Anti-CD3 Antibody Ameliorates Transfusion-Associated Graft-Versus-Host Disease in a Chemotherapy-Based Mouse Model With Busulfan and Fludarabine

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ABSTRACT

To establish a transfusion-associated graft-versus-host disease (TA-GVHD) mouse model with busulfan and fludarabine for effective treatment evaluation. BALB/c (H-2d) mice were injected with busulfan (15 mg/kg) and fludarabine (30 mg/kg) twice a day for 4 days. The mice were transfused with 10^6 T cell-depleted bone marrow (TCD-BM) and cells in different groups 3 days after chemotherapy: syngeneic BALB/c, MHC minor mismatch DBA/2 (H-2d), or MHC major mismatch C57BL/6(H2-b). Recipient BALB/c mice were injected with either blood only or blood+splenocyte. TA-GVHD was monitored in terms of body weight loss, clinical scores, and survival. Dexamethasone (50 mg/kg), cyclophosphamide (50 mg/kg), cyclosporine A (30 mg/kg), and anti-CD3 (1 mg/kg) were injected to each group to examine the treatments. Blood transfusion alone is insufficient to induce TA-GVHD in a chemotherapy-based mouse model. A MHC-mismatched TA-GVHD model can be induced by splenocyte and blood transfusion. This MHC-mismatched TA-GVHD model was resistant to dexamethasone treatment. Treatment based on anti-CD3 monoclonal antibody slightly ameliorated TA-GVHD. Treatment effectiveness was associated with T-cell depletion following activation by anti-CD3.

Busulfan and fludarabine chemotherapy regimen can be used to establish a TA-GVHD mouse model. Anti-CD3 monoclonal antibody is a potential alternative to treat TA-GVHD.

Key words: transfusion-associated graft-versus-host disease, fludarabine, busulfan, animal model, major histocompatibility complex

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INTRODUCTION

Transfusion-associated graft-versus-host disease (TA-GVHD) is a fatal complication of blood transfusion and is accounted for 90% to 100% mortality (1). TA-GVHD is a syndrome similar to GVHD induced by bone marrow transplantation. Immunosuppressive patients who receive blood transfusion containing immune-active lymphocytes are prone to serious clinical syndromes (2). With extensive applications of immunosuppressive nucleotide analogs, such as fludarabine, the incidence of TA-GVHD increases annually (3). TA-GVHD usually occurs in immunosuppressive and immunodeficient patients. The pathogenesis and mechanism of TA-GVHD are complex and likely similar to GVHD in patients who have undergone bone marrow transplantation. Infused donor lymphocytes can be activated, undergo proliferation and differentiation; these lymphocytes then attack GVHD-targeted organs and thus cause TA-GVHD (4). The severity of TA-GVHD is correlated with the number and immune activity of T lymphocytes in blood components. TA-GVHD generally occurs 2 weeks after blood transfusion (5). The main possible targets of TA-GVHD include the skin, digestive system, bone marrow, and lungs. However, the clinical manifestations of this disease are complex and atypical.

TA-GVHD progresses rapidly and presents poor prognosis. With the increasing application of immunosuppression therapy, the incidence of TA-GVHD has also significantly increased (3). This disease is commonly treated with large doses of dexamethasone (Dex), antithymocyte globulin (ATG), and immune inhibitors, such as cyclosporine A (CsA) and cyclophosphamide (CTX). However, none of these treatments can effectively reduce mortality (2). Therefore, the development of effective treatments is of high clinical value. For instance, some mouse models have been established by directly transfusing human cells into immunodeficient mice. However, these models may be unsuitable for clinical simulations. With high mortality but limited cases in clinics, mouse models are necessary to investigate TA-GVHD.

Anti-CD3 monoclonal antibody (mAb) has provided many applications in clinical practices, including treatments for renal transplantation rejection, graft-versus-host disease, and type 1 diabetes (6). Anti-CD3 mAb pretreatment can effectively separate graft versus leukemia (GVL) from GVHD (7,8). In this study, treatments with anti-CD3 antibodies and other drugs, including CsA, CTX, and Dex, were examined by using a mouse model of TA-GVHD with busulfan and fludarabine chemotherapy after splenocyte and blood transfusion was conducted.

