic reconstitution, and improved leukemia eradication [5,6]. However, PBSCT was associated with a significant increase in the development of grades 3–4 acute GVHD [7]. Consequently, researchers have been investigating alternate approaches that might alleviate acute GVHD without impairing the graft-versus-leukemia (GVL) effects [5,8–11]. Recently, attention has focused on the potentials of different cytokines for PBSC mobilization.

The regulatory effects of rhG-CSF have been attributed to its effects on T cells and other accessory immune cells in the peripheral blood stem cell grafts (PBSCs) [9–12], although the mechanisms are not completely understood. In addition, several other cytokines [13,14] have been reported to mobilize stem cells, including recombinant human granulocyte-macrophage colony-stimulating factor (rhGM-CSF), stem cell factor (SCF), recombinant human interleukin-11 (rhIL-11), and rhIL-6. Experimental data showed that rhIL-11 may mobilize stem cells and promote T cell polarization; thus it may prevent acute GVHD after Allo-HSCT [13,15,16]. Researchers in our institute found that donors treated with rhIL-11, or with a combination treatment of rhIL-11 and rhG-CSF, provided superior transplants that resulted in reduced acute GVHD. This indicated that rhIL-11 and rhG-CSF may synergistically induce immune-tolerance through their effects on T lymphocyte proliferation and function; this may be related to the signifi-

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cant reduction observed in GVHD [17,18]. Thus, combining different cytokines to mobilize hematopoietic stem cells may represent a novel approach for regulating the immunological properties of a graft and improving the outcome of allogeneic transplantation. However, it remains unclear whether (and how) GVL might be affected by the combination of rhIL-11 and rhG-CSF. Therefore, we investigated whether treating donors with a combination of rhIL-11 and rhG-CSF would protect from GVHD and maintain GVL effects in leukemic mice.

2. Materials and methods

2.1. Animals and allogeneic bone marrow transplantation

Six- to eight-week-old BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were obtained from the Animal Facility, Peking University Health Science Center. The mice were bred and maintained in a specific pathogen-free facility at the Peking University People's Hospital. Recipients (BALB/c mice) were given unsterilized food and water ad libitum. Gentamicin (32×10^4 U l^{-1}) and Erythromycin (250 ng l^{-1}) were added to the water 7 days prior to the BMT and thereafter in order to facilitate gastrointestinal preparations. The Institutional Review Board in the Institute of Hematology of Peking University approved this study.

All recipients (BALB/c mice) were injected with 1.5×10^6 EL9611 leukemia cells 4 days prior to transplantation. EL9611 is a BALB/c mouse transplantable erythroblastic leukemia induced by 7,12-dimethylbenzanthracene. Donor mice (C57BL/6, $n = 11$ in each group) were injected with optimal doses of rhIL-11 (250 μg kg^{-1} d^{-1}) or rhG-CSF (250 μg kg^{-1} d^{-1}) alone or in combination for five consecutive days. Control donors received the same vol/wt of 0.9% sodium chloride. The last dose was given 2 h before donor mice were killed by cervical dislocation. After the erythrocytes were removed, single donor bone marrow cells (BMCs) and splenic cells (SCs) were resuspended in culture medium (RPMI1640 with 10% bovine serum albumin (BSA) and 1% penicillin/streptomycin) and adjusted to suitable cell concentrations. Recipient mice were irradiated (8.0 Gy total body irradiation), rested for 2 h, and were injected with donor BMCs (1×10^7) and SCs (4×10^7). Recipients were randomly assigned as follows: group A ($n = 11$), no treatment after the injection of EL9611; group B ($n = 11$), mock transplantation; the remaining mice were given transplants (BMCs and SCs) from the following donors: group C1 ($n = 10$), syngeneic donors; group C ($n = 11$), control donors; group D ($n = 11$), donors treated with rhG-CSF alone; group E ($n = 11$), donors treated with rhIL-11 alone; group F ($n = 11$), donors treated with rhIL-11 and rhG-CSF in combination.

