

# Refractory autoimmune haemolytic anaemia following allogenic haematopoietic stem cell transplantation: successful treatment of rituximab

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## Abstract

**Objective:** To investigate the effectiveness and safety of rituximab in treating autoimmune haemolytic anaemia (AIHA) after allogenic haematopoietic stem cell transplantation (allo-HSCT).

**Methods:** Patients with refractory AIHA following allo-HSCT were treated once-weekly with rituximab 375 mg/m<sup>2</sup> for a total of four doses. In an animal study, recipient CB6F1 mice were conditioned with busulfan/fludarabine and transplanted with splenocytes and T-cell-depleted bone marrow from C57Bl/6 mice. In this animal model, anti-CD20 monoclonal antibody (mAb) was evaluated to see if it could prevent graft versus host disease (GVHD). GVHD was monitored by body weight loss, GVHD clinical scores and the survival of each group of mice. Histopathological analyses of the skin, intestine, liver and lung were used to analyse the severity of GVHD.

**Results:** After rituximab therapy, refractory AIHA was resolved in all four patients as shown by increased haemoglobin levels. B-cell proportions were reduced with a relative increase of the proportions of T-cells following rituximab treatment. None of the four patients experienced chronic GVHD. In the animal model, anti-CD20 mAb treatment reduced GVHD.

**Conclusions:** Rituximab therapy deserves consideration for the treatment of post-HSCT patients with refractory AIHA. Further studies are needed to define the therapeutic role of this anti-CD20 mAb.

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## Keywords

Haemolytic anaemia, haematopoietic stem cell transplantation, rituximab, autoimmune, graft versus host disease

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## Introduction

Autoimmune haemolytic anaemia (AIHA) is characterized by an abnormal immune system that attacks red blood cells and usually this process is mediated by autoantibodies.<sup>1</sup> AIHA can present as a primary or idiopathic disorder or secondary to other diseases, such as autoimmune disorders, malignancies or infections.<sup>2</sup> Allogeneic haematopoietic stem cell transplantation (allo-HSCT) is a curative treatment to haematological malignancies.<sup>3</sup> AIHA can present as an immune disorder secondary to allo-HSCT.<sup>4</sup> With the increasing use of allo-HSCT over the last 20 years,<sup>5</sup> more and more cases of AIHA post-transplantation have been reported.<sup>6–10</sup> AIHA usually occurs between 2 and 25 months after allo-HSCT.<sup>11</sup> Although the incidence rate of AIHA after allo-HSCT for leukaemia/lymphoma is low, it is a severe complication that is responsible for considerable morbidity (more than 50%).<sup>11</sup> Treatment involves immune modulation with corticosteroids and other agents.<sup>12</sup> Little guidance exists on the management of AIHA in post-transplantation patients. This report describes the use of rituximab in refractory AIHA in patients following allo-HSCT using cord blood or haploidentical stem cells.

## Patients and methods

### *Patient population*

This prospective study enrolled consecutive patients that underwent allo-HSCT with

cord blood transplantation (CBT) or haploidentical (peripheral blood stem cells plus bone marrow) transplantation (haplo-HSCT) in the Department of Haematology, Fujian Institute of Haematology, Fujian Provincial Key Laboratory on Haematology, Fujian Medical University Union Hospital, Fuzhou, Fujian Province, China between February 2015 and February 2017. Patients and donors were all tested for human leukocyte antigen (HLA) using high-resolution molecular typing methods for HLA-A, HLA-B, HLA-C, HLA-DRB1 and HLA-DQB1 loci. All the included patients were confirmed to have complete chimerism by short tandem repeat analysis. The prevention of graft versus host disease (GVHD) consisted of the following regime: 10 mg/kg rabbit anti-thymocyte globulin (ATG, thymoglobulin; Sanofi-Aventis, Paris, France) intravenously (i.v.) once daily from day -4 to day -1; 2.5 mg/kg cyclosporine A (Novartis, Basel, Switzerland) orally or i.v. every 12 h (100–250 ng/ml of plasma level) from day -10 to the second or third month if no GVHD was present; 5 mg/kg mycophenolate mofetil (Roche, Basel, Switzerland) orally twice daily from day +7 to the second month; with (haplo-HSCT) or without (CBT) short-term methotrexate (MTX; Pfizer, Sydney, Australia) i.v. once daily (15 mg/m<sup>2</sup> MTX, at day +1, and 10 mg/m<sup>2</sup> at days +3, +7 and +11). Accordingly, acute GVHD (Glucksberg criteria)<sup>13</sup> and chronic GVHD (revised Seattle classification)<sup>14</sup> was Antiviral, antifungal and antimicrobial

