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***In vivo* administration of recombinant BTNL2-Fc fusion protein ameliorates graft-versus-host disease in mice**

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Abstract

Although hematopoietic stem cell transplantation (HSCT) has been widely used in the treatment of many diseases, graft-versus-host disease (GVHD) remains a major complication after allogeneic HSCT. Butyrophilin-like 2 (BTNL2) protein has been reported to have the ability to inhibit T cell proliferation *in vitro*; its ability to inhibit T cell responses *in vivo* has not been determined. We show here that *in vivo* administration of recombinant BTNL2-IgG2a Fc (rBTNL2-Ig) fusion protein ameliorates GVHD in mice. This is related to the ability of rBTNL2-Ig to inhibit T cell proliferation, activation and Th1/Th17 cytokine production *in vivo*. Furthermore, rBTNL2-Ig treatment increases the generation of regulatory T cells. Our results suggest that rBTNL2-Ig has the potential to be used in the prevention and treatment of patients with GVHD.

Keywords

hematopoietic stem cell transplantation; graft-versus-host disease; Butyrophilin-like 2; regulatory T cells; T cell proliferation; activation

1. Introduction

While allogeneic HSCT has been widely used in the treatment of a variety of malignancies and non-malignant conditions, GVHD remains as a serious complication that limits its success [1–3]. GVHD occurs in up to 50% transplant recipients and accounts for 15–30% of

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deaths following allogeneic HSCT [2]. GVHD, especially acute GVHD, is primarily mediated by donor T cells in the graft [1–8]. Alloreactive donor T cells recognize disparate histocompatibility antigens in the recipients and lead to progressive damage to target organs such as the liver, gastrointestinal tract, skin, and lung. The symptoms of acute GVHD include maculopapular skin rash, nausea, anorexia, diarrhea, abdominal pain, and cholestatic hyperbilirubinemia, etc [2]. Development of new approaches to prevent or treat GVHD thus remains a major clinical goal.

T cell immune responses are regulated by T cell co-stimulatory and co-inhibitory molecules. The B7 family plays a central role in regulating immune responses. Targeting B7 family ligands and receptors has achieved great success in the treatment of autoimmune disease and cancer. For example, the recombinant fusion protein CTLA-4-Fc (Abatacept or Balatacept) has been used in the treatment of rheumatoid arthritis and kidney transplantation rejection.

Butyrophilin (BTN) and BTN-like (BTNL) molecules are emerging as potentially critical immune regulators [9–13]. BTN and BTNL molecules share sequence, structural, and functional similarity with the B7 family members so are thus considered as extended B7 family members [14–16]. It has been reported that BTNL2 can inhibit T cell proliferation and cytokine production *in vitro* [17, 18]. However, the ability of BTNL2 to inhibit T cell responses *in vivo* and to treat GVHD has not been reported.

In this study, we first clone and express the BTNL2 gene to produce a high yielding rBTNL2-Ig fusion protein. We then demonstrate that *in vivo* administration of rBTNL2-Ig attenuates GVHD in mice. This is associated with its ability to inhibit T cell proliferation, activation and cytokine production *in vivo*. In addition, rBTNL2-Ig treatment significantly increases the generation of regulatory T cells (Tregs) in the GVHD mice.

2. Methods

2.1. Cloning, expression and purification of rBTNL2-Ig

The extracellular domain of mouse BTNL2 (aa27–452) was cloned and fused into a pCMV6-AC-FC-S expression vector containing the constant region of mouse IgG2a (ORIGENE, Rockville, MD). The vectors were transfected into HEK-293 cells. The fusion protein was purified from the supernatant using Protein G Sepharose 4 Fast Flow according to the manufacturer's instructions (GE Healthcare). Purified proteins were verified by SDS-PAGE, Coomassie Staining and Western blot. Protein was quantified using the PierceTM BCA Protein Assay Kit (Pierce, Rockford, IL). The endotoxin level was determined by the Limulus Amebocyte Lysate (LAL) assay. Control Ig (recombinant mouse IgG2a Fc protein) was purchased from BXCel (West Lebanon, NH) or produced in our laboratory with the same expression vector, HEK-293 cells, and purification process as rBTNL2-Ig protein.

