Fig. 4. TYF-specific CD8^{+} T-cells recognize TYF peptide in the context of HLA-A*24:02 and effectively lyse K562/A*24:02 loaded with TYF peptide. Cytotoxicities of TYF-specific CD8^{+} T-cells against K562, K562 loaded with TYF peptide, K562/A*24:02, and K562/A*24:02 loaded with TYF peptide were evaluated in ^{51}Cr release assays. Data are representative of two independent experiments and are the mean of triplicate experiments at various E:T ratios.

TYFNLGNKF-specific CD8^{+} T-cells recognize the TYFSLNNKF peptide. TYFNLGNKF-specific CD8^{+} T-cells did not produce IFN-γ against autologous LCLs loaded with TYFSLNNKF peptide (Fig. 5), suggesting that TYFNLGNKF-specific CD8^{+} T-cells are not reactive against AdV serotype 5-infected cells.

3.5. Significance of the TYF epitope in vivo

A PE-labeled HLA-A*24:02 tetramer complexed with the peptide TYFNLGNKF (A*24:02/TYF tetramer) was produced to investigate the significance of the TYF epitope in vivo. The A*24:02/TYF tetramer bound to the TYF-specific CD8^{+} T-cells but not to the irrelevant T-cells, confirming the specificity of the tetramer constructed (Fig. 6A). We analyzed the frequency of the TYF-specific CD8^{+} T-cells using A*24:02/TYF tetramers in a HLA-A*24:02+ patient with Ad11 infection. A 64-year-old female with refractory follicular lymphoma developed Ad11-associated HC at 9 days after receiving multiagent chemotherapy. The patient received supportive care, including hydration and pain management, but did not receive any antiviral drugs. In this case, emergence of TYF-specific CD8^{+} T-cells in peripheral blood coincided with the clearance of Ad11, suggesting that the TYF epitope indeed functions as a target for Ad11-specific CTLs in vivo (Fig. 6B).

4. Discussion

The current study identified a novel HLA-A*24:02 restricted epitope of Ad11 using a reverse immunology approach. Moreover, monitoring Ad11-specific T-cells in a patient with Ad11 disease using the HLA-A*24:02 tetramer complexed with the identified epitope peptide helped to delineate the significance of the identified epitope in vivo.

TYF-specific CD8^{+} T-cells could be induced in all three Ad11-seropositive healthy volunteer donors. In addition, the emergence of TYF-specific CD8^{+} T-cells in peripheral blood coincided with the clearance of AdV in a patient with Ad11 disease. These results suggest that TYF-specific CD8^{+} T-cells can be generated from the majority of the HLA-A*24:02+ Ad11-seropositive healthy donors and that adoptively transferred TYF-specific CD8^{+} T-cells could successfully clear Ad11 in vivo. Possible drawbacks of adoptively transferring virus-specific T-cells generated by stimulating PBMCs with a single immunogenic peptide include the fact that they do not contain CD4^{+} T-cells that provide the necessary help to CD8^{+} CTLs (Moss and Rickinson, 2005) and the fact that the reconstitution of immunity to one epitope may not be sufficient to control AdV disease. Although these issues need to be addressed in future clinical studies, the in vitro and in vivo results of the present study suggests that generating TYF-specific CD8^{+} CTLs from HLA-A*24:02+ Ad11-seropositive donors is a practical and effective approach to treat Ad11 infections among HLA-A*24:02+ patients.