MATERIALS AND METHODS

1. Reagents: Busulfan was purchased from Kyowa Hakko Kylin Pharmaceutical Company. Fludarabine was obtained from Guangdong South of the Five Ridges Pharmaceutical Company. RPMI 1640 medium, fetal bovine serum, and 1× PBS were purchased from HyClone Company. Heparin was obtained from Changzhou Qianhong Technology Company. Red blood cell lysate was purchased from Beijing Soledad Bao Technology Company. Trypan Blue was obtained from Sigma Company. CD3 bead antibody was purchased from Miltenyi Company. Dexamethasone was obtained from Jinan Limin Pharmaceutical Co., Ltd., and cyclophosphamide was purchased from Shanxi Pude Pharmaceutical Company.

2. Mice: Six- to ten-week-old male C57BL/6 (H-2Kb) & DBA/2 (H-2Kd) and eight- to twelve-week-old male BALB/c mice were purchased from Shanghai Silaike Experimental Animal Company. These mice were maintained in pathogen-
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free rooms in the Animal Experimental Center of Fujian Medical University (FMU). Animal use protocols were approved by the FMU institutional review committee and performed in accordance with animal ethical standards.

3. Transfusion cell preparation: C57BL/6 or DBA/2 mice were euthanatized. In each mouse, spleen was harvested, and blood from the heart was collected. The spleen was meshed to a single-cell suspension. Blood from the heart was pretreated with heparin and rinsed five times with transfusion buffer (10% FBS in PBS). Splenocytes were counted using Trypan Blue. Cell suspension (500 µl) was transfused into the mice via tail vein injection.

4. T-cell depleted bone marrow (TCD-BM): C57BL/6 or DBA/2 mice were euthanized, and bone marrow samples were harvested from the tibia, femur, and humerus. Anti-CD3 antibody magnetic beads (Miltenyi) were used to negatively select or remove T lymphocytes from the bone marrow.

5. Chemotherapy: The mice were injected intraperitoneally with the required dose of busulfan and fludarabine (total volume of 500 µl) on the basis of their weights.

6. Scoring of TA-GVHD: After transfusion was completed, the mice were monitored in terms of TA-GVHD occurrence and survival. In general, the clinical evidence of TA-GVHD includes weight loss, poor posture, low activity, poor fur texture, poor skin integrity, and diarrhea. The assessment of clinical TA-GVHD was scored on a scale from 0 (none) to 2, as described in our previous publication (9). The scales are defined as follows: for weight loss, 0=less than 10%, 1=10% to 25%, and 2=more than 25%; for posture, 0=normal, 1=hunching noted only at rest, and 2=severe hunching impairs movement; for activity, 0=normal, 1=mild to moderately decreased, and 2=severe ruffling/poor grooming; for skin integrity, 0=normal, 1=scaling of paws/tail, and 2=obvious areas of denuded skin; for diarrhea, 0=normal, 1=mild (occurred for only 1 day), and 2=persistent diarrhea (lasted for more than 3 days).

7. Pathology scoring: Ten days after transplantation, GVHD target organs (liver, colon, lungs, and skin) were fixed with formaldehyde, dehydrated, embedded in paraffin, sliced, and stained with H&E. The assessment was scored as previously described (10,11).

8. Measurement of serum cytokine: Serum cytokine concentration was measured using ELISA kits (BD Biosciences Pharmingen) (7).

9. Flow cytometry: The following anti-mouse mAbs were purchased from BD Biosciences Pharmingen (San Jose, CA), eBioscience (San Diego, CA), BioLegend (San Diego, CA), or R&D Systems (Minneapolis, MN): TCRβ (H57-597), CD11b/Mac-1 (M1/70), H-2b (AF6-88.5), B220 (RA3-6B2), and Thy1.2(30-H12). Annexin kit was purchased from Roche Company. Cell surface staining was measured using a 4-laser MoFlo Immunocytometry System (Dako, Glostrup, Denmark). Data were analyzed using FlowJo software (Tree Star, Ashland, OR).