agents were given to all patients for prophylaxis according to the institutional transplant guidelines of Fujian Medical University Union Hospital. All patients had AIHA that was refractory to first-line conventional steroid treatment and/or immunoglobulin therapy. Blood haemoglobin levels (Hb) were monitored by routine blood tests at least 2 times a week.

This study protocol was approved by Ethics Review Committee of Fujian Medical University Union Hospital (no. FJMU-IACUC2015-0117). Written informed consent was obtained from the patients or their legal guardians for publication. Copies of the written consents are available from the corresponding author upon reasonable request.

### *Myeloablative and non-myeloablative protocols*

A myeloablative protocol containing 30 mg/m<sup>2</sup> per day fludarabine (i.v. once daily, day -13 to day -9) (Guangdong South of the Five Ridges, Guangzhou, China) and 2 g/m<sup>2</sup> per day cytarabine (i.v. once daily, day -13 to day -9) (Pfizer), followed by 1.8 g/m<sup>2</sup> per day cyclophosphamide (i.v. once daily, day -8 to day -7) (Baxter, Halle, Germany) as well as 0.8 mg/kg busulfan (i.v. four times daily, day -6 to day -4) (Kyowa Hakko Kylin, Beijing, China) (FA5-BUCY) was used to condition patients with acute leukaemia.

A non-myeloablative protocol containing 30 mg/m<sup>2</sup> per day fludarabine (i.v. once daily, day -5 to day -2) (Guangdong South of the Five Ridges) and 30 mg/kg per day cyclophosphamide (i.v. once daily, day -5 to day -2) (Baxter) and 10 mg/kg ATG (Sanofi-Aventis; 2.5 mg/kg i.v. once daily from day -4 to day -1) (FC protocol) was used to condition patients with aplastic anaemia.

### *Rituximab treatment of patients*

Patients were treated with 375 mg/m<sup>2</sup> rituximab (Roche) i.v. once weekly for a total of four doses.

### *Reagents*

The ABO blood group system is defined by the presence or absence of the A and/or B antigens on the surface of red blood cells and by the presence of antibodies in the serum corresponding to the antigen or antigens missing from the red blood cells. The determination of Rh (D) is defined by the presence or absence of the D (RH1) antigen on red blood cells. Anti-A, anti-B, anti-AB, anti-D and anti-D' reagents are used to undertake ABO and Rh blood group typing. In this current study, blood group typing was undertaken using a reverse group test (DG Gel System and Conventional Serology reagents; Grifols Diagnostic, Barcelona, Spain). Phycoerythrin (PE) mouse anti-human CD3, fluorescein isothiocyanate (FITC) mouse anti-human T-cell receptor (TCR), PE mouse anti-human CD19, allophycocyanin (APC) mouse anti-human CD4, allophycocyanin-cyanine dye (APC-Cy7<sup>TM</sup>) mouse anti-human CD8 and PE mouse anti-human CD56 antibodies were purchased from BD Biosciences (San Jose, CA, USA). All the primary antibodies were used at 1:600 dilution at 4°C for 15 min. Flow cytometry was performed on a BD FACSVers<sup>TM</sup> system (BD Biosciences).

