Protective Effect of Rituximab in Chronic Graft-Versus-Host Disease Occurrence in Allogeneic Transplant patients with Epstein Barr Virus Viremia

Shi-Meng Ji^{1,2,3,4} · Xie-Bing Bao^{1,2,3,4} · Jun Lu⁵ · Xiao Ma^{1,2,3,4} · Tao Tao⁶ ·
Ai-Ning Sun^{1,2,3,4} · De-Pei Wu^{1,2,3,4} · Sheng-Li Xue^{1,2,3,4}

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Abstract B cells are involved in chronic graft-versus-host disease (cGVHD) pathogenesis, and Rituximab may have a therapeutic effect on cGVHD in allogeneic hematopoietic stem cell transplantation (allo-HSCT) patients. Herein, we retrospectively evaluated the prophylactic effect of Rituximab on cGVHD in a group of Chinese allo-HSCT patients. A total of 102 patients, who suffered Epstein Barr virus (EBV) viremia within 100 days after allo-HSCT, were included in this study. Fifty patients received Rituximab (375 mg/m² weekly) for EBV viremia, while fifty-two patients did not receive Rituximab. A competing risk

model was adopted to compare cumulative incidence of cGVHD, cumulative incidence of relapse (CIR) and transplantation-related mortality (TRM) between two groups. Cumulative incidence of cGVHD in the Rituximab group was lower than in controls ($P = 0.0579$). Multi-variate analyses confirmed that Rituximab was an independent factor for the reduction of cumulative cGVHD incidence ($P = 0.0069$). No significant difference was observed in CIR ($P = 0.39$) or TRM ($P = 0.48$) between two groups and 2-year OS and DFS were comparable (OS, $P = 0.667$; DFS, $P = 0.571$). Administration of Rituximab in the early post-transplantation phase may protect against cGVHD in allo-HSCT patients without increasing CIR or TRM.

Shi-Meng Ji, Xie-Bing Bao, Jun Lu and Xiao Ma these authors contributed equally to this work.

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✉ De-Pei Wu
wudepei@suda.edu.cn

✉ Sheng-Li Xue
xue_shengli@sina.com

¹ Department of Hematology, The First Affiliated Hospital of Soochow University, Suzhou, China

² Institute of Blood and Marrow Transplantation, Soochow University, Suzhou, China

³ Jiangsu Institute of Hematology, The First Affiliated Hospital of Soochow University, Suzhou, China

⁴ Collaborative Innovation Center of Hematology, Soochow University, Suzhou, China

⁵ Department of Hematology, Soochow University Affiliated Children's Hospital, Suzhou, China

⁶ Department of Hematology, Loufeng Branch of the First Affiliated Hospital of Soochow University, Suzhou, China

Keywords Rituximab · Chronic graft-versus-host disease · Allogeneic transplantation · Epstein Barr virus viremia

Introduction

Chronic graft-versus-host disease (cGVHD) is the main cause of morbidity, mortality, and impairment of quality-of-life in long-term survivors treated with allogeneic hematopoietic stem cell transplantation (allo-HSCT). Moreover, the incidence of cGVHD has increased rapidly over the past several decades, because patients are treated with haploidentical HSCT or mobilized blood stem cells instead of bone marrow cells due to a lack of availability of matched donors, and older patients are receiving HSCT due to improvements in transplantation techniques [1]. Although management algorithms for cGVHD have been proposed, the treatment response is not as good as previously anticipated, especially for organs like lung and skin with deep sclerotic features manifest [2]. Only half of

cGVHD patients can be cured [3] and as high as 40% of patients may die or relapse during the treatment [4]. There are still no drugs or chemical agents that exhibit notable therapeutic effects on cGVHD. Therefore, developing prophylactic treatments is a plausible alternative strategy for cGVHD management.

Increasing evidence has implicated B cell involvement in cGVHD pathogenesis, demonstrated by the association between the presence of alloantibodies or autoantibodies and cGVHD occurrence, dysregulated B-cell reconstitution, increased B-cell activating factor levels in cGVHD patients, and the therapeutic effect of B-cell depletion therapy on steroid-refractory cGVHD [5–8].

Based on the observation that B-cell depletion in the pre-transplantation period is associated with a low incidence of cGVHD [9], researchers conducted clinical trials and revealed the prophylactic effect of Rituximab on cGVHD [10, 11]. However, until now, no related studies have been conducted in Chinese patients. In this study, we retrospectively analyzed 102 allo-HSCT patients with EBV viremia within 100 days following transplant, 50 of which were given Rituximab to eliminate EBV, to evaluate Rituximab's safety and preventive effect on cGVHD occurrence.