10. Statistical analysis: Survival was evaluated by log-rank test with GraphPad Prism version 4.0 (GraphPad). Means were compared by unpaired two-tailed Student’s t-test.

RESULTS

Establishment of MHC-mismatched TA-GVHD model by splenocyte and blood transfusion

Most reports on TA-GVHD cases indicated that patients have received whole blood transfusion in clinics. TA-GVHD patients have usually presented immunosuppression and/or immunodeficiency under chemotherapy. TA-GVHD
occurs more often after treatment with nucleotide analogs. To establish a mouse model relevant to clinical practice, we combined the chemo-reagent busulfan and the nucleotide analog fludarabine, which are common drugs used to condition patients undergoing bone marrow transplantation. Afterward, we tested whether transfused blood alone can induce TA-GVHD in mouse models. The mice were injected with busulfan (15 mg/kg) and fludarabine (30 mg/kg) twice a day for 4 days. Three days after chemotherapy, the mice were transfused with 1 ml of whole blood and $10^6$ TCD-BM from C57BL/6 (MHC-mismatched model) or with 1 ml of whole blood and $10^6$ TCD-BM from DBA/2 (MHC-matched model). We observed the mice for 80 days and did not find any signs of GVHD (Fig. 1A). All the mice survived for more than 80 days (Fig. 1B). These results indicated that blood transfusion alone in a mouse model cannot effectively induce TA-GVHD after chemotherapy.

TA-GVHD and GVHD share many similarities with each other. TA-GVHD requires activation of transfused lymphocytes to proliferate and attack target organs, such as the lungs intestines. We highly suspected that blood transfusion alone did not carry enough lymphocytes to induce observable TA-GVHD. Thus, we transfused splenocytes and blood to the chemotherapy-treated mice to establish a suitable model. Titration doses of splenocytes, blood, and TCD-BM from C57BL/6 were injected. Busulfan- and fludarabine-treated mice were then transfused with different doses of splenocytes and blood (Group A at a dose of $50 \times 10^6$ splenocytes and 1000 µl of blood; Group B at a dose of $25 \times 10^6$ splenocytes and 250 µl of blood; Group C at a dose of $12.5 \times 10^6$ splenocytes and 250 µl of blood; Group D at a dose of $6.25 \times 10^6$ splenocytes and 125 µl of blood). We found that all the mice developed TA-GVHD. The severity of TA-GVHD was determined in a dose-dependent manner. The doses of Group A and Group B mice induced the lethal severity of TA-GVHD. The mice developed severe clinical TA-GVHD, and all the recipients died immediately after transfusion. By contrast, Group C and Group D developed less severe TA-GVHD, and most of the mice survived after transfusion (Figs. 2A and 2B). Histopathology in Group D showed slight lymphocyte infiltration and low pathological score. Group A presented the highest clinical score and shortest survival. Histopathology showed vast lymphocyte infiltration and the highest pathological score (Fig. 2C). Interestingly, the mice transfused with 1 ml of whole blood and $50 \times 10^6$ splenocytes and $10^6$ TCD-BM from DBA/2 (MHC-matched model) did not show severe TA-GVHD, and all the mice survived after 80 days (Fig. 2D). These results indicated that we can induce a MHC-mismatched TA-GVHD model by splenocyte and blood transfusion.
Anti-CD3 treatment in the mouse model for TA-GVHD

Fig. 1. Only blood transfusion in mouse model showed no TA-GVHD
Recipients were transfused 1ml whole blood +10M TCD-BM from C57BL/6 or DBA/2 three days after chemotherapy (busulfan and fludarabine) treatment. (A) % body weigh (B) % survival after transfusion (n=6).