2.2. SDS-PAGE and Western blot

Purified rBTNL2-Ig was loaded on a 12% SDS-PAGE, and either stained with Coomassie blue or transferred to a polyvinylidene fluoride membrane. The protein-containing membrane was incubated with HRP conjugated anti-mouse IgG2 antibody and developed with Super Signal® West Pico chemiluminescent Substrate (Thermo Scientific).

2.3. LAL assay

The endotoxin level in the purified protein was determined by the endpoint chromogenic LAL test according to the manufacturer's instructions (Lonza, Walkersville, MD) [19].

2.4 Mice

BALB/c and C57BL/6 mice were purchased from Jackson Laboratory. The mice were used in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Connecticut.

2.5. HSCT Procedure

BM cell suspensions were harvested from mice by flushing the marrow from the femurs and tibias with cold RPMI 1640. Recipients received 900 cGy total body irradiation from a ¹³⁷Cs source (Gammator-50 Gamma Irradiator; Radiation Machinery Corporation, Parsippany, NJ). Two to four hours later, the mice were injected intravenously (i.v.) with 5×10^6 BM with or without a prescribed dose of splenic cells. Groups of mice were then injected i.v. with prescribed dose of rBTNL2-Ig or control Ig at the time of GVHD induction (day 0) and then intraperitoneally (i.p.) with rBTNL2-Ig or control Ig at indicated times.

2.6. Assessment of GVHD

The severity of GVHD was evaluated with a clinical GVHD scoring system. In brief, HSCT recipients in coded cages were individually scored every week for five clinical parameters on a scale from 0 to 2: weight loss, posture, activity, fur texture and skin integrity. A clinical GVHD index was generated by summation of the five criteria scores (maximum index = 10).

Groups of GVHD mice were euthanized 14 days after HSCT, and GVHD target organs were harvested for histopathological analysis. The organs were formalin-preserved, paraffin-embedded, sectioned and hematoxylin/eosin (H&E)-stained. Assessment of tissue damage was performed based on scoring systems previously described [20]. Briefly, liver GVHD was scored on the number of involved tracts and severity of liver cell necrosis; the maximum score is 10. Gut GVHD was scored on the basis of crypt apoptosis and lamina propria inflammation; the maximum score is 8. Lung GVHD was scored on the periluminal infiltrates, pneumonitis, and the severity of lung tissues involved; the maximum score is 9.

2.7. Flow Cytometry analysis

Single cell suspensions of organs and tumors were stained with the fluorochrome-conjugated antibodies protein as described [21–24]. For intracellular staining, the cells were first permeabilized with a BD Cytofix/Cytoperm solution for 20 minutes at 4 °C. Direct or indirect staining of fluorochrome-conjugated antibodies included: CD4, CD8, CD44, CD62L, CD69, Foxp3, and H-2K^b, IFN γ , TNF α , and IL-17A (BioLegend, or BD Biosciences, San Jose, CA, San Diego, CA). The samples were analyzed on a FACSCalibur or LSRFortessa X-20 Cell Analyzer (BD Biosciences). Data analysis was done using FlowJo software (Ashland, OR).

2.8. T cell proliferation assay

Murine CD3⁺ T cells were purified from C57BL/6 mice by an immunomagnetic system (Miltenyi, Auburn, CA), and the purity of the cells was usually >95%. T cells were stimulated with anti-CD3 antibody (Biolegend) in the presence of BTNL2-Ig or control Ig. Proliferative response was assessed by pulsing the culture with 1 μ Ci of [³H] thymidine (PerkinElmer, Inc., Downers Grove, IL) 12 hours before harvest. Incorporation of [³H] thymidine was measured by liquid scintillation spectroscopy (PerkinElmer, Inc.).

2.9. ELISA

The concentration of cytokines IFN γ , TNF α , and IL-17A was determined by the respective ELISA Kit (Biolegend) according to the manufacturer's instructions.

3.0. Statistical analysis

P-values were based on the two-sided Student's t test. A confidence level above 95% ($p < 0.05$) was determined to be significant.

