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Original Article

Influence of B7/CD28 and CD40/CD154 co-stimulation signal pathway blockage on immune function and hematopoietic stem cell transplantation in sensitized mice

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Abstract: To investigate the influence of B7/CD28 and CD40/CD154 co-stimulation signal pathway blockage on immune function as well as the regeneration of hematopoietic stem cells in sensitized mouse recipients. The co-stimulation signal pathway was blocked by CTLA4Ig and/or anti-CD154 monoclonal antibody. The numbers of B and T cells were determined using flow cytometry. The immune status and hematopoietic reconstitution in sensitized recipients were also investigated. Flow cytometry results showed that the numbers of B cells, memory T cells, and effector T cells in sensitized mice were significantly higher than that in the normal control mice. CTLA4Ig and/or anti-CD154 monoclonal antibody significantly inhibited the activation of B and T cells with a synergistic effect. The number of fluorescence-labeled HSCs from the donors in the bone marrow in control group and the combinative treatment of CTLA4Ig and anti-CD154 group gradually increased after HSCT. In addition, the chimeric rate of H-2D^{b+} cells was over 90% after the HSCT, suggesting completed bone marrow reconstruction. Blocking the co-stimulation signal pathway induced immune tolerance in sensitized mice. CTLA4Ig and anti-CD154 promoted the homing and engraftment of hematopoietic stem cells, induced immune tolerance and hematopoietic reconstitution, and increased the survival of sensitized mouse recipients.

Keywords: CTLA4Ig, anti-CD154, T cells, hematopoietic stem cell transplantation (HSCT), lymphocyte homing

Introduction

Blood transfusion is an important therapeutic approach for a number of blood disorders, such as thalassemia, aplastic anemia, glucose 6 phosphate dehydrogenase (G6PD) deficiency, and chronic nonspherocytic hemolytic anemia (CNSHA) subtypes. In clinical, hematopoietic stem cell transplantation (HSCT) is widely used for the treatment of these blood disorders [1]. However, clinical studies have identified significantly increased risk of transplant rejection in sensitized recipients of hematopoietic stem cell transplant, and blood transfusion sensitization is one of the major reasons causing implant failure [2].

In our previous study based on a sensitized animal model induced by spleen cell infusion, it is found that a number of pathways including complement-dependent cytotoxicity (CDC), antibody dependent cellular cytotoxicity (ADCC), and cytotoxicity lymphocytes (CTL) were involved in the damage of allogeneic stem cells in sensitized recipients, suggesting that inhibiting humoral and cellular immunities in sensitized recipients is critically important for the success of allogeneic stem cell transplantation [3]. In the activation of immune cells, co-stimulation is often crucial to the development of an effective immune response. Co-stimulation depends primarily on two signals, an antigen-specific first signal and an antigen nonspecific co-stimulation signal. A first signal is provided through the binding and interaction between T cell receptor (TCR)-CD3 complex and peptide-MHC molecules on the surface of antigen presenting cells

(APCs). A second signal is provided by the interaction between co-stimulation molecules expressed on the membrane of APCs and corresponding receptors on T cells. The B7/CD28 and CD40/CD154 are the most important costimulatory pathways in the activation of immune cells [4]. The B7 and CD40 molecules are expressed on the surface of APCs, while the CD28 and CD15 (CD40L) molecules are expressed on the surface of T helper cells. Under the stimulation of a first signal and a co-stimulation signal, APCs can activate T helper cells and promote the secretion of various cytokines, such as IL-2, and IFN-γ in T cells, which further stimulates the activation of B cells and cytotoxic T cells, and induces B cells to produce antibodies [5, 6]. Blocking the co-stimulation pathway can inhibit the activation of T helper cells and cytotoxic T cells as well as the production of antibodies from B cells. Therefore, blocking the co-stimulation pathway may be an effective approach for inhibiting hematopoietic stem cell transplant rejection in sensitized recipients.

CTLA4 immunoglobulin (CTLA4-Ig) is a soluble recombinant protein, which can bind with the B7 molecule on the surface of APCs to block the co-stimulation signal and inhibit graft rejection and antibody production [7-10]. As a member of the tumor necrosis factor (TNF) superfamily, CD40 is structurally expressed in B cells, macrophages, and dendritic cells (DCs). Endothelial cells can express CD40 under the condition of inflammation. The binding of CD40 with its ligand CD154 is essential for the activation of B cells and DCs and the seroconversion of antibodies. It has been reported that binding of anti-CD154 antibody with the CD154 on the surface of T helper cells blocked the CD40/CD154 co-stimulation signal, which inhibited the transplant rejection and prolonged allograft survival in both rodents and non-human mammals [11-16]. Recent studies have also shown that CTLA4Ig and/or anti-CD154 mAb effectively blocked the co-stimulation signaling pathway, inhibited the activation of immune cells, and induced immune tolerance in recipients [16-18]. In addition, CTLA4Ig and anti-CD154 mAb had a synergistic effect [16]. It has been shown in rodents and mammals that blocking the B7/CD28 and CD40/CD154 co-stimulation signal pathways in sensitized recipients prolonged the survival time of implanted allogeneic organs by inducing immune tolerance. However, the underlying mechanisms are still not fully understood.

In the present study, we investigated the immune tolerance in sensitized mice in which the B7/CD28 and CD40/CD154 co-stimulation signal pathways were blocked. The main objective of the present study was to confirm that blocking co-stimulation signal pathways can suppress immune activation in sensitized recipients. Allogeneic hematopoietic stem cells (HSCs) were labeled with 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE) and FITC-H-2D^{b} to detect their distribution and homing in different tissues, which allow us to investigate the immune status and hematopoietic reconstruction of recipients as well as improve the success of HSCT.