Patients and Methods

Patients

Between January 2012 to August 2014, 685 patients with blood disorders underwent myeloablative (MA) allo-HSCT at the Department of Hematology in the First Affiliated Hospital of Soochow University, and 172 patients were diagnosed as EBV viremia after transplantation. Among 172 patients, 110 patients with EBV viremia before day 100 after transplantation had been followed-up, however, 8 patients were excluded because of active aGVHD or cGVHD at the time of EBV viremia. Overall, 102 patients were included in the present study. The data set was locked and analyzed in October 2015. Patients were treated according to standard transplantation protocols. All patients received a myeloablative conditioning regimen and antithymocyte globulin (ATG; Genzyme, Cambridge, MA, 2.5 mg/kg/d for 4 days), including three patients who underwent matched-sibling peripheral blood stem cell transplantation. Cyclosporine (CsA), mycophenolate mofetil (MMF), and short-term methotrexate (MTX) were adopted for acute graft-versus-host disease (aGVHD) prophylaxis. Patients were categorized as 'low-risk,' 'intermediate-risk,' and 'high-risk' according to the latest classification [12]. No donor lymphocyte infusions were performed in the present study. This study was performed

according to our institutional guidelines. The institutional review board of the First Affiliated Hospital of Soochow University approved this study protocol.

EBV-DNA Load Monitoring

EBV viremia was defined as >500 EBV copies/ml according to Auger, et al. [13]. EBV-DNA load was monitored weekly using a real-time Taqman PCR assay in all allo-HSCT patients, starting on the day of neutrophil engraftment and continuing for 100 days following transplantation. Afterwards, EBV-DNA load was monitored every two weeks until 6 months post-transplantation and at every subsequent outpatient appointment until 1 year post-transplantation. Peripheral blood (PB) samples were collected in ethylenediaminetetraacetic acid (EDTA), and DNA was extracted using the DNA blood kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. DNA quantifications were performed using real-time PCR procedures (DaAn Gene co. Ltd of Sun Yat-sen University in Guangzhou, China).

Treatment of EBV Viremia

Patients with >1000 copies/ml of EBV DNA had their immunosuppressive therapy tapered, 25% reduction every week on 1 month. At the same time, fifty patients were given Rituximab infusions (375 mg/m² weekly) until their EBV-DNA PCR results were negative. The remaining 52 patients were treated using ganciclovir (10 mg/kg/d) due to their economic issues or lack of health insurance.

Cytomegalovirus Monitoring and Treatment

All patients were monitored for cytomegalovirus (CMV) antigenemia twice a week until 100 days following transplantation using PB sampling. CMVpp65 was detected as previously described [14]. Briefly, isolated PB leukocytes were obtained from EDTA-anticoagulated PB and analyzed by indirect immunofluorescence. Patients with CMV viremia were treated using ganciclovir (10 mg/kg/d) as well as foscarnet (120 mg/kg/d) depending on the kidney function.

Definitions

Acute GVHD was diagnosed and graded according to published criteria [15]. For cGVHD diagnosis and grading, the National Institutes of Health consensus criteria were used [16]. Neutrophil engraftment was defined as a blood ANC exceeding 0.5×10^9 cells/L for three consecutive days following transplantation. Platelet engraftment was defined as a platelet count exceeding 20×10^9 cells/L for

three continuous days without transfusion. Disease-free survival (DFS) was defined as survival with no evidence of relapse or progression of malignant disease. Overall survival (OS) was defined as the time from HSCT to the last follow-up or death, regardless of the cause.

Statistical Analyses

In this retrospective cohort study, all data were analyzed using the date of the last censor, with October 1, 2015 as the cut-off date. The primary end point was cumulative incidence of cGVHD. Secondary end points were transplantation-related mortality (TRM), cumulative incidence of relapse (CIR), OS, and DFS. Cumulative incidence estimates were used to evaluate cGVHD, using death and relapse without any cGVHD as competing events. Similarly, TRM and CIR were estimated using competing risks, reflecting the time to relapse and time to non-relapse death, respectively, as competing risks. Multivariate analyses were performed using the Fine and Gray competing risks regression model for cumulative incidence of cGVHD. OS and DFS were calculated using the Kaplan–Meier method, and the comparison of Kaplan–Meier curves between the two cohorts was obtained by log-rank test. Categorical variables were compared between the two groups by Chi square test or Fisher's exact test. IBM SPSS version 19 (SPSS, IBM, NY, USA) and R 3.2.2 software (<http://cran.us.r-project.org/>) were used for all analyses.