The newly identified HLA-A*24:02 restricted epitope of Ad11 (subgroup B) was located between amino acid positions 37 and 45 of the hexon protein, which was same as those of AdV serotype 5 (subgroup C) (Leen et al., 2004b). The identified epitope of Ad11, TYFNLGNKF, differed from that of AdV serotype 5, TYFSLNNKF, only in two amino acids, as expected by the fact that the location of these epitopes was within the conserved region of the hexon protein (Ebner et al., 2005). However, TYFNLGNKF-specific CD8^{+} T-cells, which were reactive against Ad11-infected cells, did not produce IFN-γ against HLA-A*24:02+ LCLs loaded with TYFSLNNKF peptide derived from AdV serotype 5. Similarly, TYFSLNNKF-specific CD8^{+} T-cells were not reactive against Ad11-infected cells (Leen et al., 2004b). Although previous studies reported that AdV-specific T-cells cross-react with AdV serotypes from different AdV subgroups (Leen et al., 2004b; Tang et al., 2006), these data indicate that AdV-specific T-cells are not necessarily cross-reactive. Thus, determining the subgroup of AdV responsible for infection in individual patients may be necessary before adoptively transferring AdV-specific T-cells.
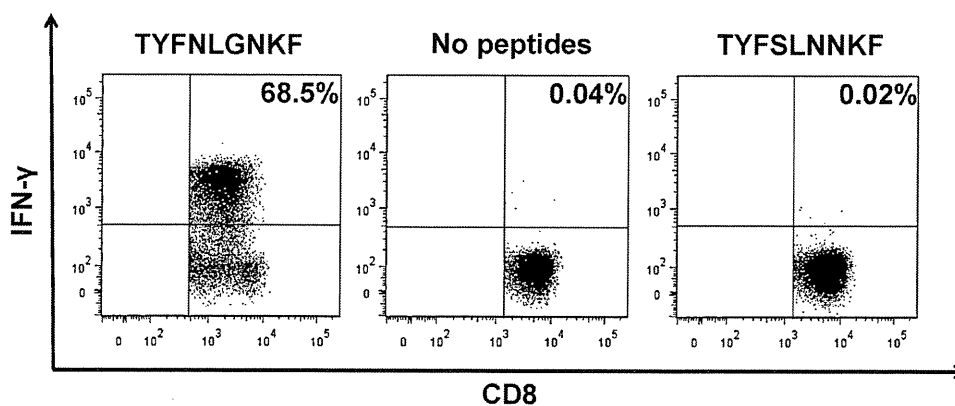

Fig. 5. TYF-specific CD8^{+} T-cells do not recognize an HLA-A*24:02 restricted epitope of AdV serotype 5. TYF-specific CD8^{+} T-cells were incubated with autologous LCLs loaded with TYFNLGNKF (a newly identified epitope of AdV serotype 11), those loaded with TYFSLNNKF (a previously identified epitope of AdV serotype 5), or peptide unloaded autologous LCLs. The numbers in the upper right quadrants are the percentage of IFN-γ-producing cells among CD8^{+} cells upon stimulation. Data are representative of two independent experiments.
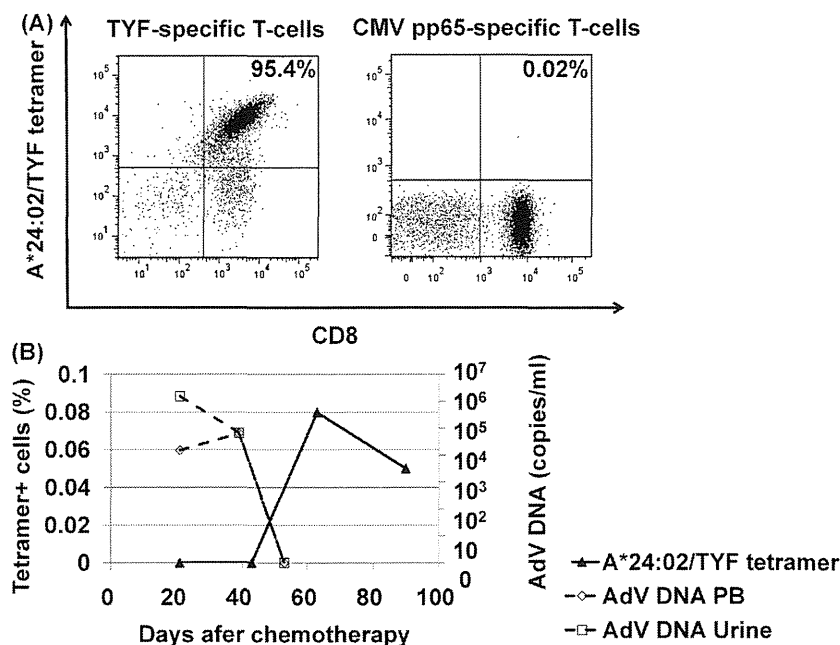
Fig. 6. Frequencies of TYF-specific CD8^{+} T-cells in a patient with Ad11 disease as determined by staining with HLA-A*24:02/TYF tetramer. (A) TYF-specific CD8^{+} T-cells and HLA-A*24:02 restricted CD8^{+} T-cells specific for CMV pp65 peptide QYDPVAALF were stained with PE-labeled HLA-A*24:02/TYF tetramer. Data are representative of two independent experiments. (B) Increase in the percentage of A*24:02/TYF tetramer-positive cells among CD8^{+} cells coincided with the decrease in adenoviral load in urine and peripheral blood in a patient with Ad11 disease.