For the animal study, pharmaceutical reagents busulfan (Kyowa Hakko Kylin) and fludarabine (Guangdong South of the Five Ridges) were used to condition the mice as described below. RPMI 1640 tissue culture medium, fetal bovine serum and 10 mM phosphate-buffered saline (PBS; pH 7.4) were purchased from Hyclone Laboratories (Logan, UT, USA). Trypan blue was purchased from Sigma-Aldrich

(St Louis, MO, USA). T-cell depletion (TCD) was performed using an anti-mouse CD3 $\epsilon$  MicroBead Kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and purified anti-mouse CD20 monoclonal antibody (mAb; isotype IgG2a, 5D2; Genentech, South San Francisco, CA, USA).

### *Measurement of blood haemoglobin levels*

Whole peripheral blood samples (2 ml) were collected into 4.5  $\mu$ mol/ml ethylenediaminetetra-acetic acid tubes. The blood samples were run immediately on a fully automated haematology analyser (XN-9000; Sysmex, Lincolnshire, IL, USA) to measure the haemoglobin concentration using a non-cyanide haemoglobin method according to the manufacturer's instructions.

### *Mouse model of GVHD*

Male C57BL/6 (8–10 weeks old; 20–26 g weight; H-2Kb as a donor marker) mice were purchased from SLRC Company (Shanghai, China) and used as donor mice. Recipient CB6F1 mice (SLRC Company; 8–10 weeks old; 20–26 g weight) were conditioned with 120 mg/kg busulfan (15 mg/kg; intraperitoneally (i.p.) twice daily from day –5 to day –2) and 80 mg/kg fludarabine (10 mg/kg; i.p. twice daily from day –5 to day –2) as a myeloablative chemotherapy, which was followed by a splenocyte (SPL) and bone marrow (BM) cell transplant to set up the GVHD model mimicking the clinical cases presented above. The C57BL/6 donor mice were euthanized, and the spleen was harvested and the BM was collected. The spleen was meshed to a single-cell suspension. The BM was flushed to a single-cell suspension. SPL and BM cells were stained with Trypan Blue and counted. The conditioned CB6F1 mice were transplanted with SPL and T-cell-

depleted BM (TCD-BM; in which the T-cells were depleted with an anti-mouse CD3 $\epsilon$  MicroBead Kit according to the manufacturer's instructions) from the C57BL/6 donor mice on day 0. The cell suspension volume (500  $\mu$ l) was transfused into the recipient mice via a tail vein injection. The CB6F1 mice were randomly assigned using a computer-generated random number table to one of two groups: mice in the BM group were preconditioned with busulfan and fludarabine and injected with  $5 \times 10^6$  TCD-BM cells; and mice in the SPL group were preconditioned with busulfan and fludarabine and transplanted with  $1 \times 10^8$  SPLs and  $5 \times 10^6$  TCD-BM cells. Control mice were CB6F1 mice treated with myeloablative chemotherapy conditioning without SPL or TCD-BM cell transplantation.

For the anti-CD20 injection experiment, on day 1, recipients were given one injection of purified anti-mouse CD20 mAb (40 mg/kg; isotype IgG2a, clone 5D2; Genentech) or 10 mM PBS (pH 7.4; 0.25 ml per mouse) as a control. GVHD was monitored by the loss of body weight, GVHD clinical scores and the survival of each group. In general, the clinical signs of GVHD include weight loss, poor posture, low activity, poor fur texture, poor skin integrity and diarrhoea. The assessment of clinical transfusion associated-GVHD was scored on a scale from 0 (none) to 2 as follows: for weight loss, 0 = <10%, 1 = 10–25%, and 2 = >25%; for posture, 0 = normal, 1 = hunching noted only at rest, and 2 = severe hunching that impairs movement; for activity, 0 = normal, 1 = mild to moderately decreased, and 2 = stationary unless stimulated; for fur texture, 0 = normal, 1 = mild to moderate ruffling, and 2 = severe ruffling/poor grooming; for skin integrity, 0 = normal, 1 = scaling of paws/tail, and 2 = obvious areas of denuded skin; for diarrhoea, 0 = normal, 1 = mild (occurred for only 1 day), and 2 = persistent