Materials and methods

Ethics statement

This research does not involve human subjects, human materials. All animal research protocols for this work were reviewed and approved by The Institute Research Medical Ethics Committee of Sun Yat-Sen University and conducted the National Institutes of Health (NIH) Guide and Declaration of Helsinki.

Establishment of a sensitized mouse model

Specific-pathogen-free (SPF) BALB/c (H-2D^{d}) and C57BL/6 (H-2D^{b}) mice (male, 6-8 weeks of age, 18-20 g) were provided and bred by the Experimental Animal Center of Sun Yat-Sen University. The C57BL/6 mice were sacrificed by decapitation and immersed in 75% alcohol for 5 min for disinfection. Spleen was dissected from the sacrificed C57BL/6 mice under sterile conditions. The spleen was washed repeatedly using PBS to collect spleen cells, which were passed through a 200-mesh metal filter to prepare spleen cell suspension. After centrifugation at 1200 rpm for 5min, the supernatant was removed and 1 ml of erythrocyte lysis buffer (Sigma-Aldrich, St. Louis, MO, USA) was added to the cell pellet to lyse spleen cells for 8-10 mins before the lysis reaction was terminated by the addition of 5 ml of PBS. The spleen cells were re-suspended in RPMI-1640 culture medium after centrifugation at 1200 rpm for 5 min. The cells vitality was evaluated using trypan blue staining (>99%). The density of spleen

cells obtained from C57BL/6 mice was regulated to 1×10^6 and the C57BL/6 spleen cells were intravenously infused into BALB/c mice 7 days prior to bone marrow transplantation surgery to establish a moderate sensitized mouse model for follow-up experiments [19].

Animal grouping

A total of 20 BALB/c (H-2D^d) mice were used to establish the sensitized mouse model. The 20 mice were randomly divided into four groups (5 mice/group) according to different interventions. The sensitized group mice received murine isotype Ig. The anti-CD154 group mice received anti-CD154 and murine isotype Ig. The CTLA4Ig group mice received murine CTLA4Ig and murine isotype Ig. The CTLA4Ig plus anti-CD154 group mice received a combinative treatment of CTLA4Ig and anti-CD154. In addition, a group of mice with non-sensitization and received murine isotype control Ig were used as the normal control. A total of 500 μg of CTLA4Ig, anti-CD154, or murine isotype control Ig (Abcam, UK) were infused into each mouse through tail vein seven days after the HSTC.

In addition, 45 sensitized BALB/c were randomly divided into three groups to investigate the influence of the blockage of the co-stimulation signal pathway on graft rejection of allogeneic bone marrow transplantation. No treatment was given to the sensitized mouse group (n= 15). The CTLA4Ig plus anti-CD154 group mice received the combinative treatment of CTLA-4Ig and anti-CD154 (n=15). BALB/c mice without sensitization and received murine isotype Ig were used as the control. Gentamicin and erythromycin were added to the drinking water since the fifth day after HSCT. A lethal dose (8 Gy) irradiation was given to the mice on the same day of HSCT using a linear accelerator radiation. The bone marrow in the femoral and tibial in C57BL/6 mice was isolated to determine the vitality of bone marrow cells (>99%). Some bone marrow cells were labeled with CFSE to evaluate the homing of HSCs. Bone marrow cells (1×10^7) in 0.2 ml were infused into recipients through the tail vein after irradiation for 4-6 hours.

Determination of the numbers of B and T cells using flow cytometry

The sensitized BALB/c mice were sacrificed by decapitation and immersed in 75% alcohol for 5 min for disinfection. Spleen was dissected from the sacrificed BALB/c mice under sterile conditions, homogenized in a 1ml syringe, and passed through a 200-mesh stainless steel filter. The spleen cells were lysed using erythrocyte lysate, washed using PBS twice, and suspended in PBS for flow cytometry analysis (FACScan, Becton Dickinson). The number of B cells ($CD19^+CD69^+$) in 10^6 spleen cells, which were labeled with anti-CD19-FITC and anti-CD69-PE, was determined by flow cytometry. In addition, 10^6 spleen cells were labeled with anti-CD4-PerCP, anti-CD69-PE, anti-CD8-APC, anti-CD62L-PE, and anti-CD44-FITC. The expressions of CD62L and CD44 in T cells ($CD4^+CD8^+$) were determined. Memory and effector T cells were defined as $CD4^+CD44^{high}/CD62L^{high}$ and $CD8^+CD44^{high}/CD62L^{low/-}$, respectively.

Mixed lymphocyte reaction (MLR)

The proliferation ability of spleen cells that were labeled with BrdU (Millipore, USA) was evaluated in a mixed lymphocyte reaction in which the spleen cells from C57BL/6 mice were used as stimulator cells and mouse spleen lymphocytes isolated by the Ficoll method were used as effector cells. BrdU-labeling of spleen cells was conducted according to the manufacture's instruction.

Determination of the serum levels of a number of cytokines, IgM and IgG antibodies, and sensitized antibodies

The mice received intervention treatments mentioned above were sacrificed by decapitation. Blood collected from the ophthalmic venous plexus was stored in an anticoagulant tube with EDTA. After standing at room temperature for 1 hour, serum was collected by centrifugation. The levels of a number of cytokines including IL-2, IL-4, IL-10, and IFN-γ and IgM and IgG antibodies in the serum from sensitized recipients were determined using a Platinum ELISA kit according to the manufacturer's instruction (Ebioscience, USA). In order to determine the levels of sensitized antibodies in the serum of BALB/c mice, 5 μl of BALB/c mouse serum was mixed with 5×10^5 C57bL/6 mouse spleen cells in a 96-well culture plate, and incubated in an incubator at 37°C with 5% CO_2 for 30 min. After incubation with FITC-labeled goat anti-mouse Ig secondary antibody at 4°C in dark for 30 min, the cells were washed

with PBS and re-suspended in 400 μL PBS. After washed with PBS, the cells were incubated with FITC-labeled goat anti-mouse IgG for flow cytometry assay.