As preparation of virus-specific T-cells requires several weeks (Leen et al., 2009), early identification of patients at the risk of developing disseminated AdV disease is crucial. Although previous studies suggested that monitoring AdV-specific cellular immunity using Enzyme-linked immunosorbent spot assays or intracellular cytokine assays by flow cytometry might identify these patients (Guerin-El Khourouj et al., 2011; Myers et al., 2007), these methods are too laborious and complex for routine clinical use. In this regard, the MHC tetramer assay is attractive, because it can rapidly quantify virus-specific CD8^{+} T-cells using very simple procedures. Previous studies that monitored CMV-specific CD8^{+} T-cells for prediction of recurrent or persistent CMV infection described the utility of the tetramer assays (Gondo et al., 2004; Gratama et al., 2010). Thus, it is worth exploring whether monitoring Ad11-specific CD8^{+} T-cells with the A*24:02/TYF tetramer can identify patients at high risk for severe Ad11 disease. In addition, the A*24:02/TYF tetramer can be used to monitor the kinetics of adoptively transferred Ad11-specific T-cells, which is essential for the evaluation of the efficacy of the transferred cells. Taken together, these data suggest that the A*24:02/TYF tetramer is a very useful tool with multiple important applications.

CD8^{+} T-cells specific for VYS and LYA peptides were induced from one healthy donor. However, these T-cells were not reactive against HLA-A*24:02+ cells infected with Ad11, indicating that VYS and LYA peptides were not naturally processed and presented by HLA-A*2402+ cells infected with Ad11. These peptides are located in the conserved region of the hexon protein (Ebner et al., 2005), and AdV other than serotype 11 have the same or similar amino acid sequence as these peptides in their hexon protein. In addition, the donor from whom VYS- and LYA-specific CD8^{+} T-cells were induced was seronegative for Ad11. Thus, induced VYS- and LYA-specific CD8^{+} T-cells might be T-cells specific for other serotypes of AdV.

In the current study, as in the previous studies (Kuzushima et al., 2001), the binding affinity of peptides to MHC molecules determined by a computer algorithm (BIMAS) and that determined by a MHC stabilization assay did not correlate very well. One of the

caveats of MHC stabilization assays is that the results would be affected by culture conditions. In this regard, cell free assays capable of directly measuring the binding affinity of peptides to MHC molecules may be beneficial (Liu et al., 2011). In addition, although BIMAS was used to identify potential HLA-A24-binding peptides in the current study, several other epitope prediction algorithms such as one offered at the IEDB website (http://www.iedb.org/) are available. Application of these tools may allow more efficient identification of T-cell epitopes.

In conclusion, we identified a novel HLA-A*24:02 restricted epitope of Ad11, TYFNLGNKF, that could be used to generate Ad11-specific CD8^{+} T-cells for adoptive immunotherapy. A*24:02/TYF tetramers can be used to monitor Ad11-specific CD8^{+} T-cell responses in immunocompromised patients at risk for developing Ad11 disease and following adoptive transfer of Ad11-specific CD8^{+} T-cells.