2.2. Assessment of engraftment and phenotype of donor grafts

Engraftment was assessed by examining the percentage of H-$2D^b$ (donor) cells in the bone marrow of recipients by fluorescence-activated cell sorter (FACS) analysis with a FACScalibur (Becton-Dickinson) at +14 d and +30 d after transplantation. In addition, cell compositions of the donor spleen grafts were investigated by labeling with the following antibodies: 145-2C11 (CD3e-FITC), 145-2C11 (CD3e-PE-CY5), GK-1.5 (CD4-PE), and 53-6.7 (CD8a-APC) (eBioscience, USA). Briefly, 2×10^6 splenic cells were incubated with the appropriate antibody combination for 15–20 min. Erythrocytes were lysed in lysing solution (Becton-Dickinson) for 10 min. After washing in PBS with 1% BSA and 0.1% sodium azide (NaN_3) (PBS/BSA), cells were resuspended in PBS/BSA buffer and analyzed by FACS. A minimum of 20,000 nucleated cell-gated events were acquired and analyzed with cellQuest software (Becton-Dickinson). T cell subgroups were identified by antibodies and expressed as a percent of positive cells within the nucleated cell population.

2.3. Assessment of GVHD, GVL, and graft rejection [19,20]

All animals were examined daily and peripheral blood counts were performed every 3 days from day 7 to either full recovery of hematopoiesis or death. The degree of systemic GVHD was assessed by a scoring system that summed changes in five clinical parameters: weight loss, posture (hunching), activity, fur texture, and skin integrity. Death caused by GVHD was defined as death with recovery of hematopoiesis (leukocytes $>1 \times 10^9$ l^{-1}) and clinical signs of GVHD. Death caused by leukemic relapse was defined as death with macroscopic evidence of a tumor, for example, splenomegalia and megalohepatia with infiltration of leukemic cells. Death caused by graft failure/graft rejection was defined as death between day 6 and 30 after transplantation with leukocytopenia (leukocytes $<0.5 \times 10^9$ l^{-1}).

T-cell function was assessed by the proliferation of T cells in response to stimulation by phyohemaglutinin (PHA). On day 10 after BMT the recipient splenic cells were separated by Ficoll-Hypaque centrifugation. Cells from the interphase (mononuclear splenic cells) were isolated, washed twice, resuspended in culture medium, and adjusted to a cell concentration of 1×10^6 ml^{-1}. These cells were plated in 96-well flat bottom plates at a concentration of 5×10^5 cells/well with an equal number of irradiated cells from the donor C57BL/6 mice. At 68 h, cultures were pulsed with 10 μl of a stock solution (5 mg ml^{-1}) of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT; Sigma, St. Louis, MO). Cells were incubated at 37 °C for 4 h. Next, 10% sodium dodecyl sulfate (SDS) (Sigma) was added to all wells and mixed thoroughly to dissolve the dark blue MTT crystals. Cells were stored at 37 °C overnight to ensure that all the crystals were dissolved. Then the optical density (OD) values were read on a Microllisa reader, using a test wavelength of 570 nm. The stimulation index (SI) was calculated for each individual experiment as follows:

$$\mathrm{SI} = \frac{\mathrm{OD(T\ cell\ responders + PHA)}}{\mathrm{OD(T\ cell\ responders)}}$$

T cell cytotoxicity was examined using the EL9611 cell line as targets, and effector-target ratios ranging from 40:1 to 10:1. We used commercial cytotoxicity assays, based on lactate dehydrogenate (LDH) detection, according to the manufacturer's instructions (Cytotox 96, Promega, Madison, WI).

T cell stimulation was analyzed by incubation with phorbol myristate acetate (PMA). The recipient splenic cells were adjusted to a concentration of 1×10^6 ml^{-1} with culture medium in the presence of 50 ng ml^{-1} PMA and cultured for 48 h at 37 °C in a 5% CO_2 incubator. Culture supernatants were then harvested and cryopreserved at −30 °C until IL-4 and IFN-gamma production were determined. Cytokine production (IL-4 and IFN-gamma) was measured with a sandwich ELISA kit according to the manufacturer's recommendations. (Jing Mei, China).

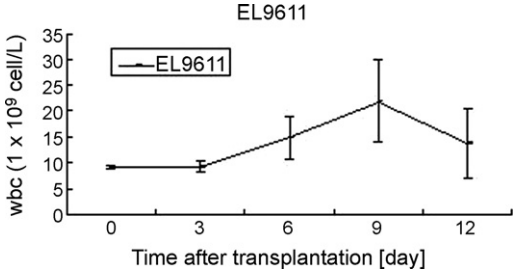

Fig. 1. The kinetics of white blood cell reconstitution in recipients after BMT in each treatment group.