Bone marrow transplant

Drinking water containing antibiotics gentamicin (32×10^4 U/L) and erythromycin (250 mg/L) were provided daily to all mice 5 day prior to the bone marrow transplant surgery. In addition, the mice undergone radiation pre-treatment in a Siemens primus H type linear accelerator (500 cGy/min, a total dose of 8 Gy) prior to the bone marrow transplant surgery. Femoral and tibial bone marrows were dissected from C57BL/6 mice. Red blood cells in the collected bone marrows were lysed using erythrocyte lysate. After washing, the concentration of bone marrow cells were regulated to 5×10^7/ml and the cell viability of bone marrow cells was evaluated using trypan blue staining (>99%).

Evaluation of lymphocyte homing

Allogeneic donor HSCs were labeled with carboxyfluorescein succinimidyl ester (CFSE) (10 μmol/L). Transplant recipients were sacrificed after two, 12, and 24 hours of bone marrow transplant surgery. Eye blood smear and frozen sections of femur, spleen, liver, and lungs were prepared to examine and count fluorescent cells under a fluorescence microscopy (×100). The results were classified into four grades: no fluorescent cells observed (-), 1-5 fluorescent cells per field (+), 5-10 fluorescent cells per field (++), and 10-100 fluorescent cells per field (+++). In addition, cell suspension of the spleen, femur, and ocular blood from the sensitized mice was prepared to quantify the fluorescent cells using flow cytometry assay.

Evaluation of the growth of graft in sensitized recipients

The living conditions and time of death of sensitized recipients of allogeneic bone marrow transplantation were examined and recorded daily to plot survival curves. Blood collected from tail veins of sensitized recipients was used to count WBC, RBC, (hemoglobin) Hb, and platelet (PLT) weekly. Recipient femurs were dissected from the sensitized recipients and both ends of the femurs were removed. Femoral bone marrow cells were washed off by PBS using a syringe, filtrated with a 200 mesh metal filter, and suspended in PBS to determine the number of bone marrow cells in recipient femurs. Bone marrow cells (1×10^6) from recipient femurs were labeled with 1 μl FITC-H-$2D^b$ to evaluate the fitting of hematopoietic stem cells in recipient femurs using flow cytometry. In addition, to evaluate the growth and proliferation of bone marrow cells in recipient femurs, fresh femurs were fixed in 4% paraformaldehyde, decalcified, and embedded in paraffin to prepare histopathological slides for HE staining.

Statistical analyses

Statistical analyses were conducted using the SPSS19.0 software, measurement data were presented as mean $\bar{x} \pm SD$. Comparison between groups was analyzed using ANOVA test. A P value less than 0.05 was considered as statistically significant.

Results

CTLA4Ig and anti-CD154 reduced the number of B and T cells in sensitized BALB/c mice

Based on flow cytometry assay, the percentage of B cells in sensitized BALB/c mice was significantly higher than that in normal control mice ($P<0.01$) (**Figure 1A**). CTLA4Ig, anti-CD154, or the combination of CTLA4Ig and anti-CD154 significantly reduced the percentage of B cells in sensitized BALB/c mice ($P<0.01$). In addition, the percentage of B cells in sensitized BALB/c mice received the combinative treatment of CTLA4Ig and anti-CD154 was significantly lower than that in sensitized BALB/c mice received CTLA4Ig or anti-CD154 treatment alone ($P<0.01$).

As shown in **Figure 1B**, the percentage of $CD4^+CD44^{high}/CD62L^{high}$ T cells in sensitized BALB/c mice was significantly higher than that in normal control mice ($P<0.01$). CTLA4Ig or the combination of CTLA4Ig and anti-CD154 significantly reduced the percentage of $CD4^+CD44^{high}/CD62L^{high}$ T cells in sensitized BALB/c mice ($P<0.01$). However, no significant influence of anti-CD154 treatment on the percentage of $CD4^+CD44^{high}/CD62L^{high}$ T cells in sensitized BALB/c mice was observed ($P>0.05$). In addition, the percentage of $CD4^+CD44^{high}/CD62L^{high}$ T cells in sensitized BALB/c mice received the