4. Results

4.1 Production and characterization of rBTNL2-Ig fusion protein

We cloned the extracellular domain of mouse BTNL2 to an expression vector that contains the constant region of mouse IgG2a. The vector was then transfected into HEK-293 cells to generate rBTNL2-Ig fusion protein. We found that this system expressed rBTNL2-Ig protein at a high level. This protein was the dominant band in SDS-PAGE for the supernatant before purification. We purified rBTNL2-Ig protein from the supernatant using Protein G. As shown in Figure 1A, a relatively high purity of rBTNL2-Ig protein was obtained, as determined by Coomassie blue-stained SDS-PAGE (lane 2). The identity of the fusion protein was verified by Western blot using an anti-mouse IgG2a antibody (lane 3). The actual molecular weight (MW) of the rBTNL2-Ig protein was higher than the predicted MW, suggesting that the recombinant protein was glycosylated. According to the NetNGlyc 1.0 server prediction, BTNL2 has 3 potential N-glycosylated sites. The endotoxin level was less than 0.01 EU/ml of 1 μ g of purified rBTNL2-Ig. *In vitro* assays show that rBTNL2-Ig protein inhibits anti-CD3-induced T cell proliferation in a dose-responsive manner (Figure 1B), consistent with the previous reports [17, 18].

4.2. rBTNL2-Ig treatment ameliorates GVHD

To determine whether BTNL2 treatment affects GVHD, we used a well-defined MHC-mismatched HSCT model [C57BL/6 (H2^b) $\rightarrow$ BALB/c (H2^d)] [25]. GVHD in this model is mediated by both donor CD4 and CD8 T cells [25]. BALB/c mice were lethally irradiated and injected i.v. with BM and splenic cells from allogeneic C57BL/6 mice. The recipient mice were then treated with rBTNL2-Ig or control Ig. As shown in Figure 2A-C, control Ig-treated recipients revealed gradual body weight loss and all had succumbed by day 35 after HSCT (Figure 2A-C). In contrast, rBTNL2-Ig reduced the mortality and morbidity of GVHD with 50% of the mice still surviving at day 90 post HSCT (Figure 2A-C). GVHD severity was confirmed with pathologic analysis, showing that pathology scores of the liver,

lung and small intestine (SI) in rBTNL2-Ig-treated recipients were significantly lower than those in control Ig-treated recipients (Figure 2D, E). It is worth noting that there were not significant differences in the survival, weight change, and clinical GVHD scores between control Ig-treated recipients and untreated recipients (data not shown). The results suggest that *in vivo* administration of rBTNL2-Ig ameliorates GVHD in mice.

4.3 rBTNL2-Ig inhibits T cell proliferation and activation *in vivo*

We then investigated the mechanisms by which rBTNL2-Ig ameliorates GVHD. Since the severity of GVHD is associated with the proliferation, survival and alloactivation of donor T cells [6, 26–28], we first analyzed the effect of BTNL2 on T-cell proliferation *in vivo*. Splenocytes from C57BL/6 mice were labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE), and adoptively transferred into lethally irradiated BALB/c recipients that were also injected i.v. at day 0 and i.p. at day 2 with 50 µg rBTNL2-Ig or control Ig protein. The recipients were analyzed for the proliferation of donor T cells in the spleen at day 4. CFSE dilution analysis revealed that rBTNL2-Ig decreased the percentage of CFSE^{lo} proliferating CD4⁺ and CD8⁺ T cells (Figure 3A, B), indicating that rBTNL2-Ig inhibits the proliferation of the donor T cells. We then analyzed Annexin V⁺ 7-ADD-apoptotic donor CD4⁺ and CD8⁺ T cells, and found that rBTNL2-Ig treatment did not significantly affect T cell survival, as compared to control Ig treatment (data not shown).

We next examined the expression of activation markers by CD4⁺ and CD8⁺ T cells. CD69 is an activation marker. We observed that rBTNL2-Ig decreased the percentages of CD69⁺ in donor CD4⁺ and CD8⁺ T cells (Figure 3C, D). Since naïve T cells are CD44^{lo}CD62L^{hi}, whereas effective memory T cells are CD44^{hi}CD62L^{lo}, we analyzed the percentages of CD44^{hi} and CD62L^{lo}. As shown in Figure 3E-H, rBTNL2-Ig treatment decreased the percentages of CD44^{hi} and CD62L^{lo} cells in CD4⁺ and CD8⁺ T cells, although the percentages of CD62L^{lo} cells in CD8⁺ T cells were not significantly different between control Ig- and rBTNL2-Ig-treated groups. Collectively, our data suggest that rBTNL2-Ig inhibits the proliferation and activation of donor T cells in response to alloantigens in mice.