2.4. Statistical analysis

Biological data were summarized with mean ± standard deviation (S.D.). Survival data were plotted and analyzed according to the method of Kaplan–Meier. The experimental groups were compared using the log-rank test. The calculations were carried out with "SPSS" statistical software. Statistical comparisons were performed using the *t*-test for two samples or ANOVA for more than two samples; $P < 0.05$ was considered statistically significant.

3. Results

3.1. Engraftments and graft cell compositions

Complete donor engraftment was determined by the percent of H-2D^b cells in recipient bone marrow. Percents at +10 d and +30 d were: group C, 94.31 ± 4.77% and 94.31 ± 4.77%; group D, 95.55 ± 5.79% and 94.94 ± 3.03%; group E, 95.12 ± 6.87% and 95.91 ± 7.84%, group F, 94.37 ± 7.51% and 95.77 ± 8.51%, respectively. No relevant differences were detected in hematopoietic reconstitution kinetics in the four groups of animals that received allografts from donors treated with 0.9% sodium chloride, rhG-CSF, rhIL-11, or rhG-CSF and rhIL-11 in combination ($P \pm > 0.05$), indicating that complete donor engraftment were obtained in the above four groups.

In group A mice received EL9611 leukemic cells without transplantation. In these mice, the white blood cells continuously increased until death; maximum white blood cell counts (WBCs) ranged from 18 to 43 ± 10^9 l^{-1} (Fig. 1). There were no significant differences in the kinetics of WBC for recipient groups B, C1, C2, D, E, and F (data not shown).

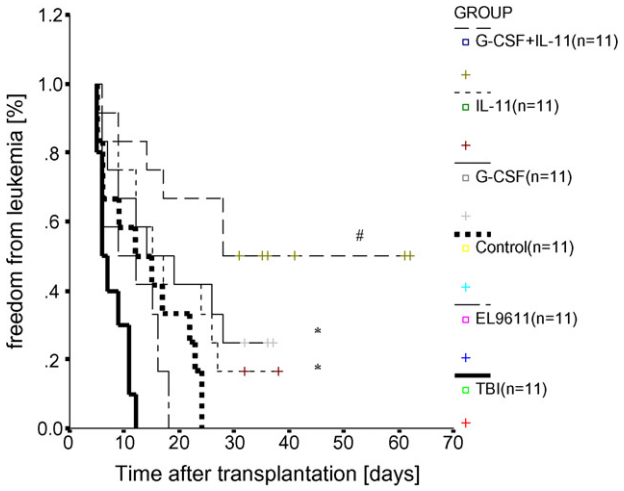

Fig. 2. Kaplan–Meier survival plot of recipients in each treatment group. The combination of rhIL-11 and rhG-CSF treatment to donor significantly improve leukemia-free survival after transplantation. *$P < 0.01$ compared with the control mice; $^{\#}P < 0.01$ compared with mice in rhG-CSF and rhIL-11 group.

As shown in Table 1, the percent of lymphocytes and T cell subgroups decreased significantly in grafts from donors treated with rhG-CSF and with the combination of rhIL-11 and rhG-CSF compared to controls ($P < 0.05$). Furthermore, the percent of lymphocytes and CD3$^+$, CD4$^+$, and CD8$^+$ T cells were significantly lower in grafts from donors treated with the combination of rhIL-11 and rhG-CSF compared to other groups ($P < 0.05$). The ratios of CD4$^+$ cells/CD8$^+$ cells were also significantly lower in the grafts from donors treated with the combination of rhIL-11 and rhG-CSF (1.52 ± 0.47) compared to those treated with rhIL-11 (2.01 ± 0.75), rhG-CSF (2.04 ± 0.54), or untreated controls (1.82 ± 0.33) ($P < 0.05$).