diarrhoea (lasted for >3 days). Histological samples of the skin, intestine, liver and lung were harvested at day 10 to analyse the severity of GVHD. The histological samples were fixed in 10% formalin, embedded in paraffin blocks and routinely sectioned. Tissue sections (5 µm) were stained with haematoxylin (Tianlian Huagong, Shanghai, China) and eosin (Sanaisi, Shanghai, China). The tissue sections were visualized with an Olympus microscope (CX31; Olympus America, Melville, NY, USA) and a Pixera camera (150CL; Pixera, Los Gatos, CA, USA). Lymphocyte infiltration and tissue damage were scored. Sections were scored by a histopathologist (J.F.H.) blinded to the experimental groups on the basis of the following criteria. Skin sections: epidermis (0, normal; 1, foci of interface damage in <20% of the section with occasional necrotic keratinocytes; 2, widespread interface damage in ≥20% of the section); dermis (0, normal; 1, slightly altered with mild increased collagen density; 2, marked increased collagen density); inflammation (0, none; 1, focal infiltrates; 2, widespread infiltrates); fat (0, normal; 1, reduced number of normal adipocytes; 2, serous fat atrophy); and follicles (0, normal number of hair follicles, >5 follicles per linear mm; 1, 1–5 follicles per linear mm; 2, <1 follicle per linear mm). Intestinal sections: crypt apoptosis (0, rare to none; 1, occasional apoptotic bodies per 10 crypts; 2, few apoptotic bodies per 10 crypts; 3, the majority of crypts contain an apoptotic body; 4, the majority of crypts contain >1 apoptotic body); inflammation (0, none; 1, mild; 2, moderate; 3, severe, without ulceration; 4, severe, with ulceration). Liver sections: bile duct injury (manifest by nuclear hyperchromasia, nuclear crowding, infiltrating lymphocytes and cytoplasmic vacuolation) and inflammation (infiltration with lymphocytes, neutrophils and eosinophils). Lung sections: disease was scored between 0 and

4 based on the number of involved tracts and the severity of disease in each tract (0, none; 1, few involved tracts with mild involvement; 2, numerous involved tracts but with only mild disease; 3, injury in the majority of tracts; 4, severe involvement of most tracts). Chimerism was studied by flow cytometry as follows. Peripheral blood was collected on day 10 and the cells stained with PE rat anti-mouse H2Kd/FITC rat anti-mouse H2Kb, APC rat anti-mouse B220 and PE-Cy7<sup>TM</sup> rat anti-mouse TCR (all primary antibodies from BD Biosciences). All the primary antibodies were used at 1:600 dilution at 4°C for 15 min. Flow cytometry was performed on a BD FACSVerser<sup>TM</sup> system (BD Biosciences).

All animals were maintained in a pathogen-free room in the Animal Experimental Centre of Fujian Medical University. The mice were exposed to a 12-h light/12-h dark cycle with free access to standard rodent chow and water. Animal use protocols were approved by the Institutional Review Committee of Fujian Medical University (no. SYXK2012-0001) and the experiments were undertaken in accordance with the ethical standards for animal research.

### Statistical analyses

All statistical analyses were performed using GraphPad Prism software version 5.0 (GraphPad Software, San Diego, CA, USA). Clinical scoring and survival in the different groups were compared using rank sum test or log-rank test. Comparison of two means was undertaken using an unpaired two-tailed Student's *t*-test. A *P*-value < 0.05 was considered statistically significant.

### Results

Four patients with refractory AIHA following allo-HSCT were treated with rituximab

**Table 1.** Clinical characteristics of four patients with refractory autoimmune haemolytic anaemia subsequent to allogeneic haematopoietic stem cell transplantation (allo-HSCT) that participated in this study of the effects of rituximab.