Fig. 2. A dose dependent manner of the TA-GVHD severity in MHC mismatched mouse model.
Chemotherapy (busulfan and fludarabine) treated mice were transfused with different dose of splenocyte and blood (Group A at a dose of 50X10^6 splenocyte & 1000ul blood; Group B at a dose of 25X10^6 splenocyte & 250ul blood;
Group C at a dose of $12.5 \times 10^6$ splenocyte&250ul blood; Group D at a dose of $6.25 \times 10^6$ splenocyte&125ul blood. After transfusion, recipients were monitored for clinical TA-GVHD 1-2 times a week. (A) Clinical score (mean ± SE, N=6). (B) %Survival (n=6). (C) Pathology score (n=4). (D) %Survival of DBA/2 model (n=6)

### Treatment for TA-GVHD in the established mouse model

Given that mice in Group A died immediately, we decided to use Group B for further testing of TA-GVHD treatment. Gamma irradiation for blood components effectively prevented TA-GVHD (12). However, gamma irradiation is time consuming and costly. In some developing countries, few patients at high risk of developing TA-GVHD, such as those who underwent bone marrow transplantation, have received blood components through gamma irradiation. Increasing studies on TA-GVHD have been reported in recent years, but the treatment for this disease remains an obstacle. Therefore, we tested the treatment of TA-GVHD in the established mouse model. In clinical practice, large doses of glucocorticoids and immune inhibitors (such as CsA and CTX) were used to treat TA-GVHD. Recently, anti-CD3-based conditioning regimen was reported to separate GVL from GVHD in animal models (13). Subsequently, we tested these treatments. Mitogenic anti-CD3 can cause cytokine storm (14). To avoid such side effects and determine the safe dose to treat TA-GVHD, we titrated down the dose from 5 mg/kg as described in animal models (13). Serum concentrations of cytokines (IL-2, IL-6, TNF-α, and IFN-γ) were measured at 0.5, 1, 4, and 24 h after treatments. We found that the peak concentration was dependent on the dosage. When anti-CD3 was injected at 1 mg/kg, the peak concentration markedly decreased (Fig. 3A). Accordingly, we used anti-CD3 (1 mg/kg) to treat TA-GVHD. Dexamethasone (Dex, 50 mg/kg) was also used to compare the treatment effects.

Afterward, the mouse models were set up as described in Group B (Fig. 2). All the mice were randomized into PBS group, Dex group, or anti-CD3 group. One week after transfusion, when the mice began to present TA-GVHD, PBS (0.5 ml), Dex (50 mg/kg), or anti-CD3 (1 mg/kg) was injected to treat the disease. All the groups were followed up for 80 days. We found that dexamethasone treatment slightly affected the mouse model, and this finding indicated that this model was steroid refractory. A significant difference existed between the survival of animals treated with anti-CD3 and Dex, and anti-CD3-treated mice showed slightly prolonged survival compared with Dex/PBS controls (p < 0.05, Fig. 3B). Recipients treated with anti-CD3 developed milder TA-GVHD, and about 30% of the mice survived for more than 80 days; by contrast, recipients treated with PBS developed severe TA-GVHD, and majority of the mice died 80 days after transfusion (p < 0.05, Fig. 3B). These results indicated that anti-CD3-mAb treatment ameliorated TA-GVHD in a chemotherapy-based MHC-mismatched transfusion mouse model.
Anti-CD3 treatment in the mouse model for TA-GVHD

Fig. 3. Treatment of TA-GVHD in the mouse model. (A) BALB/c mice were injected with different doses of anti-CD3 (5mg/kg, 3mg/kg, 1mg/kg) or PBS. The serum concentrations of cytokines (IL-2, IL-6, TNF-α, IFN-γ) were shown (n=3); (B) The mice were treated with busulfan&fludarabine and transfused with 25X10^6 splenocyte&250ul blood as described in Group B. All the mice were randomized to PBS group or anti-CD3 group. One week after the transfusion, 0.5ml PBS, Dex (50mg/kg) or anti-CD3 (1mg/kg) were injected i.v. to treat TA-GVHD. All the groups were monitored for clinical TA-GVHD 1-2 times a week and followed up for 80 days. %Survival was shown (n=24). (C) The mice were treated with busulfan&fludarabine and transfused with 25X10^6 splenocyte&250ul blood as described in Group B. All the mice were randomized into different groups. One week after the transfusion, when the mice began to show up TA-GVHD, CTX&Dex (50 mg/kg), CsA (30 mg/kg) and Dex (50 mg/kg), CTX (50 mg/kg) & Anti-CD3 (1 mg/kg) or CsA (30 mg/kg) & Anti-CD3 (1 mg/kg) were injected to treat the disease. All the groups were monitored for clinical TA-GVHD 1-2 times a week and followed up for 80 days. %Survival was shown (n=20-24).