3.2. Combined rhIL-11 and rhG-CSF treatment effects on survival

As shown in Fig. 2 and Table 2, the mean survival time for untreated animals (group A) was 10.7 ± 1.02 d. In addition, pathological analyses revealed infiltration of leukemic cells in both the spleen and liver, indicating that the mice died of leukemia. The mice in group B survived for 3–9 d, and the majority died of hematopoietic failure. The mice in group C1 survived for 8–16 d, and the majority died of leukemia. The

Table 1
Composition of lymphocytes and T cells subsets in the grafts from donors treated with cytokines in combination or alone ($\chi \pm s$)

	Number	Lmphocytes	CD3$^+$ cells	CD4$^+$ cells	CD8$^+$ cells
Controls	11	74.44 ± 3.29	23.54 ± 3.44	15.47 ± 0.73	7.52 ± 0.65
Donor treated with rhG-CSF	11	57.66 ± 3.86*	16.45 ± 0.66*	10.02 ± 1.19*	5.50 ± 1.73*
Donor treated with rhIL-11	11	73.07 ± 2.74	18.69 ± 2.22*	13.19 ± 0.89	6.06 ± 0.43
Donor treated with rhIL-11 and rhG-CSF	11	59.45 ± 5.04*	11.49 ± 0.49*,#	7.77 ± 0.48*,#	4.62 ± 0.59*,#

Data are expressed as percent positive cells within the nuclear cells (mean ± S.D.).
* $P < 0.05$, compared with control.
# $P < 0.05$, compared with donor treated with rhIL-11 or rhG-CSF alone.

Table 2
Transplant outcomes of mice with leukemia after transplantation

Group	n	Survival time (d, $x \pm s$)	Cause and number of death		Number of mice survival more than 30 d
			Cause	n	
A	11[a]	10.70 ± 1.02	Leukemia	10	0
B	11[a]	7.31 ± 1.89	Hematopoietic failure/leukemia	7/3	0
C1	10	12.66 ± 5.53	Leukemia/hematopoietic failure	6/4	0
C	11[a]	15.34 ± 7.86	GVHD	10	0
D	11[a]	23.14 ± 13.66[*]	GVHD	6	4
E	11[a]	27.88 ± 11.33[*]	GVHD	7	2
F	11[a]	41.54 ± 16.15[*,#]	GVHD	3	7

[a] Indicates that there are 1, 1, 1, 1, 2, and 1 mice in groups A, B, C, D, E, and F, respectively, which died of other causes and neither GVHD nor leukemia.
[*] $P < 0.01$, compared with group C.
[#] $P < 0.05$ compared with groups D and E.

mice in group C (control group; donors treated with 0.9% sodium chloride) gradually showed signs of severe clinical GVHD on day +4, and survived for 6–21 d; all died of GVHD. Animals in groups D and E showed similar survival times (23.14 ± 13.66 d and 27.88 ± 11.33 d, respectively). In both groups GVHD began to occur on day +7, and mice survived for 10 or 12 d, respectively, to over 30 d. The mean survival times in these two groups were significantly extended compared with mice in groups A, B, C1, and C ($P < 0.01$). In addition, the mean survival time in group F (donors treated with the combination of rhIL-11 and rhG-CSF) was statistically longer than those of the other six groups ($P < 0.01$). Only three mice in group F acquired GVHD on day +7, and they died of GVHD within 20 d. The remaining seven mice survived more than 30 days without any signs of clinical GVHD. The incidences of GVHD were 100%, 60%, 78%, and 30% in groups C, D, E, and F, respectively. Significant differences were found in the 30 d leukemia-free survival between group F (combination treatment; 70%) and groups C (the control group; 0%), D (rhG-CSF treatment; 40%), and E (rhIL-11 treatment; 20%) ($P < 0.01$). This indicated that the effects of GVL were substantially maintained in group F.

To evaluate the effects of cytokine treatment on donor T cell responses to host tissue, we assessed the growth of mixed lymphocytes in culture (MLC) 14 days after BMT. Similar reductions in the MLC were observed from mice treated with rhG-CSF, rhIL-11, and the combination of rhG-CSF and rhIL-11 compared to MLC from mice treated with 0.9% sodium chloride ($P < 0.01$). The ability of lymphocytes to proliferate in response to host alloantigens significantly decreased after BMT, especially in MLC from mice treated with rhIL-11 plus rhG-CSF compared to MLC from mice treated with rhIL-11 or rhG-CSF alone ($P < 0.01$). Thus hyporesponsiveness was facilitated by the combination of rhG-CSF and rhIL-11 compared to either alone (Fig. 3).