Conflict of interest

Shingo Toji is a current employee of Medical & Biological Laboratories Co., Ltd. Susumu Suzuki is an advisory role of Medical & Biological Laboratories Co., Ltd.

Acknowledgments

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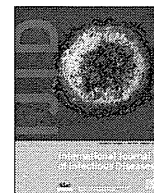
Randomized controlled trial comparing ciprofloxacin and cefepime in febrile neutropenic patients with hematological malignancies

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ARTICLE INFO

SUMMARY

Background: Ciprofloxacin (CPFX) is a potential alternative in patients with febrile neutropenia (FN) because of its activity against Gram-negative organisms. We conducted a non-inferiority, open-label, randomized controlled trial comparing intravenous CPFX and cefepime (CFPM) for FN patients with hematological malignancies.
Methods: Patients aged from 15 to 79 years with an absolute neutrophil count of $<0.500 \times 10^9$/l were eligible, and were randomized to receive 300 mg of CPFX or 2 g of CFPM every 12 h. Initial treatment efficacy, overall response, and early toxicity were evaluated.
Results: Fifty-one episodes were included in this trial, and 49 episodes (CPFX vs. CFPM: 24 vs. 25) were evaluated. Treatment efficacy at day 7 was significantly higher in the CFPM group (successful clinical response: nine with CPFX and 19 with CFPM; $p = 0.007$). The response was better in high-risk patients with neutrophil counts of $\leq 0.100 \times 10^9$/l ($p = 0.003$). The overall response during the study period was similar between the CPFX and CFPM groups ($p = 0.64$). Adverse events were minimal, and all patients could continue the treatment.
Conclusions: We could not prove the non-inferiority of CPFX in comparison with CFPM for the initial treatment of FN. CFPM remains the standard treatment of choice for FN.

1. Introduction

The goal of initial empiric antibiotic therapy for febrile neutropenia (FN) with hematologic malignancies is to prevent serious morbidity and mortality due to bacterial pathogens, until the results of blood cultures are available to guide more precise antibiotic choices. Although Gram-positive bacteria have increased as pathogens in FN during the past 20 years, Gram-negative bacteria are associated with a greater mortality.[1] In particular, *Pseudomonas aeruginosa* infection is associated with a higher mortality,[2] and coverage of this organism remains an essential component of the initial empiric antibiotic regimen. A commonly used therapy for FN is a combination of β-lactam antibiotic and aminoglycoside, which offers a broad spectrum of initial coverage, including *P. aeruginosa*.[3,4]

Although combination therapy with a β-lactam antibiotic and an aminoglycoside has been reported to be highly effective for neutropenic patients,[3,4] aminoglycosides have some serious adverse effects such as renal dysfunction and ototoxicity. Antibiotics as monotherapy are generally less toxic, less costly, and more convenient to administer to patients than combination therapy,[5] so monotherapy with a fourth-generation cephem or carbapenem has been applied and compared to combination therapy in randomized controlled trials; these did not show diminished effectiveness of monotherapy.[6–9] Monotherapy is now also recommended as standard therapy in the Infectious Diseases Society of America (IDSA) guidelines 2010.[10]

However, the incidence of drug-resistant bacterial species in the institute should be taken into consideration when using monotherapy, because resistant bacteria would tend to result in

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treatment failure in the case of monotherapy compared with combination therapy.[5] In fact, extended-spectrum β-lactamase (ESBL)- and metallo-β-lactamase-producing Gram-negative bacteria are emerging at an increasing rate, and these cause significant mortality.[11–13] In this context, alternative effective regimens other than β-lactams are warranted for neutropenic patients to overcome the resistant bacteria.