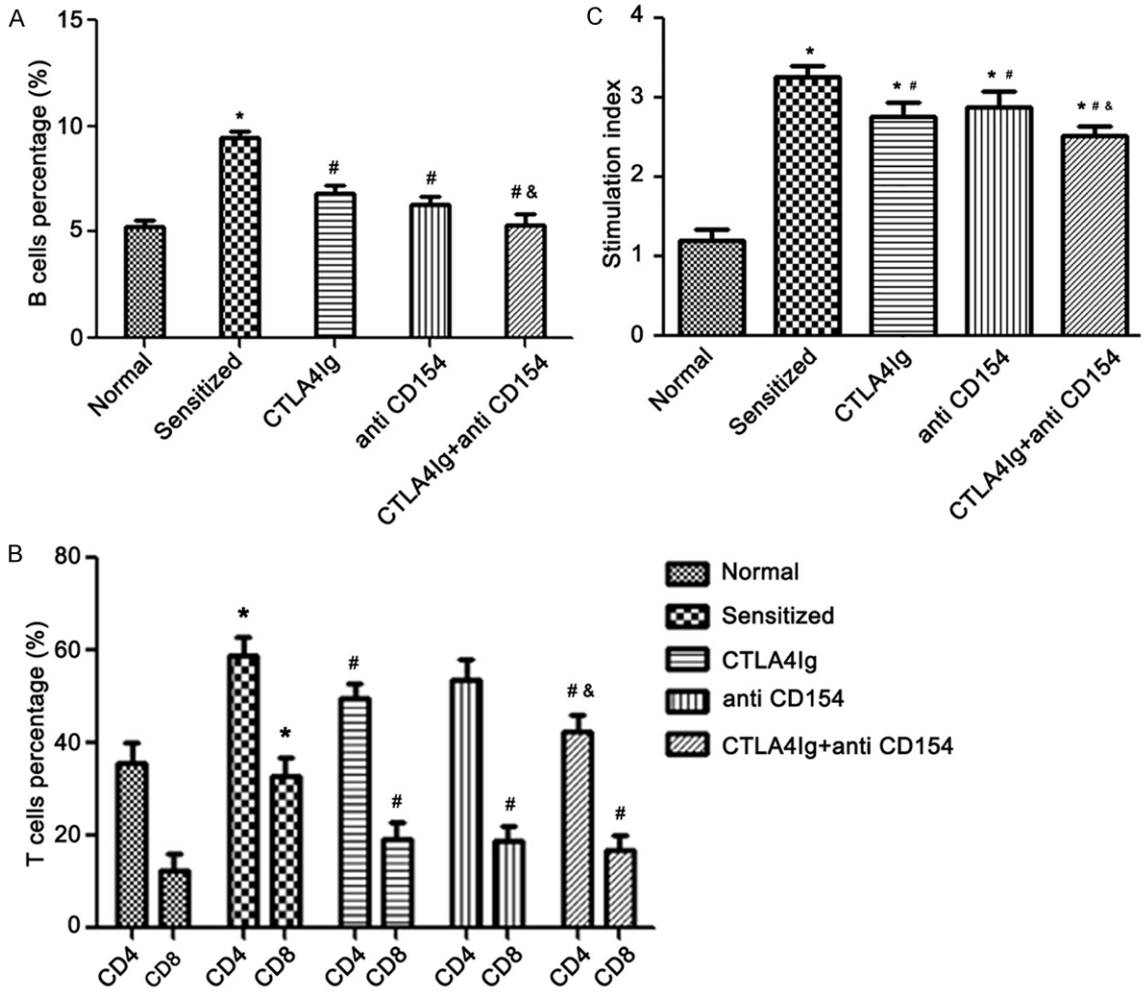

Figure 1. A: Comparison of the numbers of $CD19^+CD69^+$ B cells between different groups based on flow cytometry analysis (±s, %, n=5). B: Comparison of the numbers of memory and effector T cells between different groups based on flow cytometry analysis (±s, %, n=5). CD4: $CD4^+CD44^{high}/CD62L^{high}$ T cells, CD8: $CD8^+CD44^{high}/CD62L^{low/-}$ T cells. C: Comparison of the stimulation index (SI) between different groups based on mixed lymphocyte reaction (MLR) experiment. $^*P<0.01$, compared with the normal control group; $^\#P<0.01$, compared with the sensitized mouse group; $^\&P<0.01$, compared with the single treatment group in A and C, compared with the CTLA4Ig group in B.

combinative treatment of CTLA4Ig and anti-CD154 was significantly lower than that in sensitized BALB/c mice received CTLA4Ig treatment alone ($P<0.01$).

The percentage of $CD8^+CD44^{high}/CD62L^{low/-}$ T cells in sensitized BALB/c mice was significantly higher than that in normal control mice ($P<0.01$) (**Figure 1B**). CTLA4Ig, anti-CD154, or the combination of CTLA4Ig and anti-CD154 significantly reduced the percentage of $CD8^+CD44^{high}/CD62L^{low/-}$ T cells in sensitized BALB/c mice. However, no significant difference in the percentage of $CD8^+CD44^{high}/CD62L^{low/-}$ T cells was observed among the three groups of sensitized BALB/c mice received the treatment of CTLA4Ig, anti-CD154, and the combination of CTLA4Ig and anti-CD154 ($P>0.05$).

CTLA4Ig and anti-CD154 reduced the stimulation index (SI) of spleen cells from the sensitized BALB/c mice

In the mixed lymphocyte reaction (MLR) experiment, the spleen cells from normal or sensitized BALB/c mice were used as effector cells and C57bL/6 spleen cells were used as stimulating cells. We found that the stimulation index (SI) of spleen cells from the sensitized BALB/c mice was significantly higher than that of

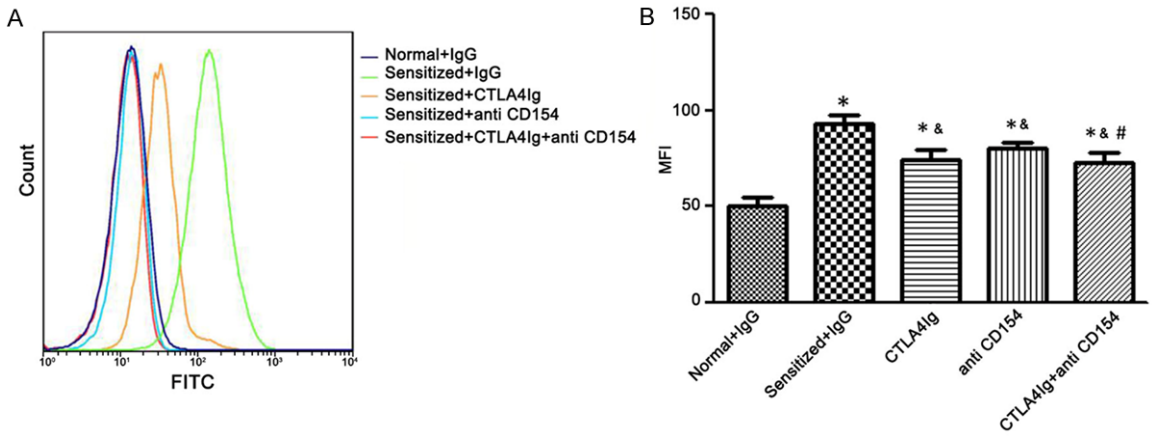

Figure 2. A: Goat-anti mouse FITC antibodies in each group detected by flow cytometry. B: Comparison of the median fluorescence intensity (MFI) between different groups. *P<0.01, compared with the normal control group; &P<0.01, compared with the sensitized group; #P<0.01, compared with the single treatment group.