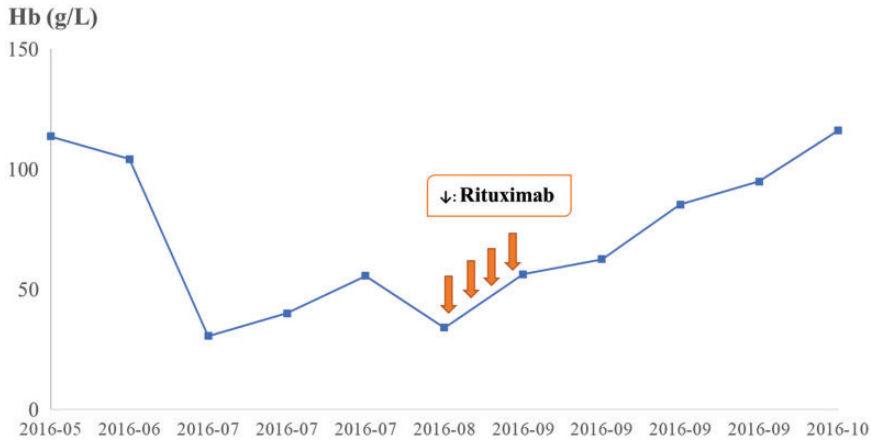
Case number	Disease	Type of allo-HSCT	Virus infection	Other complications	Immunosuppressant agents used
Case 1	ALL	CBT	CMV + BKV + HBV	Haemorrhagic cystitis	ATG + MMF + CsA
Case 2	AA	Haplo-HSCT	CMV	Graft versus host disease	ATG + MMF + CsA + MTX
Case 3	AML	CBT	CMV + BKV	Renal insufficiency	ATG + MMF + CsA
Case 4	AML	CBT	CMV + BKV + JCV	Haemorrhagic cystitis	ATG + MMF + CsA

ALL, acute lymphocytic leukaemia; CBT, cord blood transplantation; CMV, cytomegalovirus; BKV, BK virus; HBV, hepatitis B virus; ATG, anti-thymocyte globulin; MMF, mycophenolate mofetil; CsA, cyclosporine A; AA, anaplastic anaemia; Haplo, haploidentical; MTX, methotrexate; AML, acute myeloid leukaemia; JCV, JC virus.

in this current study. The clinical details of the four cases are presented in Table 1. Three of the patients underwent myeloablative CBT with either the same blood type or a different blood type between the donor and recipient using a myeloablative FA5-BUCY protocol. All three patients were diagnosed with an acute leukaemia: one had acute lymphocytic leukaemia and two had acute myeloid leukaemia. One patient with aplastic anaemia underwent non-myeloablative haplo-HSCT with the same blood type donor and a reduced-intensity conditioning FC protocol. All four patients developed AIHA within 1 year of their allo-HSCT. Treatment with 1 mg/kg methylprednisolone i.v. once daily for 14 days was administered as first-line treatment in all cases. All patients were refractory to steroid treatment. In three cases, 0.4 g/kg immunoglobulin i.v. once daily for 5 days was also administered, but these cases showed no response to immunoglobulins. Therefore, monoclonal antibody therapy with rituximab was reserved for treatment failure and it was given as described in the Methods section. After rituximab therapy, the AIHA in all four patients was resolved as determined by blood haemoglobin levels as shown for one case in Figure 1. The distribution of lymphocyte subsets is presented

for patients that underwent CBT (Table 2) and haplo-HSCT (Table 3). The proportions of T-cells increased and the proportion of B-cells decreased after rituximab treatment. None of the four patients experienced chronic GVHD.

To further study the effect of anti-CD20 in reducing GVHD, the current study aimed to establish an animal model of GVHD. The majority of GVHD mouse models are based on total body irradiation.<sup>15</sup> As radiation was not involved in the development of AIHA in the four patients studied, a more clinically relevant mouse model was required. The current study demonstrated that 120 mg/kg busulfan (15 mg/kg; i.p. twice daily from day -5 to day -2) and 80 mg/kg fludarabine (10 mg/kg; i.p. twice daily from day -5 to day -2) administered to recipient CB6F1 mice, which were then transplanted with SPL and TCD-BM cells 48 h later, could induce GVHD. None of the mice in the BM group had signs of GVHD, with GVHD scores of 0 and they survived for > 120 days. In the SPL group, the mice developed diarrhoea, hair loss and weight loss (Figures 2A and 2B). All of the mice in the SPL group died within 1 month (Figure 2C). Mice in the SPL group had a higher GVHD score and shorter survival