Ciprofloxacin (CPFX) is an attractive drug that has wide coverage against Gram-negative organisms including *P. aeruginosa*, good pharmacokinetic characteristics, and an absence of the need for drug level monitoring.[14,15] A number of studies have demonstrated that CPFX combined with a β-lactam is effective for neutropenic patients.[16–18] Furthermore, CPFX inhibits DNA gyrase of prokaryotic organisms,[14] and the drug mechanism is completely different from that of β-lactams. Therefore, CPFX may be active for some organisms resistant to β-lactams and it would be acceptable for those who are allergic to β-lactams.[19] In this context, CPFX is a potential alternative for the empiric treatment of patients with FN. However, monotherapy with CPFX has been less well reported and is not well established in the treatment of FN patients.

To assess the possibility of increasing the choice of initial treatment for FN, we designed a randomized controlled trial of intravenous CPFX vs. cefepime (CFPM) in FN patients. This trial aimed to prove its non-inferiority compared to CFPM, a standard therapy for FN.

2. Materials and methods

From January 2005 to December 2009, a non-inferiority, open-label, randomized, multicenter trial was conducted to evaluate the efficacy of intravenous CPFX for FN. The study was approved by both the protocol committee and the institutional review board of each institution. Informed consent was obtained from all patients before registration in this study. The study was registered in the University Hospital Medical Information Network Clinical Trials Registry (UMIN-CTR number: UMIN C000000083) and at Clinical-Trials.gov (identifier: NCT00137787). Randomization was performed automatically, stratified by primary disease and balanced in each institute, at the time of enrollment, on a website operated by the Center for Supporting Hematology-Oncology Trials (C-SHOT) data center.

3. Definitions

Fever was defined as an axillary temperature of not less than 38 °C, or of 37.5–38 °C sustained for more than 1 h. Resolution of fever was defined as a maximum temperature of less than 37.5 °C sustained for three successive days, and the first day was defined as the date the fever disappeared. Fever was considered to be worse when at least one of the following criteria was met: more than 1 °C elevation in maximum body temperature, change from remittent fever to continued fever, emergence of new infectious foci, blood culture positivity after administration of antibiotics, more than 10% fall in arterial O_2 pressure or oxygen saturation, and a decline of performance status.

Episodes of fever were classified as microbiologically documented infection, clinically documented infection, or fever of unknown origin (FUO). Microbiologically documented infection was defined as the isolation of microorganisms. Clinically documented infection was considered when there were foci of infection on physical examination or clinical data, without microbiological documentation. FUO was considered when there was no clinical or microbiological evidence of infection in a febrile episode.

Neutropenia was defined as an absolute neutrophil count (ANC) of $<0.500 \times 10^9/l$ or that from $0.500 \times 10^9/l$ to

$0.100 \times 10^9/l$ showing a decline compared with the level at the last examination. Recovery of neutropenia was defined as an ANC of $\geq 0.500 \times 10^9/l$ sustained for 24 h after ANC had dropped to $<0.500 \times 10^9/l$. The first day was considered to be the recovery date.

3.1. Patients

Patients had to meet all of the following criteria for inclusion in the study: age 15–79 years, at least one episode of fever, neutropenia within 72 h, total bilirubin of 2.0 times the upper limit of normal (ULN) or less, creatinine of 1.5 times ULN or less, and giving informed consent. Patients were excluded if they had a history of allergic reaction to antibiotics, HIV infection, were pregnant or lactating, had a family history of deafness, had received antibiotics in the last 14 days, had received an antifungal or antiviral agent, ketoprofen, or sodium valproate, were infected with bacteria resistant to agents used in this study, were in septic shock, or other inappropriate cases as judged by a physician. If the ANC did not recover to $\geq 1.000 \times 10^9/l$ after the last episode of fever, the patient was also ineligible for this study.

3.2. Treatment

Patients received 300 mg of CPFX or 2 g of CFPM intravenously every 12 h immediately upon the development of FN. Treatment was continued until patients met the criteria for treatment discontinuation as follows: fever absent for more than 48 h (ANC of $\geq 0.500 \times 10^9/l$) or for more than 5 days (ANC from $0.100 \times 10^9/l$ to $0.500 \times 10^9/l$) without any symptoms. If the associated symptoms worsened or were sustained during the study period, the treatment was modified according to the study protocol (Figure 1). From 72 h to 120 h after the study started, an aminoglycoside was added to the treatment if fever symptoms worsened. From 120 h to 168 h, the initial antibiotic was discontinued and the combination therapy of carbapenem (meropenem or imipenem), aminoglycoside, and antifungal agents was started. After 168 h, patients were allowed to receive any treatment as required if fever persisted. Patients could receive granulocyte colony-stimulating factor, if required, at any time.