Table 1. The fluorescence intensity in frozen sections and blood smears*

Group	Liver	Lung	Spleen	Femur	Blood
Normal 2 h	+++	+++	+++	+	+
Sensitized 2 h	+++	+++	+++	+	+
CTLA4Ig+anti-CD154 2 h	+++	+++	+++	+	+
Normal 12 h	++	++	+++	++	+
Sensitized 12 h	++	++	++	+	+
CTLA4Ig+anti-CD154 12 h	++	++	+++	++	+
Normal 24 h	+	+	+++	+++	+
Sensitized 24 h	+	+	-	+	-
CTLA4Ig+anti-CD154 24 h	+	+	+++	+++	+

*The fluorescent intensity were defined as the intensity per high-powered (HP, ×100) field as follows: no fluorescent cells/HP was considered as (-), 1 to 5 fluorescent cells/HP as (+), 5 to 10 fluorescent cells/HP as (++), and 10 to 100 fluorescent cells/HP as (+++).

spleen cells from normal BALB/c mice (P<0.01). CTLA4Ig, anti-CD154, or the combination of CTLA4Ig and anti-CD154 significantly reduced the SI of spleen cells from the sensitized BALB/c mice (P<0. 01). In addition, the SI of spleen cells from the sensitized BALB/c mice received the combinative treatment of CTLA4Ig and anti-CD154 was significantly lower than that of spleen cells from the sensitized BALB/c mice received the treatment of CTLA4Ig or anti-CD154 alone (P<0. 01) (**Figure 1C**).

The changes of serum levels of cytokines, IgG, IgM, and median fluorescence intensity (MFI)

The serum levels of IL-2, IL-4, IL-10, IFN-γ, IgM, and IgG in all mice were extremely low or undetectable. No significant differences in the serum levels of IL-2, IL-4, IL-10, and IFN-γ between different groups of mice (**Figure 2A**). The median fluorescence intensity (MFI) in the sensitized BALB/c mice was significantly higher than that in the normal BALB/c mice (P<0.01). CTLA4Ig, anti-CD154, or the combination of CTLA4Ig and anti-CD154 significantly reduced the MFI in the sensitized BALB/c mice (P<0.01). However, no significant difference in the MFI between the sensitized BALB/c mice received the combinative treatment of CTLA4Ig and anti-CD154 and the sensitized BALB/c mice received CTLA4Ig or anti-CD154 treatment alone (P>0.05) (**Figure 2B**).

Distribution of donor bone marrow cells in the tissue of recipients

The distribution of fluorescence-labeled allogeneic bone marrow cells in the peripheral blood, lung, liver, spleen, and femur in recipients was evaluated after two, 12, and 24 hours of hematopoietic stem cell transplant (HSCT) (**Table 1**). The number of fluorescence-labeled allogeneic bone marrow cells in the lung and liver in all groups decreased gradually with the time. However, the number of fluorescence-labeled allogeneic bone marrow cells in the femur in normal mice and sensitized mice received the combinative treatment of CTLA4Ig and anti-CD154 increased gradually with the time. After two hours of bone marrow cell transplantation,

Table 2. The numbers of CFSE-labeled cells in different tissues based on FCM ($\bar{x} \pm SD$, %, n=3)

Group		Time		
		2 h	12 h	24 h
Peripheral blood	Normal control	1.262±0.234*	0.817±0.136**	0.685±0.174**
	Sensitized	0.418±0.205	0.053±0.021	0.010±0.003
	CTLA4IgG+Anti-CD154	0.869±0.186*	0.626±0.228**	0.315±0.138**,#
Spleen	Normal control	0.676±0.124	0.328±0.121*	0.195±0.084
	Sensitized	0.473±0.212	0.066±0.021	0.048±0.013
	CTLA4IgG+Anti-CD154	0.673±0.225	0.324±0.131*	0.144±0.087
Femur	Normal control	0.296±0.088	0.473±0.126*	0.679±0.132***
	Sensitized	0.292±0.112	0.113±0.076	0.042±0.021
	CTLA4IgG+Anti-CD154	0.268±0.143	0.424±0.134*	0.615±0.136***

*P<0.05, compare with the sensitized mouse group; **P<0.01, compare with the sensitized mouse group; ***P<0.001, compare with the sensitized mouse group; #P<0.01, CTLA4IgG+Anti-CD154 group compare with the normal control group.

no significant difference in the distribution of fluorescence-labeled bone marrow cells in the peripheral blood, femur, spleen, and liver was observed between different groups. After 12 hours of bone marrow cell transplantation, the distribution of fluorescence-labeled bone marrow cells in the peripheral blood, femur, and spleen of normal control recipients and recipients received the combinative treatment of CTLA4Ig and anti-CD154 were all (+), (++), and (+++) which were increased after 24 hours. However, after 12 hours of bone marrow cell transplantation, the distribution of fluorescence-labeled bone marrow cells in the peripheral blood, femur, and spleen of sensitized recipients were (+), (+), and (++) which were decreased after 24 hours.