**Figure 1.** Representative changes in blood haemoglobin concentration in one patient with refractory autoimmune haemolytic anaemia subsequent to allogeneic haematopoietic stem cell transplantation (HSCT) before and after treatment with rituximab (375 mg/m<sup>2</sup> once weekly for a total of four doses). The colour version of this figure is available at: <http://imr.sagepub.com>

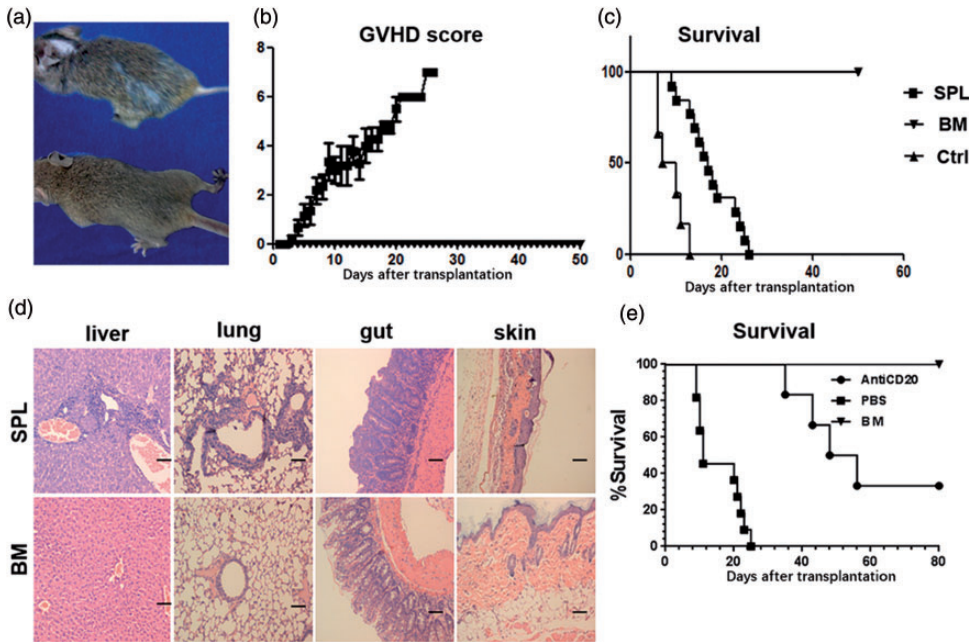
time than mice in the BM group. Histopathology of the mice in the BM group showed almost no lymphocyte infiltration and a lower pathological score. The histopathology of the mice in the SPL group showed higher levels of lymphocyte infiltration and higher mean  $\pm$  SD pathological scores compared with the BM group (liver: SPL  $5.9 \pm 0.2$  versus BM  $0.7 \pm 0.1$ ; lung: SPL  $3.6 \pm 0.2$  versus BM  $1.1 \pm 0.4$ ; gut: SPL  $5.5 \pm 0.2$  versus BM  $0.3 \pm 0.1$ ; skin: SPL  $3.4 \pm 0.5$  versus  $0.0 \pm 0.0$ ;  $P < 0.01$ ) (Figure 2D). Injection of anti-CD20 mAb on the day after the transplant improved the survival rate of GVHD (Figure 2E).

## Discussion

Autoimmune haemolytic anaemia can occur after HSCT or solid organ transplantation.<sup>16</sup> It can be caused by autoimmunity, by passenger lymphocyte syndrome or by an ABO blood mismatch.<sup>17</sup> Antibodies against donor red blood cells (RBCs) derived from recipient lymphocytes can persist after allo-HSCT.<sup>18</sup> This usually happens in ABO major-mismatched HSCT.<sup>19</sup>

The antibodies can also be derived from donor lymphocytes as well.<sup>20</sup> The antibodies can attack host ABO type RBCs (passenger lymphocyte syndrome).<sup>17</sup> It can also happen in ABO minor-mismatched transplantation.<sup>21</sup> Donor plasma cells (developed and differentiated from donor B-cells) produce autoimmune antibodies.<sup>22</sup> These mechanisms contribute to the cause of haemolysis of donor ABO RBCs after engraftment.<sup>17,19,22</sup> AIHA caused by this mechanism can be seen in all types of allo-HSCT including CBT and haplo-HSCT, and even in ABO-matched transplantations.<sup>23</sup> The above mechanisms show that suppression of the plasma cells that are producing antibodies is the requirement for a successful transplantation.