Considering that the use of anti-CD3 antibody slightly improved the model, we further examined whether anti-CD3 antibodies can improve the treatment effect of cyclosporin A (CsA) and cyclophosphamide (CTX), which are drugs used to treat TA-GVHD. Given the created steroid refractory model of TA-GVHD, all the mice were randomized into different groups. CTX and Dex (50 mg/kg), CsA (30 mg/kg) and Dex (50 mg/kg), CTX (50 mg/kg) and Anti-CD3 (1 mg/kg), or CsA (30 mg/kg) and Anti-CD3 (1 mg/kg) were injected as treatments one week after transfusion or when the mice manifested symptoms of TA-GVHD. All the groups were followed up for 80 days. Anti-CD3-treated animals showed prolonged survival compared with Dex-treated mice (p < 0.05, Fig. 3C). However, CsA showed no additive effect to other drugs on this mouse model.

Anti-CD3 mAb treatment of TA-GVHD is associated with apoptosis and T-cell receptor internalization of donor T cells

Given that the treatment effect of anti-CD3 was detected in a chemotherapy-based MHC-mismatched transfusion mouse model, we should determine whether the donor or recipient T cells bound to the antibodies. Thereafter, we checked the chimeric status on the day we injected anti-CD3 (d7) by using several markers (TCR as a T-cell marker; B220 as a B cell marker; Gr-1/Mac1 as a myeloid marker). We found that majority of the T cells, B cells, and myeloid cells were H2-b-positive donor type (Fig. 4A). At 24 h after anti-CD3 injection, T-cell receptor (TCR) internalization was found on the T cells (Figs. 4B and 4C). The T cells also underwent apoptosis in ex vivo culture (Fig. 4D). Overall, anti-CD3 mAb treatment of TA-GVHD is associated with T-cell depletion following activation with anti-CD3. This finding indicated the immunosuppression mechanism of anti-CD3 in treating TA-GVHD by blocking T
cells.

**Fig. 4.** Anti-CD3 mAb treatment of TA-GVHD is associated with the apoptosis and TCRs internalization of donor T cells

(A) The mice were treated with busulfan&fludarabine and transfused with 25X10^6 splenocyte&250ul blood as described in Group B. One week after the transfusion, mononuclear cells(MNCs) from peripheral blood (Orbital blood) were stained for H-2Kb (donor marker), TCR, B220 and Mac-1/Gr-1. One representative FACS pattern from 3 recipients is shown. (B&C) The mice were injected with anti-CD3. (B) mononuclear cells(MNCs) from peripheral blood (Orbital blood) were stained for Thy1.2 (T cell marker) and TCR. One representative FACS pattern of TCRs internalization is shown. (C) Mean fluorescence of anti-TCR-FITC on T cells was measured at 24 hours after antibody injection. One representative FACS pattern of TCRs internalization is shown. (D) Spleen cells (0.5x10^6/well) were stimulated with anti-CD3 in a U-bottom 96-well plate for 48 hours. Cell apoptosis as measured by staining of DAPI versus Annexin V. One representative FACS pattern is shown.