3.3. Clinical and laboratory evaluations

Clinical symptoms were monitored daily. Blood cell counts were obtained at least twice a week, and biochemical parameters were measured at least once a week. Blood culture, serum endotoxin, β-D-glucan, and chest radiographs were obtained before starting antibacterial therapy and in the case of a sustained or worsened pattern of fever.

3.4. Response criteria

The primary endpoint of this study was the rate of the initial treatment success at day 7. Response to treatment at day 7 was divided into four groups as follows: very effective: fever disappeared with a temperature below 37.5 °C within 4 days and an afebrile state remained for more than 3 days; effective: maximum temperature decreased 1 °C or more within 4 days and an afebrile (below 37.5 °C) state persisted for 7 days; partial response: maximum temperature decreased 1 °C or more within 7 days accompanied by the improvement of clinical symptoms; not effective: maximum temperature did not decrease by 1 °C or more within 7 days and/or no improvement of febrile symptoms. The response to treatment was categorized as a success if patients were
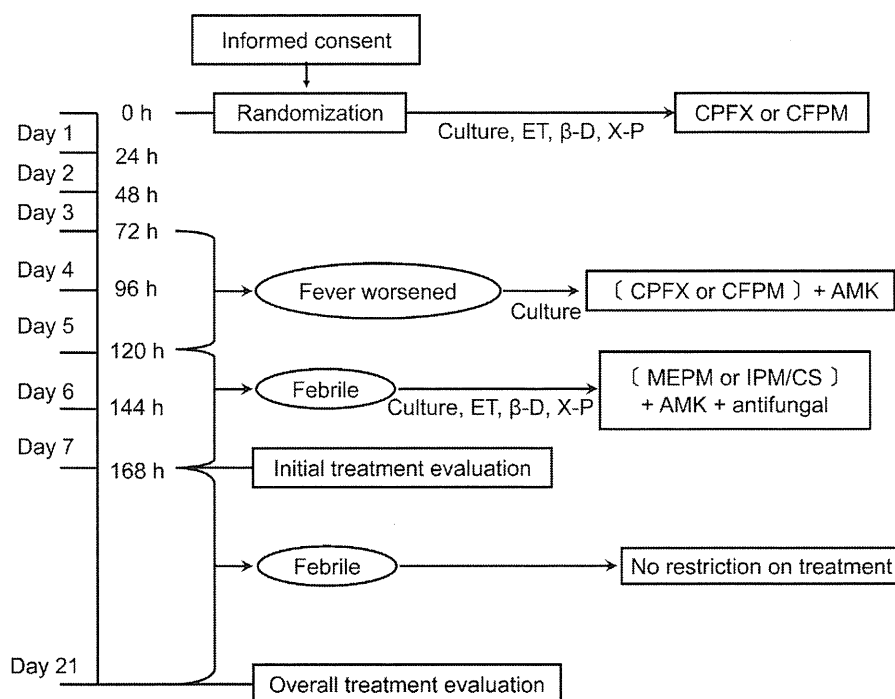
Figure 1. Treatment algorithm for febrile neutropenia. For febrile neutropenia, we treated patients according to the treatment algorithm. Treatment evaluation and treatment modification were performed as shown (CPFX, ciprofloxacin; CFPM, cefepime; ET, endotoxin; β-D, β-D-glucan; X-P, X-ray picture; AMK, aminoglycoside; MEPM, meropenem; IPM/CS, imipenem/cilastatin).

included in either the very effective group or the effective group at day 7.

We also evaluated the overall response rate at day 21 as a secondary endpoint. If patients were able to discontinue the treatment according to the criteria described above, it was considered to be successful.