Results

Patient Characteristics

Patients consisted of 58 males and 44 females with a median age of 26 years (range 7–57 years). Acute myeloid leukemia (AML) was the most common diagnosis ($n = 37$), followed by acute lymphoid leukemia (ALL; $n = 28$), non-Hodgkin's lymphoma (NHL; $n = 10$), myelodysplastic syndrome (MDS; $n = 10$), severe aplastic anemia (SAA; $n = 8$), chronic myeloid leukemia-blastic phase (CML-BP; $n = 5$), hybrid acute leukemia (HAL; $n = 3$) and primary myelofibrosis (PMF; $n = 1$). Fifty-six patients were classified as high-risk, thirty-one as intermediate-risk, and fifteen as low-risk (including nine patients with nonmalignant disease). Overall, 76 (74.5%) patients were in complete remission (CR) at the time of HSCT, and the remainder were in partial remission or no remission (PR/NR). 67 patients without matched donors underwent HLA-haploidentical HSCT to improve long-term survival, and 35 patients received matched related/unrelated donor HSCT. In addition, 38 patients (37.3%) received stem cells harvested from peripheral blood

(PBSCs); 17 patients (16.7%) received bone marrow stem cells (BMSCs); and 47 patients (46.1%) received both PBSCs and BMSCs. The median dose of MNCs and CD34⁺ cells was 9.26×10^8 cells/kg (range 2.10 – 26.43×10^8 cells/kg) and 3.89×10^6 cells/kg (range 1.21 – 11.01×10^6 cells/kg), respectively. One hundred one patients achieved neutrophil reconstitution and 93 got platelet reconstitution with a median period of 12 days (range 9–24 days) and 14 days (range 9–30 days), respectively. The peri-transplant parameters of the two groups were similar, with the exception of the distribution pattern for gender. The baseline characteristics of all patients are shown in Table 1.

EBV Viremia After Transplantation

In our study, the EBV DNA was negative in 102 patients before transplantation, and they all suffered EBV viremia within 100 days following transplantation with a median time of 54 days (range 14–99 days). In the Rituximab group, the median time of EBV viremia was 53 days following transplantation (range 14–84 days), in the control group, the median time was 55 days (range 25–99 days). The range of the number of EBV DNA copies of 102 patients was 3.05×10^3 – 3.97×10^6 copies/ml. Fifty patients received Rituximab (375 mg/m², weekly) with a median cycle of administration of 2 (range 1–4), and the remaining 52 patients received ganciclovir (10 mg/kg/d) during the same period. All the patients had their immunosuppressive tapered, 25% reduction every week on 1 month, at the same time. The median Rituximab administration time was 64 days after transplantation (range 23–100 days). After treatment, all patients achieved EBV-DNA negativity, and the median time of achieving negativity in the Rituximab and control groups was 14 days and 13 days after the first EBV viremia, respectively. After negativity, 10 patients had EBV viremia again (4 patients in Rituximab group and 6 patients in control group). Three patients in the Rituximab group developed post-transplant lymphoproliferative disorder (PTLD).

Incidence of GVHD

Firstly we assessed the incidence of GVHD between different types of transplantation. Because of the limited numbers of sibling transplant this group of patients were excluded in this comparison. The incidence of aGVHD and cGVHD in matched unrelated donor and haploidentical donor transplantation was comparable (aGVHD, 59.3 vs. 67.2%, $P = 0.504$; cGVHD, 40.6 vs. 46.2%, $P = 0.668$). Acute GVHD developed in 62.0% and 67.3% of Rituximab and control group of patients, respectively ($P = 0.443$). In addition, there was a trend toward reduction of overall

Table 1 Characteristics of patients (N = 102)