In order to determine whether reduced T cell proliferation results in decreased donor T cells in rBTNL2-Ig-treated mice, we analyzed the percentages of donor T cells in the allogeneic recipients 14 days after HSCT. At this time point, almost no BM-derived donor T cells were generated. Therefore, the injected T cells could be measured exclusively [20]. As shown in Figure 3I and J, the percentages of CD4⁺ and CD8⁺ donor T cells in the spleen of rBTNL2-Ig-treated mice was significantly lower than that in control-treated mice. The results suggest that the reduced T cell expansion results in reduced donor T cells in rBTNL2-Ig-treated mice.

4.4. rBTNL2-Ig reduces Th1 and Th17 cytokine production *in vivo*

Inflammatory T cells such as Th1/Th17 cells are thought to play a critical role in the development of GVHD. We measured Th1/Th17 cytokine production in the serum of rBTNL2-Ig or control Ig-treated GVHD recipients. We found that the production of Th1 cytokines IFN γ and TNF α , as well as Th17 cytokine IL-17A was significantly reduced in rBTNL2-Ig-treated mice (Figure 4A).

We also analyzed intracellular cytokine profiles in CD4 and CD8 T cells from rBTNL2-Ig and control Ig-treated GVHD recipients. Similar to the serum results, significantly lower percentages of IFN γ -, TNF α -, and IL-17A-producing CD4 and CD8 T cells were observed in rBTNL2-Ig-treated mice, as compared to that in control Ig-treated mice (Figure 4B-F). Collectively, the results suggest that rBTNL2-Ig inhibits Th1 and Th17 cytokine production in the GVHD mice.

4.5 rBTNL2-Ig-treated mice have an increased percentage of Treg cells

Studies have shown that Tregs also play an important role in the prevention of GVHD [29–36]. It has been reported that BTNL2 can induce the development of Foxp3-expressing T cells with a regulatory cell phenotype and function *in vitro* [37]. We thus analyzed Tregs in rBTNL2-Ig or control Ig-treated GVHD recipients. As shown in Figure 5, rBTNL2-Ig treatment resulted in a significantly higher percentage of Tregs in the spleen.

5. Discussion

We show here that *in vivo* administration of rBTNL2-Ig attenuates GVHD in mice. This is related to the ability of rBTNL2-Ig to inhibit T cell proliferation, activation and Th1/Th17 cytokine production, and to enhance the generation of Tregs *in vivo*. To the best of our knowledge, this is the first report that rBTNL2-Ig fusion protein is able to inhibit T cell responses and GVHD *in vivo*. The results are consistent with our *in vitro* data and those from others that rBTNL2-Ig inhibits the proliferation and cytokine production of effective T cells, and enhances the generation of Tregs *in vitro* [17, 18, 37].

The B7 family members typically contain IgV and IgC domains in the extracellular portion. BTNL2 shares sequence and structural similarity with B7 family members. The extracellular region of BTNL2 contains two IgV-IgC pairs (IgVa-IgCa and IgVb-IgCb) [17, 18]. Human and mouse BTNL2 share 63% identity in amino acid sequence. Although human BTNL2 has an isoform that lacks the IgCa domain [17, 38], it is likely that human and mouse BTNL2 proteins function similarly, because in the B7 family it is the IgV domain that mediates receptor binding [39]. BTN molecules typically contain an intracellular B30.2 domain, whereas B7 molecules do not. BTNL2 does not have the B30.2 domain, suggesting that BTNL2 is most similar to B7 molecules. The lack of the B30.2 domain also suggest that BTNL2 may not be capable of signaling itself; rather it may act via delivery of a signal into cells expressing its cognate receptor [40]. However, since BTNL2 has an unusual structure, it is not clear whether it represents a *bona fide* gene or pseudogene in humans.