3.5. Adverse events

Adverse events, regardless of whether they appeared to be related to the use of the study medication, were carefully recorded throughout the study. Causal relationships between the study drugs and adverse events were analyzed using six stages: definitive, probable, possible, unlikely, not related, and not assessable. Adverse events were considered related to the study drug if the stage was definitive, probable, or possible. The severity of the adverse events was classified according to the National Cancer Institute Common Toxicity Criteria version 2.0 (http://ctep.cancer.gov/).

3.6. Statistical analysis

Percentages of comparability of the treatment arms, treatment response, and treatment modification were compared by Chi-square test or Fisher's exact test. Quantitative variables were analyzed by Mann–Whitney test.

The success rates of the CFPM and CPFX arms were estimated to be 50% and 60%, respectively.[7] The δ value of non-inferiority was set to be −15% in accordance with previous reports. The CFPM arm was the reference. To prove the non-inferiority of the CPFX arm, the lower limit of the 95% confidence interval (CI) of the difference of efficacy should exceed the δ value. With a statistical power of 90% and a one-sided type I error of 2.5%, the number of patients required for this study was calculated to be 82 in each arm using a binominal analysis method. Therefore, the total number of accrual was planned to be 100 patients in each arm.

4. Results

4.1. Characteristics of the study population

From January 2005 to December 2009, 51 patients were registered from seven participating institutes in Japan. Forty-nine patients (24 in the CPFX arm and 25 in the CFPM arm) were eligible for assessment, but two patients were excluded because they did not meet the inclusion criteria. Ten patients were enrolled in the study more than once via different episodes of FN. Although we planned to include 200 patients, this study was closed in December 2009 due to slow accrual.

The clinical characteristics of the patients in both treatment arms are listed in Table 1. The distribution of patient sex, diagnosis, treatment for primary disease, neutrophil count at randomization, and duration of neutropenia did not differ between the arms. Acute leukemia was the most common disease in this study (55.1%). Patient age was younger in the CPFX arm than in the CFPM arm (median age 53 vs. 61 years; $p = 0.02$). Four patients were excluded from further analysis of the duration of neutropenia because their neutrophil counts did not exceed $0.500 \times 10^9/l$ ($n = 3$), or their neutrophil counts did not drop below $0.500 \times 10^9/l$ ($n = 1$).

4.2. Type of infection and microbiological outcomes

Of 49 episodes, the responsible bacterium was identified in 11 (22.4%). A Gram-positive coccus was cultured in eight episodes, consisting of one each of methicillin-sensitive *Staphylococcus aureus*, *Staphylococcus haemolyticus*, and *Staphylococcus epidermidis*, and five *Streptococcus* species (Table 2). A Gram-negative bacillus was isolated in three episodes: one each for *P. aeruginosa*, *Klebsiella pneumoniae*, and Pasteurella (Table 2). Ten of the 11 episodes were diagnosed with sepsis and one with meningitis. The other two clinically documented episodes were diagnosed with pneumonia and peritonitis, but no responsible organisms were identified.

Table 1
Characteristics of patients enrolled in the study

Characteristic	CPFX (n = 24)	CFPM (n = 25)	p-Value
Patient sex			
Male	16 (67%)	14 (56%)	0.44
Female	8 (33%)	11 (44%)	
Patient age			
Median	53	61	0.02
Range	21–65	21–79	
Diagnosis			
AML	9 (38%)	11 (44%)	0.72
ALL	4 (17%)	3 (12%)	
CML	3 (13%)	2 (8%)	
MDS	1 (4%)	0	
ML	5 (21%)	5 (20%)	
MM	1 (4%)	3 (12%)	
ATLL	0	1 (4%)	
Myeloid sarcoma	1 (4%)	0	
Treatment for primary disease			
HSCT	0	1 (4%)	0.32
Chemotherapy	24 (100%)	24 (96%)	
Neutrophil count at start of study			
<0.100 × 10^9/l	15 (63%)	18 (72%)	0.19
0.100–0.500 × 10^9/l	6 (25%)	7 (28%)	
0.501–1.000 × 10^9/l	3 (13%)	0	
Duration of neutropenia			
≤7 days	5 (24%)	7 (29%)	0.75
>7days	16 (76%)	17 (71%)	

CPFX, ciprofloxacin; CFPM, cefepime; AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CML, chronic myelogenous leukemia; MDS, myelodysplastic syndrome; ML, malignant lymphoma; MM, multiple myeloma; ATLL, adult T-cell leukemia/lymphoma; HSCT, hematopoietic stem cell transplantation.