Two, 12, and 24 hours after the bone marrow cell transplantation, single cell suspensions of the peripheral blood, femur, and spleen of recipients were prepared to evaluate the distribution of CFSE-labeled donor bone marrow cells in these tissues using flow cytometry. As shown in **Table 2**, no significant difference in the distribution of fluorescence-labeled bone marrow cells in the femur and spleen was observed among the normal control recipients, the recipients received the combinative treatment of CTLA4Ig and anti-CD154, and the sensitized recipients after two hours of bone marrow cell transplantation (P>0.05). However, the number of fluorescence-labeled bone marrow cells in the peripheral blood of the recipients received the combinative treatment of CTLA4Ig and anti-CD154 was significantly higher than that in the peripheral blood of sensitized recipients after two hours of bone marrow cell transplantation (P<0.05). After 12 and 24 hours of bone marrow cell transplantation, the number of fluorescence-labeled bone marrow cells in the peripheral blood of the recipients received the combinative treatment of CTLA4Ig and anti-CD154 was more significantly higher than that in the peripheral blood of sensitized recipients (P<0.01). After 12 hours of bone marrow cell transplantation, the number of fluorescence-labeled bone marrow cells in the femur in the normal control recipients and the recipients received the combinative treatment of CTLA-4Ig and anti-CD154 was 0.473±0.126% and 0.424±0.134%, respectively, which was significantly higher than the sensitized recipients group (0.113±0.076% and P<0.05). After 24 hours of bone marrow cell transplantation, the number of fluorescence-labeled bone marrow cells in the femur of the recipients received the combinative treatment of CTLA4Ig and anti-CD154 was more significantly higher than that in the femur of sensitized recipients (P<0.001).

After 2 and 12 hours of bone marrow cell transplantation, no significant difference in the number of fluorescence-labeled bone marrow cells in the peripheral blood was observed between the normal control recipients and the recipients received the combinative treatment of CTLA4Ig and anti-CD154 (P>0.05). After 24 hours of bone marrow cell transplantation, the number of fluorescence-labeled bone marrow cells in the peripheral blood in the recipients received the combinative treatment of CTLA4Ig and anti-CD154 was 0.315±0.138%, which is significantly lower than that in the peripheral blood in

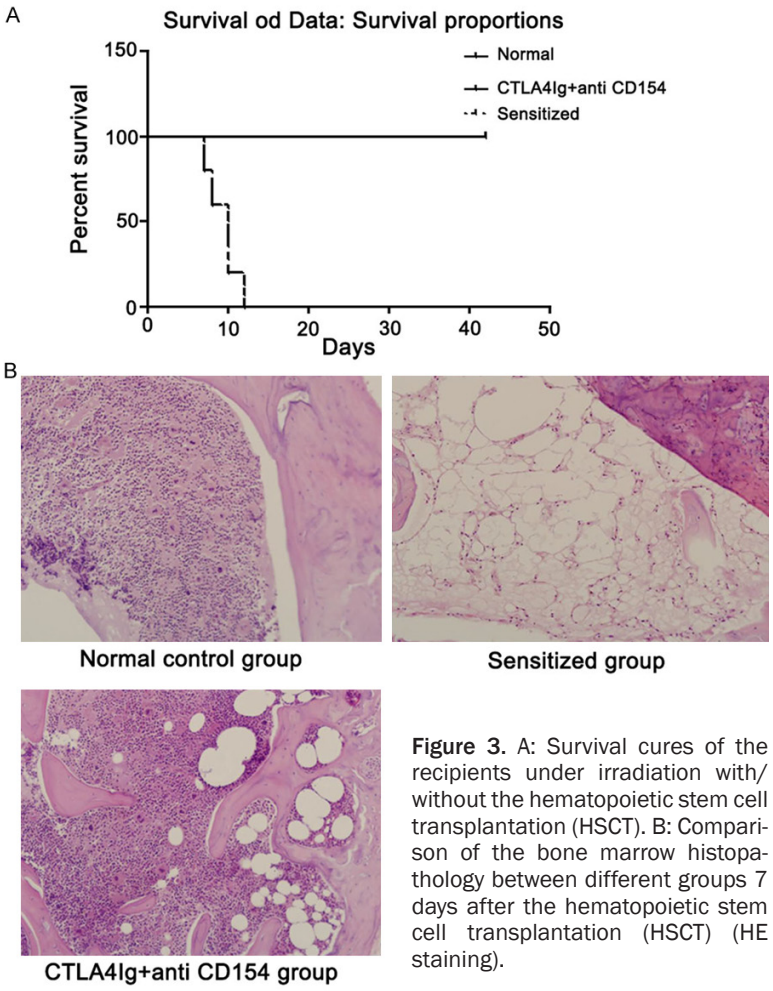

Figure 3. A: Survival cures of the recipients under irradiation with/without the hematopoietic stem cell transplantation (HSCT). B: Comparison of the bone marrow histopathology between different groups 7 days after the hematopoietic stem cell transplantation (HSCT) (HE staining).

the normal control recipients (0.685±0.174%) (P<0.01) (**Table 2**), suggesting that some transplanted bone marrow cells were destroyed by antibodies in the blood in the recipients.

Homing of donor bone marrow cells *in* sensitized mouse *recipients*

As shown in **Figure 3A**, after 42 days of bone marrow cell transplantation, all normal control mice and mice received radiation survived, however, the sensitized mice died after 7-12 days of bone marrow cell transplantation (median survival time: 10 days). Log-rank test suggests that sensitized mice had significantly short survival time than normal control mice and mice received radiation (P<0.001).