The cytotoxicity of donor-derived T cells from groups D, E, and F were significantly higher than those of group C (controls) on day 12 after transplantation ($P < 0.05$, Fig. 4). There were no statistical differences in the cytotoxicity of T cells among groups D, E, and F ($P > 0.05$, Fig. 4). This indicated that GVL effects could be retained after donor treatments with rhG-CSF and rhIL-11 alone or in combination.

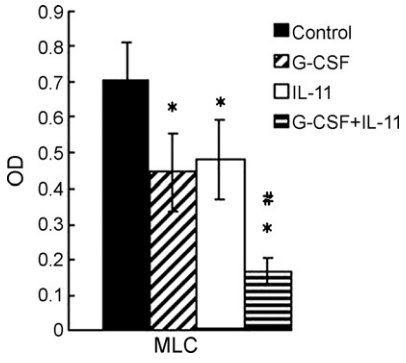

Fig. 3. Effects of different cytokines treatment on the recipients' splenic T cells proliferation activity in response to host alloantigens +14 d after BMT. $P < 0.01$ compared with the control mice, [#]$P < 0.01$ compared with mice in rhG-CSF and rhIL-11 group.

3.3. Th1 to Th2 polarization of T cells in BMT recipients

We found a persistently significant decrease in IFN-gamma production ($P < 0.01$) and a prominent increase in IL-4 production ($P < 0.01$) in mice that received allografts from donors treated with rhG-CSF plus rhIL-11 compared to control mice (Fig. 5A and B). Thus polar-

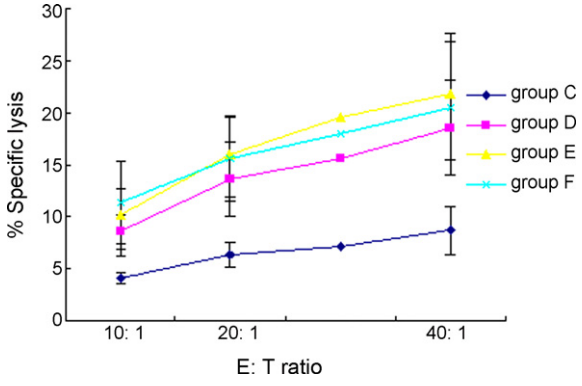

Fig. 4. The cytotoxicity of T cells after transplantation. The cytotoxicity capability of T cells against EL9611 cells by day 12 after transplantation was assessed by LDH release assay. The killing potentials of donor-derived T cells from groups D, E, and F were significantly higher than those of group C by day 12 after transplantation ($P < 0.05$); there were no statistic difference in the cytotoxicity of T cells among groups D, E, and F ($P > 0.05$).

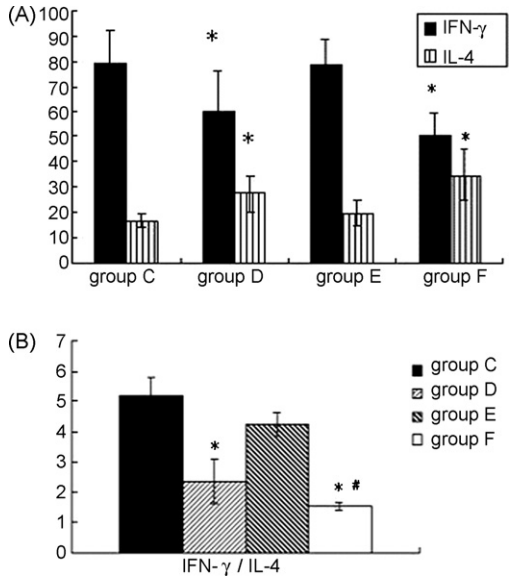

Fig. 5. (A, B) Concentrations of IFN-gamma and IL-4 in the supertant of the splenic T cells cocultured with PHA. Quantities of IFN-gamma and IL-4 secreted by splenic T cells in recipients and the ratios of IFN-gamma/IL-4 of groups C, D, E, and F. $^{*}P < 0.01$ compared with group C, $^{\#}P < 0.05$ compared with groups C, D, and E.

ization in T cell cytokine secretion from Th1 to Th2 cytokines was maintained in transplant recipients after BMT.

4. Discussion

In this study, we demonstrated that the combination of rhIL-11 and rhG-CSF had synergistic effects on the incidence of acute GVHD. Our most important findings were that donors treated with rhIL-11 and rhG-CSF in combination resulted in recipients with significantly reduced GVHD and unimpaired graft-versus-leukemia effects. However, the mechanisms that drive T cells to reduce the incidence of acute GVHD without impairing the GVL effects should be investigated further.