**DISCUSSION**

Transfusion-associated graft-versus-host disease (TA-GVHD) is a rare but almost universally fatal complication of blood transfusion. The risk factors of this disease include the number and immunoreactivity of contaminating lymphocytes in transfusion components. The dominant mechanism of TA-GVHD in immunocompetent and compromised hosts is exposure to viable donor lymphocytes that are responsive against recipients but are not recognized as foreign substances \(^{(3,15)}\). Similar to GVHD, TA-GVHD requires an immunoactive component, difference in MHC between a donor and a recipient, and susceptibility of a patient’s immune system to the engraftment of donor lymphocytes. TA-GVHD is unresponsive to immunosuppressive therapy, and the associated mortality exceeds 90%. Leukoreduction cannot completely eliminate the risk of TA-GVHD. Irradiation (with a preferred irradiation dose of 2500 cGy) of blood components, which inhibit the proliferation of donor lymphocytes, is necessary to prevent TA-GVHD. Therefore, gamma irradiation for blood components remains the main strategy to prevent TA-GVHD in clinical practice \(^{(16)}\). Whole blood treatment with riboflavin and ultraviolet light can also be an alternative to gamma irradiation \(^{(17)}\). Animal models should be established to investigate diseases because of high mortality, low incidence, and unsatisfactory treatment. In several mouse models, human lymphocytes have been transfused into NOD/SCID/IL-2R^null^ mice \(^{(16)}\). TA-GVHD exhibits similar clinical features to GVHD after allogeneic hematopoietic stem cell transplantation, which is characterized by a multisystem and cutaneous involvement. In the current study, we established an animal model by transfusion of
MHC-mismatched splenocytes and blood to recipients treated with combination of busulfan and fludarabine. The mice presented hunch back appearance, diarrhea, hair loss, skin damage, weight loss, and other typical symptoms of GVHD. These observations are partly consistent with those of TA-GVHD patients suffering from rashes, fever, liver dysfunction, and gastrointestinal disorders. Interestingly, blood transfusion alone is insufficient to induce TA-GVHD in the MHC-mismatched mouse model. The mice developed TA-GVHD after the addition of splenocytes. This finding might result from the lymphocyte insufficiency in peripheral blood, probably because that donor lymphocytes were easier to be rejected as foreign bodies (18).

Immunosuppressive regimens involving corticosteroids, antithymocyte globulin (ATG), and immune inhibitors (CsA, CTX) have yielded poor results with few documented survivors (19,20). Some scholars even believe that “there is currently no treatment” (2). In our study, we found that anti-CD3 antibody treatment ameliorated TA-GVHD in the established mouse model. A significant difference existed between the anti-CD3 group and the PBS group, and this finding was consistent with the observation on human patients (21). We hypothesized that the mechanism by which anti-CD3 ameliorated TA-GVHD is immunosuppression. Still, some other mechanisms might also play a role. Anti-CD3 can regulate the expression of chemokine receptors, as well as target organ protective molecules, such as B7H1 and indoleamine 2,3-dioxygenase (9,13). Further research is necessary to determine the mechanisms underlying the improvement of TA-GVHD.

In our study, a classical anti-CD3 (145-2C11) ameliorated TA-GVHD in a chemotherapy-based MHC-mismatched transfusion mouse model. The classical anti-CD3 causes cytokine storm, which can affect the clinical progression of TA-GVHD. In our study, 1 mg/kg anti-CD3 did not cause a clinically observable cytokine storm in TA-GVHD mice. This cytokine storm issue can also be addressed and settled by testing non-mitogenic anti-CD3 mAbs, which have been extensively reported. In our study, one antibody injection was administered, and the anti-CD3 treatment effect is limited. The survival rate of the anti-CD3 group was increased from 10% to 30% in the mouse model. As a consequence, the outcome of TA-GVHD remains very poor. Nevertheless, several injections may enhance the treatment effect. Autologous peripheral blood progenitor cell infusion is also applied in clinical practice (22). In our future study, this therapy combined with anti-CD3 will be used to determine the optimal and clinically applicable regimen and thus improve the survival of patients with TA-GVHD.

CONCLUSION

Busulfan and fludarabine chemotherapy regimen can be used to establish a TA-GVHD mouse model. Anti-CD3 monoclonal antibody is a potential alternative to treat TA-GVHD.

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Compliance with Ethical Standards
Ethical approval: All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

Disclosures
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REFERENCE


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