Post-transplantation AIHA poses an extraordinary challenge to transplant surgeons. Despite the advancements in diagnostic tools, therapeutic challenges remain due to the myriad of interacting pathways in AIHA. However, whether AIHA contributes to increased mortality remains controversial. For example, a paediatric study investigated the incidence, risk



**Figure 2.** Investigations into the effects of anti-CD20 monoclonal antibody (mAb) on graft versus host disease (GVHD) and mortality in a mouse model. Busulfan and fludarabine-treated CB6F1 mice were transplanted with  $5 \times 10^6$  T-cell depleted bone marrow (TCD-BM) cells in the BM group and with  $1 \times 10^8$  splenocytes +  $5 \times 10^6$  TCD-BM in the SPL group. Control mice (Ctrl) were CB6F1 mice treated with myeloablative chemotherapy conditioning without SPL or TCD-BM cell transplantation. After transplantation, recipients were monitored for clinical GVHD 1–2 times per week. (A) Representative of photographs of mice from the two treatment groups: upper mouse from the SPL group showing hair loss, weight loss and a hunched posture; and the lower mouse from the BM group showing normal physical status and activity. (B) The GVHD scores for each group of mice ( $n = 6$  each for the SPL and BM groups). The results of the BM group are on the zero line. As the control group was not transplanted, their GVHD scores were not applicable. (C) Percentage survival of the mice ( $n = 13$  per group) in the Ctrl, BM and SPL groups. (D) Representative photomicrographs of histological specimens from mice in the SPL and BM groups (haematoxylin and eosin; scale bar 50  $\mu\text{m}$ ). (E) Survival rates following treatment of mice with anti-CD20 mAb on the day after transplantation ( $n = 12$  per group). The colour version of this figure is available at: <http://imr.sagepub.com>

**Table 2.** Lymphocyte subset distribution in three patients with refractory autoimmune haemolytic anaemia (AIHA) subsequent to allogeneic haematopoietic stem cell transplantation (allo-HSCT) via cord blood transplantation that participated in this study of the effects of rituximab.

Lymphocyte subset	At 3 months after allo-HSCT	At 6 months after allo-HSCT	During AIHA	At 2 months after rituximab treatment
CD3+ lymphocytes	8.94 ± 3.96	18.82 ± 4.07	30.11 ± 5.45	76.58 ± 7.11
CD4+ lymphocytes	5.92 ± 1.45	12.59 ± 4.62	17.62 ± 7.52	20.98 ± 4.01
CD8+ lymphocytes	2.61 ± 1.89	5.85 ± 0.62	10.8 ± 5.59	54.68 ± 3.33
Natural killer cells	51.27 ± 1.15	29.01 ± 14.33	16.97 ± 7.25	12.29 ± 5.04
B-lymphocytes	25.48 ± 5.06	39.57 ± 10.16	51.64 ± 3.67	4.72 ± 1.52

Data presented as mean ± SD proportion of blood cells (%).



**Table 3.** Lymphocyte subset distribution in one patient with refractory autoimmune haemolytic anaemia (AIHA) subsequent to allogeneic haematopoietic stem cell transplantation (allo-HSCT) via haploidentical transplantation that participated in this study of the effects of rituximab.