The BTNL2 mutation has been associated with inflammatory autoimmune diseases [38, 41, 42]. For example, the sarcoidosis-associated polymorphism rs 2076530 has over-activated T cells and overt inflammation that are caused by a G–A transition in BTNL2 resulting in the loss of its inhibitory function [38]. Studies have also linked BTNL2 polymorphism to increased risk of ulcerative colitis [43–45], tuberculosis [46], rheumatoid arthritis, and systemic lupus erythematosus [47]. It has been reported that BTNL2 is highly expressed in lymphoid tissues including the lymph nodes, spleen and thymus, as well as in immune cells, such as B cells, T cells, and macrophages [17, 18]. BTNL2 is also expressed in some of the GVHD target organs, such as intestine and lung [17, 18, 48]. In addition, BTNL2 expression

was upregulated in inflamed colon [17]. Therefore, endogenous BTNL2 may play a role in the prevention of autoimmune disease in physical conditions, but may not be sufficient to prevent GVHD and autoimmune disease in pathological conditions.

The BTNL2 putative receptor is expressed on activated T and B cells [17, 18]. Although the nature of the receptor remains unknown at this stage, it is distinct from CD28, CTLA4, ICOS, BTLA and PD-1 [17, 18]. Like other T cell inhibitory molecules, BTNL2 inhibits proximal TCR signaling events [18], suggesting that BTNL2 may bind to an ITIM-containing receptor [40]. Our results and those from others show that BTNL2 inhibits the proliferation of purified T cells, suggesting that BTNL2 directly acts on T cells. Although the putative BTNL2 receptor is also expressed on B cells, it does not affect B cell proliferation [17, 18]. It remains to be determined whether BTNL2 also indirectly acts on T cells by affecting antigen presenting cells, such as dendritic cells.

In addition to the inhibition of the proliferation, activation and cytokine production of effective T cells, the effect of BTNL2 on Tregs may also play a role in ameliorating GVHD. We have shown that rBTNL2-Ig treatment significantly increased the generation of Tregs in GVHD mice. This is consistent with the results that BTNL2 induces the development of Tregs *in vitro* [37]. It has also been shown that the BTNL2-induced Tregs are similar to thymus-derived Tregs [37]. It remains to be determined whether the increased production of Tregs in BTNL2-Ig-treated mice is caused by enhanced development of naturally Tregs in the thymus and/or induced Tregs in the periphery.

In summary, we have demonstrated that *in vivo* administration of rBTNL2-Ig reduces T cell immune responses and ameliorates GVHD *in vivo*. Our results suggest that rBTNL2-Ig may offer a new tool to prevent and treat GVHD in patients. In addition, targeting the BTNL2 pathway may represent a new strategy to modulate T cell-mediated immunity for the treatment of autoimmune disease and cancer.

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Abbreviations:

HSCT	hematopoietic stem cell transplantation
GVHD	graft-versus-host disease
BTNL2	Butyrophilin-like 2
rBTNL2-Ig	recombinant BTNL2-IgG2a Fc
BTN	Butyrophilin
BTNL	BTN-like
Tregs	regulatory T cells

SI	small intestine
CFSE	Carboxyfluorescein diacetate succinimidyl ester
LAL	Limulus Amebocyte Lysate

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Highlights

- *In vivo* administration of rBTNL2-Ig protein ameliorates GVHD in mice
- rBTNL2-Ig protein inhibits T cell proliferation, activation and Th1/Th17 cytokine production *in vivo*
- rBTNL2-Ig protein treatment increases the generation of regulatory T cells

Figure 1. Characterization of purified rBTNL2-Ig protein. (A) Gel and blot show purified rBTNL2-Ig protein; Lane 1: MW markers; 2: Coomassie blue-stained SDS-PAGE; 3: Western blot with an anti-mouse IgG2a antibody. (B) rBTNL2-Ig protein inhibits T cell proliferation *in vitro*. T cells were purified from splenocytes of C57BL/6 mice by magnetic separation. The cells were cultured on plates pre-coated with anti-CD3 antibody (1 μ g/ml) and indicated doses (ng/ml) of rBTNL2-Ig or control Ig for 3 days. [3 H] thymidine (1 μ Ci/well) was added to the cultures 12 hours before harvest. T cell proliferation was measured by [3 H] thymidine incorporation. Results are expressed as counts per minute (CPM). The data are expressed as mean $\pm$ SD and representative of 3 independent experiments. * P<0.05 compared with control Ig.