The WBC, Hb, and PLT in the normal control mice and mice received the combinative treatment of CTLA4Ig and anti-CD154 increased gradually after the bone marrow cell transplantation. However, the WBC, Hb, and PLT in the sensitized mice decreased after the bone marrow cell transplantation. Seven days after the bone marrow cell transplantation, the numbers of WBC and PLT in the normal control mice and mice received the combinative treatment of CTLA4Ig and anti-CD-154 were significantly higher than that in the sensitized mice (P<0.01). No significant difference in the WBC, Hb, and PLT on different time points between the normal control mice and mice received the combinative treatment of CTLA4Ig and anti-CD154 (P>0.05).

Based on HE staining, the number of bone marrow cells in the femur in the normal control mice and mice received the combinative treatment of CTLA4Ig and anti-CD154 increased gradually after radiation. However, the number of bone marrow cells in the femur in the sensitized mice decreased significantly after seven days and vacuolization was observed in the bone marrow, suggesting bone marrow depletion (**Figure 3B**).

Chimerism analysis suggests no significant difference in the number of H-2D^{b+} cells after seven days of bone marrow cell transplantation between the normal control mice (53.4±2.3%) and mice received the combinative treatment of CTLA4Ig and anti-CD154 (46.7±2.5%) (P>0.05) (**Figure 4**). After 28 days of bone marrow cell transplantation, the chimeric rates in both the normal control mice and mice received the combinative treatment of CTLA4Ig and anti-CD154 were over 90%, suggesting successful bone marrow reconstruction. Bone marrow cells in the sensitized mice were limited to be detected.

Discussion

Immune rejection against Implanted HSCs in sensitized recipients depends mainly on the

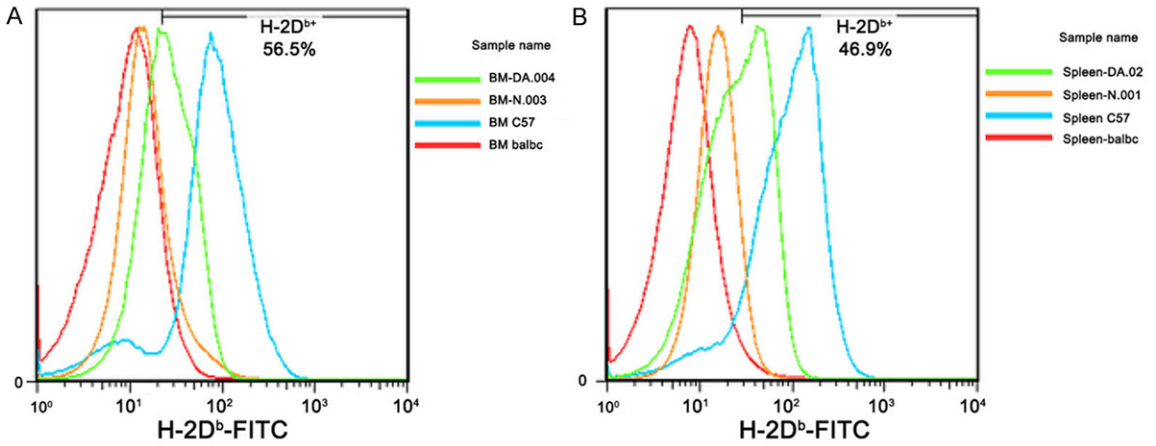

Figure 4. Comparison of the numbers of H-2D^b positive cells in the bone marrow (BM, A) and spleen (B) between recipients of different groups 7 days after the hematopoietic stem cell transplantation (HSCT). DA: CTLA4Ig+anti-CD154; N: the normal control; C57: the positive control; Balbc: the negative control.

activation of immune cells after the sensitization and various antibodies produced before the sensitization. These antibodies destroy HSCs and results in graft failure through complement-dependent cell cytotoxicity (CDC), antibody-dependent cell-mediated cytotoxicity (ADCC), and cytotoxic lymphocytes (CTLs). A previous study have reported that the absolute number of T cells (CD4$^+$orCD8$^+$ T cells) and B cells (CD19$^+$) was not significantly changed by the sensitization, however, the number of memory T cells (CD44^{high}/CD62L^{high}) and effector T cells (CD44^{high}/CD62L$^{low/-}$) significantly increased after the sensitization, suggesting that sensitization induced immune memory in recipients [20]. Our results in the present study are consistent with the previous study. In addition, we found that blocking the B7/CD28 and/or CD40/CD154 co-stimulation pathways synergistically reduced the numbers of B, memory T, and effector T cells. Mixed lymphocyte reaction assay also suggests that CTLA4Ig and anti-CD154 inhibited the immune responses to donor cells in recipients in a synergistic manner. We also found that anti-CD154 significantly inhibited the production of effector T cells, but had no effects on the amount of memory T cells. These observations suggest that anti-CD154 did not completely inhibit the activation of CD4$^+$ T cell and immune memory, and other pathways independent of CD40 are involved in the activation of CD4$^+$ T cells, which are consistent with the study conducted by Xu et al. [20]. The activation of CD4$^+$ memory T cell causes graft rejection [21, 22]. Taken together, blocking the CTLA4Ig and anti-CD154 co-stimulation pathways can effectively inhibits cellular immunity, the activation of B cells, and Immunological memory in sensitized recipients.