Characteristics	Total N = 102	Rituximab N = 50	Controls N = 52	P value
Median, y (range)	26(7–57)	28.5(7–57)	24.5(13–50)	0.115
Sex				0.009
Male	58	35	23	
Female	44	15	29	
Diagnosis				
AML	37	18	19	
ALL	28	10	18	
HAL	3	1	2	
NHL	10	6	4	
MDS	10	9	1	
CML-BP	5	2	3	
SAA	8	4	4	
PMF	1	0	1	
Risk				0.739
Low	15	7	8	
Intermediate	31	17	14	
High	56	26	30	
Disease status				0.751
CR	76	37	39	
PR/NR	17	9	8	
Donor type				0.466
MUD	32	18	14	
MRD	3	2	1	
MMRD	67	30	37	
Sex match				0.158
Matched	56	31	25	
Mismatched	46	19	27	
ABO blood type match				0.703
Matched	55	26	29	
Mismatched	47	24	23	
Stem cell source				0.268
PBSC	38	21	17	
BMSC	17	10	7	
BMSC + PBSC	47	19	28	
Median MNC $\times 10^8/\text{kg}$ (range)	9.26(2.1–26.43)	9.71(2.1–26.43)	9.05(2.48–20.15)	0.428
Median CD34 $^{+} \times 10^6/\text{kg}$ (range)	3.89(1.21–11.01)	3.68(1.90–11.01)	4(1.21–9.18)	0.428
aGVHD prophylaxis				
CsA + MTX + MMF	102	50	52	
aGVHD	66	31	35	0.443
Grade 0–I	27	14	13	
Grade II–IV	39	17	22	
cGVHD	46	18	28	0.07
Mild	22	8	14	
Moderate	16	6	10	
Severe	8	4	4	

AML acute myeloid leukemia; ALL acute lymphoid leukemia; HAL hybrid acute leukemia; NHL non-Hodgkin's lymphoma; MDS myelodysplastic syndrome; CML-BP chronic myeloid leukemia–blastic phase; SAA severe aplastic anemia; PMF primary myelofibrosis; CR complete remission; PR partial remission; NR no remission; MUD matched unrelated donor; MRD matched related donor; MMRD mismatched related donor; PBSC peripheral blood stem cell; BMSC bone marrow stem cell; MNC mononuclear cell; CsA cyclosporine; MMF mycophenolate mofetil; MTX methotrexate; aGVHD acute graft-versus-host disease; cGVHD chronic graft-versus-host disease

Fig. 1 A comparison of cumulative incidence of cGVHD between two groups ($n = 102$). Cumulative incidence of cGVHD in the Rituximab group ($n = 50$) was lower than that of the control group ($n = 52$) at each time point (14.0 vs. 32.7%, 34.0 vs. 50.0%, and 36.1 vs. 54.1% at 6, 12, and 24 months, respectively, $P = 0.0579$)

cGVHD incidence in the Rituximab group compared with the control group; however, this did not reach statistical significance (36.0 vs. 53.8%, $P = 0.07$). The median onset time of cGVHD tended to be later in the Rituximab-treated group (218.5 days [range 107–454 days]) than in the control group (164 days [range 80–539 days], $P = 0.07$). When compared using a competing risk model, Rituximab patients showed a lower cumulative incidence of cGVHD compared with the control group. Cumulative incidence of cGVHD at 6, 12, and 24 months was lower (14.0, 34.0, and 36.1%, respectively vs. 32.7, 50.0 and 54.1%, respectively, in the control group, $P = 0.0579$) (Fig. 1). Furthermore, using multivariate competing risks regression analysis, Rituximab was found to play a protective role in developing cGVHD (Hazard ratio [HR] = 0.37, 95% confidence interval [CI] = 0.1795–0.761, $P = 0.0069$) (Table 2). Consistent with previous studies [17, 18], aGVHD was a risk factor for cGVHD (HR = 2.779, 95% CI = 1.2609–6.126, $P = 0.011$). Notably, patients undergoing transplant at a disease status of PR/NR developed cGVHD less frequently (HR = 0.038, 95% CI = 0.0046–0.314, $P = 0.0024$), which may be due to a higher relapse rate than that of patients of CR status (47.1 vs. 15.8%, $P = 0.009$). The organ predominantly affected by cGVHD was skin ($n = 32$), followed by mouth ($n = 14$), liver ($n = 9$), and gastrointestinal (GI) tract ($n = 4$) and no significant differences between the two groups were observed. The severity of cGVHD made no difference between two groups (data not shown).

Prognosis

The median time of follow-up was 17.6 months (range 1.3–41.8 months). To determine whether the reduction of cumulative incidence of cGVHD was associated with an

increase of disease relapse as reported in previous studies [19, 20], we estimated the CIR. The two-year CIR was 15.1% in the Rituximab group and 17.4% in the control group ($P = 0.39$) (Fig. 2a). Moreover, the 2-year OS was the same (67.0 vs. 67.0% for Rituximab and control patients, respectively, $P = 0.667$), and the 2-year DFS was 78.0% for Rituximab patients and 74.0% for controls ($P = 0.571$) (Fig. 3). Among the whole population, the main cause of death was relapse ($n = 17$). Causes of death are shown in Supplementary Table 1.