Figure 2.

rBTNL2-Ig ameliorates GVHD. Lethally irradiated BALB/c recipients were injected with 5×10^6 BM and 2.5×10^6 spleen cells from C57BL/6 mice, as well as $50 \mu\text{g}$ rBTNL2-Ig or control Ig at day 0. The recipients were then injected i.p. with $50 \mu\text{g}$ rBTNL2-Ig or control Ig at 3-day intervals for 30 days. (A-C) Recipients were monitored for (A) survival (A Kaplan-Meier survival curve is shown), (B) weight change, and (C) clinical GVHD. (D, E) In separate experiments, recipients given $50 \mu\text{g}$ rBTNL2-Ig or control Ig at 3-day intervals from days 0–12 were euthanized 2 weeks after HSCT. The SI, liver and lung were analyzed for histologic damage. (D) Representative photomicrographs (the magnification was X200), and (E) mean $\pm$ SD of histopathology scores. Pooled data from 3 separate experiments are represented; with 4–5 mice per group in each experiment. * $P < 0.05$ compared with control Ig-treated mice.

Figure 3.

rBTNL2-Ig inhibits T-cell proliferation and activation in response to alloantigens in mice. (A-H) Lethally irradiated BALB/c mice were injected i.v. with 5×10^6 BM cells and 10×10^6 splenic cells labelled (A, B) with or (C-H) without CFSE from C57BL/6 mice. The recipients were injected i.v. at day 0 and i.p. at day 2 with 50 μ g rBTNL2-Ig or control Ig. At Day 4 post-transplant, the percentage of (A, B) CFSE^{lo} (shaded histograms: splenic cells labelled with CFSE before injection into mice), (C, D) CD69⁺, (E, F) CD44^{hi}, and (G, H) CD62L^{lo} cells in donor CD4 and CD8 T cells (H-2K^b+CD4⁺, or H-2K^b+CD8⁺) in the spleen

were examined by flow cytometry. Dot lines: control Ig; solid lines: rBTNL2-Ig. (I, J) Lethally irradiated BALB/c mice were injected with BM and splenic cells, and then given 50 μ g rBTNL2-Ig or control Ig as in Figure 2D. The percentages of donor CD4⁺ and CD8⁺ T cells were analyzed 2 weeks after HSCT. (A, C, E, G, I) Representative flow cytometric profiles and (B, D, F, H, J) mean $\pm$ SD from one of three independent experiments with similar results. * P<0.05 compared with control Ig group.

Figure 4. rBTNL2-Ig reduces Th1 and Th17 cytokine production *in vivo*

(A) Serum cytokine profile in control- or BTN-treated recipients. Lethally irradiated BALB/c mice were injected with BM and splenic cells, and then injected with 50 μ g rBTNL2-Ig or control Ig as in Figure 2D. Serum was harvested at day 14 after HSCT and indicated cytokines were measured by ELISA. (B-F) Lethally irradiated BALB/c recipients were injected i.v. with 5×10^6 BM and 2.5×10^6 spleen cells from C57BL/6 mice. The recipients were then injected i.p. with 50 μ g rBTNL2-Ig or control Ig at 3-day intervals for 27 days. At Day 30 after BMT, intracellular cytokine profiles of splenic CD4⁺ and CD8⁺ T cells were analyzed. (B, D) Representative flow cytometry profiles, (C, E, F) statistical analysis showing the percentages of IL-17A⁺, TNFα⁺ and IFNγ⁺-producing CD4⁺ or CD8⁺ T cells. * P<0.05 compared with control Ig group.

Figure 5. rBTNL2-Ig treatment increases the percentage of Tregs in GVHD recipients. Lethally irradiated BALB/c recipients were injected i.v. with 5×10^6 BM and 2.5×10^6 spleen cells from C57BL/6 mice. The recipients were treated with 50 μ g rBTNL2-Ig or control Ig at 3-day intervals from days 0–12 as in Figure 2D. Fourteen days after BMT, the spleens were harvested and analyzed for CD4⁺CD25⁺Foxp3⁺ Tregs. (A) Flow cytometry files showing the expression of CD25 and Foxp3 in gated donor CD4⁺ cells; (B) Mean $\pm$ SD for the percentage of Tregs from one of three independent experiments with similar results. * $P < 0.05$ compared with control Ig group.