Th1 type cytokines (IFN-γ and IL-2) and Th2 cytokines (IL-4 and IL-10) are primarily secreted by activated immune cells. The previous study reported that increasing Th2 cytokine levels significantly inhibited Th1 cytokine levels in CTLA4Ig-induced immune tolerance in allogeneic heart transplantation [23]. Recently, Zhu et al. [24] reported that blocking the co-stimulation pathways using CTLA4Ig and anti-CD154 inhibited the production of Th1 and Th2 cytokines and induced immune tolerance of allogeneic heart transplantation. Zhang et al. [25] also observed reduced levels of Th1 cytokines in CTLA4Ig and CDL transgenic mouse with immune tolerance of allogeneic pancreas transplantation. However, no significantly variations in the levels of Th1 and Th2 cytokines were detected in the present study, which may be explained by the undetectable levels of FN-γ, IL-2, and IL-4 in the serum of most mice [26]. In addition, the limited number of activated immune cells and the potential inhibitory effects of CTLA4Ig and anti-CD154 may reduce the number of both Th1 cytokines.

Antibody-mediated humoral immunity is the major factor causing HSC damage in sensitized recipients and HSCs are mainly destroyed by antibodies produced prior to the sensitization [27, 28]. However, we found in the present

study that the serum levels of total IgM and IgG were not significantly changed after sensitization and the combinative treatment of CTLA4Ig and anti-CD154. Blocking the co-stimulation pathways using CTLA4Ig and/or anti-CD154 significantly effectively inhibited the production of sensitized antibodies. Typically, IgM antibodies are produced seven days after the first antigen stimulation and IgG production is later than the production of IgM. In addition, the antibodies specifically against antigens of allogeneic mice account only a small part of the antibody pool; therefore, the changes of antibodies specially against antigens of allogeneic mice had no significant influence on the total amount of antibody.

Homing of hematopoietic stem/progenitor cells from donors in the bone marrow into recipients depends mainly on complicated molecular interactions between hematopoietic stem/progenitor cells and other cells. Successful homing of hematopoietic stem cells into the bone marrow in recipients through the circulatory system is critically important to HSCT [29]. During the homing of hematopoietic stem cells, hematopoietic stem cells enter bone marrow cavity through gaps between endothelial cells, proliferate and differentiate in the bone marrow stromal microenvironment to recover bone marrow hematopoietic function. Therefore, the number of homing hematopoietic stem cells can be used for the evaluation of HSCT [30].

In the present study, we used the CFSE fluorescent dye to label and track allogeneic bone marrow cells from C57BL/6 mice in recipients to evaluate the homing of hematopoietic stem/progenitor cells. We found that the number of CFSE-labeled cells in the liver and lungs in sensitized recipients gradually decreased after the HSCT. However, the number of CFSE-labeled cells in the liver and spleen in the control mice and mice received the combinative treatment of CTLA4Ig and anti-CD154 increased after the HSCT. While no significant difference in the number of CFSE-labeled cells in the peripheral blood, femur, spleen, and liver was identified between different groups of mice two hours after HSCT, we observed significantly increased number of CFSE-labeled cells in the peripheral blood, femur, spleen, and liver in the normal control mice and mice received the combinative treatment of CTLA4Ig and anti-CD154 12 and 24 hours after HSCT. These observations suggest that a dynamic homing of the bone marrow cells from donors in recipients and most bone marrow cells from donors were destroyed before homing in the bone marrow in sensitized recipients, which are further confirmed by the results of flow cytometry. It has been reported that the homing of hematopoietic stem cells from donors in recipients is a short process, which completes ~1-2 days after HSCT [31]. Transplanted hematopoietic stem cells migrate to the peripheral blood 48 hours after HSCT and many factors may affect the homing of hematopoietic stem cells in the bone marrow in recipients, therefore, we did not evaluate the homing of hematopoietic stem cells in the bone marrow in recipients after 48 hours of HSCT.

Based on survival analysis, all mice received radiation (8 Gy) died 1-12 days after HSCT, suggesting that the 8 Gy radiation is the lethal dose. Given that all sensitized recipients died after 1-12 days of HSCT (median survival time: 10 days), complete graft rejection occurred in sensitized recipients. However, the normal control mice and mice received the combination treatment of CTLA4Ig and anti-CD154 survived after 42 days of HSCT and radiation, suggesting that CTLA4Ig and anti-CD154 inhibited the destruction and promoted the homing of hematopoietic stem cells in sensitized recipients.

We found that the serum levels of WBC, Hb, and PLT in sensitized recipients decreased seven days after the HSCT. Anemia, reduced activity, and weight loss were observed in these mice and they died of bone marrow failure after 7-12 days of HSCT. However, the serum levels of WBC, Hb, and PLT in the control mice and mice received the combinative treatment of CTLA4Ig and anti-CD154 increased and reached the normal levels after 28 days of HSCT. No significant difference in the serum levels of WBC, Hb, and PLT was detected between the normal control mice and mice received the combination treatment of CTLA4Ig and anti-CD154 at all time points, suggesting that CTLA4Ig and anti-CD154 effectively promoted the homing and proliferation of hematopoietic stem cells in recipients, and induced immune tolerance and hematopoietic reconstitution, which were supported by the chimerism analysis. In the present study, X-ray linear

accelerator was used for lethal irradiation for recipients [32]. Compared with the 60Co γ ray irradiation, mice received X-ray irradiation survived for a short time and the serum levels of WBC, Hb, and PLT recovered more slowly, suggesting that the damage caused by X-ray irradiation was more serious than that caused by γ-ray irradiation. This observation is consistent with the previous study [33].

Taken together, our results demonstrated that CTLA4Ig and anti-CD154 block the co-stimulation pathways to inhibit both cellular and humoral immunities, which can suppress immune activation in recipients, effectively promote the implantation and homing of hematopoietic stem cells in sensitized recipients, and induce immune tolerance to extend the survival of grafts.

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Disclosure of conflict of interest

None.

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