Lymphocyte subset	At 3 months after allo-HSCT	At 6 months after allo-HSCT	During AIHA	At 2 months after rituximab treatment
CD3+ lymphocytes	86.73	94.76	78.76	95.41
CD4+ lymphocytes	10.91	35.39	39.22	30.55
CD8+ lymphocytes	75.39	42.25	36.77	57.92
Natural killer cells	3.87	2.32	0.93	3.03
B-lymphocytes	9.01	3.06	20.10	0.91

Data presented as proportion of blood cells (%).

factors and outcomes of post-transplant AIHA and found that no significant risk factor for post-transplant AIHA had emerged.<sup>24</sup> Multiple agents for treatment were required, with 12 of 15 (80%) patients achieving complete resolution of AIHA.<sup>24</sup>

It is difficult to treat those AIHA patients post-HSCT who are refractory to steroid treatment. Rituximab, a monoclonal antibody against the CD20 protein, can be used as a treatment.<sup>25</sup> Rituximab has been reported to be an effective treatment for immune haemolytic anaemia in the nontransplant setting.<sup>26</sup> Usually, AIHA occurs concomitantly with Epstein-Barr virus (EBV) and/or monoclonal gammopathy.<sup>23</sup> Rituximab can also be used for treating EBV and monoclonal gammopathy after transplantation.<sup>27</sup> Treating patients with rituximab can result in AIHA and monoclonal gammopathy resolving as well as EBV-DNA becoming undetectable.<sup>28</sup> Rituximab therapy deserves consideration for the treatment of patients with AIHA following allo-HSCT, especially those that cannot be given immunosuppressive therapy. It is also worth noting that patients with immune haemolytic anaemia that persists after the administration of steroids, immunoglobulins and rituximab, require less immunosuppressive treatment that is more specifically targeted to plasma

cells because many patients with immune haemolytic anaemia die from opportunistic infections during immunosuppressive drug therapy.<sup>29</sup>

It is interesting that the proportion of T-cells increased in the four patients with AIHA following rituximab therapy in the current study. In contrast, the proportion of B-cells was reduced after rituximab treatment. T-cells are mainly responsible for acute GVHD, which is why immunosuppressants are widely used when it develops.<sup>30</sup> Chronic GVHD (cGVHD) can be mediated by autoreactive T-cells and B-cells after acute GVHD.<sup>31,32</sup> In these current four clinical patients, the rituximab treatment was given 1 year after transplantation, which means that acute GVHD was unlikely. In this setting, it was not surprising that the increased proportion of T-cells after depleting the B-cells with rituximab treatment did not increase GVHD.

Chronic GVHD is a major cause of late post-transplant morbidity and mortality.<sup>33</sup> It is also interesting that in the present study, none of the patients developed cGVHD. B-cells are thought to play a substantial role in the pathogenesis of cGVHD.<sup>34</sup> In an animal model, the administration of anti-CD20 mAb before the development of any signs of cGVHD prevented the induction of autoimmune-like cGVHD whilst preserving a graft versus leukaemia (GVL)

effect, but there was little effect if the mAb was administered after the onset of cGVHD.<sup>35</sup> The current study found similar findings in the mouse model and the mechanisms involved are still being investigated. There was a clinical response to the anti-CD20 mAb rituximab in the four patients with post-HSCT AIHA described in this current study. Some scholars believe that, in some patients that do not respond to rituximab, the limited therapeutic success of anti-CD20 blockade is associated with altered peripheral B-cell homeostasis and excess levels of the B-cell activating factor of the tumour necrosis factor family.<sup>36</sup> Ongoing research on several small molecule inhibitors is attempting to reduce pathological B-cell populations in an attempt to restore B-cell homeostasis.<sup>37</sup> Taken together, these findings can provide new insights into the prevention and treatment of cGVHD with B-cell-depleting reagents.<sup>36,37</sup>

In conclusion, rituximab therapy deserves consideration for the treatment of patients with post-HSCT refractory AIHA. Further research is needed to better define the optimal treatment schedule and the therapeutic role of other monoclonal antibody immunosuppressive agents.

### Authors' contributions

X.F.L. and N.N.L. participated in data collection, interpretation, drafting and review of article. X.F.L., J.F.H. and N.N.L. participated in the laboratory work. X.F.L. and N.N.L. contributed to data interpretation and revised the manuscript. All authors revised and approved the final version of the manuscript.

### Declaration of conflicting interest

The authors declare that there are no conflicts of interest.

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