Safety of Rituximab

During Rituximab infusion, no adverse events were observed. It has been reported that Rituximab increases post-transplant CMV disease in lymphoma patients [21], so we evaluated the incidence of CMV viremia in our cohort. Fifty-one patients had CMV viremia in the whole population (29 patients in the Rituximab group and 22 patients in the control group). The median time of CMV viremia was 46 days following transplantation in the Rituximab group and 48 days in the control group, respectively. In the Rituximab group, 55.2% (16 of 29 patients) had CMV viremia after Rituximab administration. All patients received ganciclovir with/without foscarnet until CMVp-p65 was negative. We observed that all patients achieved CMV viremia negative status, and the median time of negativity was 14 days following CMV infection in the Rituximab group and 16 days in the control group. Further, transplantation-related mortality (TRM) was estimated, and there was no significant difference in terms of 2-year TRM between the two groups (18.3% in the Rituximab group vs. 12.8% in the control group, $P = 0.48$) (Fig. 2b). In addition, no patients developed neutropenia during or after the treatment course, and the incidence and grade of infection of the two groups were not different.

Discussion

In the present study, we have firstly retrospectively analyzed the prophylactic effect of Rituximab on cGVHD after allo-HSCT in China. Cumulative incidence of cGVHD in patients receiving Rituximab treatment for EBV viremia 100 days following transplantation versus patients with EBV viremia who did not use Rituximab was estimated. We observed that cumulative incidence of cGVHD was lower in the Rituximab group, although the P value did not reach statistical significance, which may be due to the low infusion dose (the median infusion frequency was 2 [range 1–4] with 375 mg/m² administered intravenously weekly). However, multivariate analysis confirmed that Rituximab is an independent protective factor for the development of

Table 2 Multivariable analysis of risk factors for cGVHD

Variable	Hazard ratio (HR)	95% Confidence interval (CI)	P value
Rituximab (no vs. yes)	0.37	0.1795–0.761	0.0069
Age (>26 vs. ≤26 years)	2.009	0.9697–4.163	0.06
Sex (male vs. female)	0.507	0.2453–1.046	0.066
Risk			
Low versus intermedia	0.602	0.2188–1.654	0.32
Low versus high	1.609	0.7086–3.653	0.26
Disease status (CR vs. PR/NR)	0.038	0.0046–0.314	0.0024
HLA match (matched vs. mismatched)	1.337	0.3594–4.977	0.66
ABO blood type match (mismatched vs. matched)	1.354	0.7124–2.574	0.35
Stem cell source			
BMSC versus PBSC	0.547	0.1178–2.540	0.44
BMSC versus BMSC + PBSC	0.396	0.1309–1.2	0.10
CMV viremia (negative vs. positive)	0.792	0.4199–1.494	0.47
aGVHD (no vs. yes)	2.779	1.2609–6.126	0.011

CMV cytomegalovirus

Fig. 2 Cumulative incidence of relapse and transplantation-related mortality. (a) Cumulative incidence of relapse (CIR). The two-year CIR of the Rituximab group and the control group was 15.1% and 17.4%, respectively ($P = 0.39$). (b) Transplantation-related mortality (TRM). The TRM was comparable between two groups ($P = 0.48$)

cGVHD, which suggested that Rituximab may be a candidate for the prevention of cGVHD as demonstrated by previous studies [10, 11].

Fig. 3 Survival analysis of Rituximab and control groups. (a) Overall survival (OS). There was no difference in overall survival between the two groups ($P = 0.667$). (b) Disease-free survival (DFS). The 2-year DFS was 78.0% for the Rituximab group and 74.0% for the control group ($P = 0.571$)

Traditionally, T cells are considered to be the main functioning cells of immunological attack and tissue damage in both aGVHD and cGVHD following allo-HSCT, however, the use of T-cell depletion has impaired overall survival because of excess mortality associated with opportunistic infections and possibly malignant