Several potential mechanisms may be related to the alteration of T cell function and graft compositions after donor treatments with different cytokines [5,8–12,15–18,21], including: T cell hyporesponsiveness; polarization of T cells from Th1 to Th2; preferred increases in the counts of monocytes and regulatory T cells; increases in type 2 dendritic cells, which are believed to promote T cell differentiation from Th0 to Th2; and downregulation of co-stimulatory molecules expressed on antigen-presenting cells. We previously reported that donor treatments with cytokines caused polarization of T cells from Th1 to Th2 in human and mice experiments [11,17,18]. To investigate whether this polarization was maintained in recipients after BMT, we further analyzed the IL-4 and IFN-gamma production of splenocytes from recipients 10 d after transplantation. We found that a persistent, significant decrease in IFN-gamma production and a prominent increase in IL-4 production were associated with rhG-CSF plus rhIL-11 treatment. This suggested that the combined rhG-CSF and rhIL-11 treatment polarized the T cell cytokine secretion from Th1 to Th2 in donors and this tendency was maintained in transplant recipients after BMT.

T cells, especially $CD8^{+}$ cytotoxic T cells, represent the most important effector cell population in promoting GVL effects [14,22,23]. Montagna et al. [24] found that the anti-tumor cytolytic T cells are associated with maintenance of hematologic remission in patients with hematological malignancies after autologuous or allogeneic hematopoietic stem cell transplantation. The higher killing potentials that we found in T cells from donors treated with rhIL-11 and rhG-CSF in combination suggested that GVL could be efficiently retained. Therefore, our results are consistent with previous reports that demonstrated T-cell polarization and hyporesponsiveness did not impair the cytotoxicity of T cells; moreover, this may play a major role in the maintenance of GVL effects in mice transplant models. Schmaltz et al. [25] suggested that GVHD and GVL may be mediated by distinct cytotoxic T lymphocyte mechanisms. Further studies should be carried out to clarify whether our finding of the donor T-cell polarization caused by rhIL-11 and rhG-CSF in combination might be associated with these distinct cytotoxic mechanisms in mediating GVHD and GVL.

We found that donor-derived T cells obtained from recipients 14 days after allo-BMT showed reduced responses to host alloantigens and retained higher cytotoxicity to EL9611 cells. This indicated that specific T cell tolerance was induced in our transplant model, and may partly explain the results observed above. Our previous studies demonstrated that CD28 expression on T cells, and B7 expression on B cells and monocytes were downregulated after donors were treated with rhG-CSF alone or rhIL-11 and rhG-CSF in combination. Some researchers have proposed that B-7 molecules expressed on APC bind to CD28, thereby providing a second co-stimulus signal for T cell activation and colony expansion [26,27]. Moreover, the absence of the co-stimulatory signal during T cell priming may lead to T cell anergy [26]. Taken together, these results suggest that the low proliferative ability we observed in splenic T cells derived from recipient mice may be a consequence of the lower expression of CD28 molecules and a suppression of CD28/B7-mediated signal transduction.

In summary, although some of the mechanisms remain unclear, our data confirm that a combination treatment of rhIL-11 and rhG-CSF is markedly superior to treatments of rhIL-11 or rhG-CSF alone for both the maintenance of GVL effects and the reduction of GVHD. These effects caused a significant extension of survival of recipient mice following allogeneic BMT. Moreover, our results suggest that cytokines may induce antigen-specific tolerance of donor-derived T cells. Therefore, further exploration of the underlying mechanisms might reveal separate pathways involved in GVHD and GVL and pave a road for improving transplant outcomes.

Conflict of interest

The authors report no potential conflicts of interest.

Acknowledgements

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Contributions. Xiao-Jun Huang involved in conception and design, critical manuscript revision, and approval of the final version to be published. Jie Zhao performed research, data analysis and interpretation of data, manuscript drafting, and approval of the final version to be published. Xiang-Yu Zhao performed research, data analysis and interpretation of data, manuscript drafting, and approval of the final version to be published. Ying-Jun Chang performed research, data analysis and interpretation of data, manuscript drafting, and approval of the final version to be published.

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