disease relapse [22, 23]. Following allogeneic hematopoietic stem cell transplantation, donor B-cell reconstitution occurs which results in the aberrant persistence of allo- and autoreactive B cells [24]. Thus several studies have compelled interest in pathological role of B cell in cGVHD, which are implicated as the following several lines of evidence: (1) the H-Y alloantibody, an alloreactive antibody against minor histocompatibility antigens, has a predictive potential for cGVHD development [25]. These antibodies cannot be detected in PB of sex-mismatched patients who receive Rituximab following transplant, and none of these patients developed cGVHD during the course of the study [10]; (2) B cell-activating factor (BAFF), a vital factor for B-cell survival and maturation, is significantly elevated in patients with active cGVHD and leads to failure of physiological negative selection and promotes the survival of autoreactive B cells, which produce auto- and alloantibodies that can lead to cGVHD [11, 26]; (3) B-cell depletion therapy using Rituximab effectively improves several autoimmune diseases, such as rheumatoid arthritis, systemic sclerosis, and systemic lupus erythematosus, whose manifestation features partly share with cGVHD [27]. Based on these results, it is logical to treat cGVHD with B cell ablation.

In year 2000, Voravit's group successfully treated a patient diagnosed as thrombocytopenia associated with cGVHD using Rituximab, which indicates that B cells might play an important role in the pathogenesis of cGVHD [28]. Since then, several groups reported that Rituximab had a therapeutic effect on cGVHD [6, 8]. Afterwards, Suzanne et al. reported that patients receiving Rituximab within six months prior to a reduced intensity conditioning regimen HSCT have a lower incidence of extensive cGVHD [9]; and in 2012 and 2013, two clinical trials demonstrated that Rituximab decreased the incidence of cGVHD and corticosteroid-requiring cGVHD, respectively [10, 11]. Herein, we observed that cumulative incidence of cGVHD in 50 patients receiving Rituximab treatment for EBV viremia was lower than the control group, and multivariate analysis confirmed that Rituximab is an independent protective factor for the development of cGVHD. However, we have to notice that it has been reported that mainly in patients those who capable to generate sufficient numbers of naïve and transitional B cells, B-cell depletion could improve or prevent cGVHD [29]. In this light, Rituximab may have limited efficacy in cGVHD improvement and prevention and this may be an explanation for the unsatisfactory results in the present study.

Notably, in our study, the median onset of cGVHD in the Rituximab group (218.5 days [range 107–454 days]) was later than that of the control (164 days [range 80–539 days]), and we speculate that this is due to a

Rituximab-mediated delay in recovery of donor derived B cells that react with recipient tissues. In addition, a previous study has shown that donor B-cell reconstitution occurs approximately 60 days after total lymphoid irradiation-antithymocyte globulin (TLI-ATG) transplantation [30], and our median time for Rituximab infusion was 64 days, which is coincident with donor B-cell recovery. In a study conducted by A Dominiotto et al. Rituximab (200 mg) was given 5 days after transplantation as prophylaxis to prevent EBV viremia [31]. They observed that the incidence of aGVHD was lower in the treatment group than that of the control group but the incidence of cGVHD was not different. This may be because early Rituximab therapy only affected the donor B cells in the graft, which take part in the pathogenesis of aGVHD, but not the later donor-derived alloreactive B cells [11]. However, in the present study, we did not analyze the effect of Rituximab on aGVHD because the median time of Rituximab administration (64 days, range [23–101 days]) was later than the median onset of aGVHD (41 days, range [3–101 days]).

Differences in grade and affected organs of cGVHD were also compared between the two groups; however, no statistical significance was observed (data not shown). To evaluate whether the use of Rituximab affects the graft-versus-leukemia (GVL) effect in the Rituximab cohort, we also assessed CIR. We did not note any increase in the CIR in the Rituximab group. This suggests that CD20⁺ B cells may not be the critical cell in the GVL effect. But we have to admit that the evaluation of GVL effect may not be accurate because of the disease heterogeneity which may impact diseases control.

In this study, we did not observe any adverse events during or after Rituximab infusion. The incidence of infection and TRM was comparable, and neutropenia was not observed. This may be because the low dose of Rituximab is not sufficient to induce hematological toxicity or antibodies against infectious targets are not affected by Rituximab [6]. However, further immunology studies are warranted to illustrate this fully.

We acknowledge the limitations of this study, such as its retrospective nature, patient heterogeneity, and the shortness of the follow-up time. However, no increase in TRM or Rituximab infusion-associated negative events were observed. In summary, Rituximab can partly reduce cumulative incidence of cGVHD without impairment of overall survival in patients after allogeneic hematopoietic stem cell transplantation, and it should be tested in a prospective randomized trial in a large cohort.

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Compliance with Ethical Standards

Conflict of interest All the authors declare no conflict of interest.

Ethical Approval All procedures performed in studies involving human participants were in accordance with the ethical standards of our institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

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