4.3. Treatment modification

Ten patients (41.7%) treated with CPFX and 15 patients (60.0%) treated with CFPM received the same treatment without modification (Table 3). For the patients who were judged as febrile by physicians, treatment modifications were performed according to the algorithm described in Figure 1. In the CPFX arm, an aminoglycoside was added to the treatment regimen for 13 patients (54.2%) and CPFX was replaced by other antibiotics for 10 patients (41.7%). In the CFPM arm, an aminoglycoside was added for eight patients (32.0%) and CFPM was replaced for five patients (20.0%). Vancomycin was added for four patients (16.7%) in the CPFX arm, but not in the CFPM arm.

4.4. Efficacy of CPFX and CFPM

The treatment was effective in nine patients (37.5%) in the CPFX arm and 19 (76.0%) in the CFPM arm at day 7 (Figure 2). The difference of the effective proportion was −38.5% (95% CI −64% to −13%), and the lower limit (−64%) did not exceed the δ value of −15%. Furthermore, the efficacy was significantly lower in the CPFX arm (p = 0.007). However, the overall efficacy at day 21 was similar between the two arms (CPFX 83.3% vs. CFPM 88.0%,

Table 2
Microbiological blood culture results on day 0

Infecting microorganisms	CPFX (n = 24)	CFPM (n = 25)
Gram-positive organisms	5 (21%)	3 (12%)
Coagulase-positive Staphylococcus	1 (4%)	-
Coagulase-negative Staphylococcus	2 (8%)	-
Streptococcus	2 (8%)	3 (12%)
Gram-negative organisms	-	3 (12%)
Pseudomonas aeruginosa	-	1 (4%)
Klebsiella pneumoniae	-	1 (4%)
Pasteurella species	-	1 (4%)

CPFX, ciprofloxacin; CFPM, cefepime.

Table 3
Treatment modification

Treatment modification	CPFX (n = 24)	CFPM (n = 25)	p-Value
Initial treatment continued	10 (42%)	15 (60%)	0.20
Modification			
Initial treatment replaced	10 (42%)	5 (20%)	0.10
Add aminoglycoside	13 (54%)	8 (32%)	0.12
Add vancomycin	4 (17%)	0	0.05
Add antifungal agents	8 (33%)	7 (28%)	0.69
Add antiviral agents	2 (8%)	1 (4%)	0.61

CPFX, ciprofloxacin; CFPM, cefepime.

p = 0.64). Patients for whom treatment failed were rescued by treatment modification.

For patients from whom the responsible bacteria were isolated, a treatment response at day 7 was achieved in 20.0% in the CPFX arm and 66.7% in the CFPM arm (p = 0.12, Table 4). Gram-positive coccus infection (16.3%) was more common than Gram-negative bacillus infection (6.1%). The efficacy was better in the CFPM arm (66.7%) than in the CPFX arm (20.0%), but the difference was not statistically significant (p = 0.12). For patients retaining FUO, a treatment response was achieved in 47.1% of patients in the CPFX arm and 78.9% of patients in the CFPM arm (p = 0.05, Table 4).

Since patients with prolonged neutropenia of more than 7 days or profound neutropenia (ANC of ≤0.100 × 10^9/l) are regarded as at high risk in the IDSA guidelines 2010,[10] a subgroup analysis of this population was also conducted. Fewer patients in the CPFX arm than in the CFPM arm had a good clinical response (Table 4).

4.5. Adverse events

Table 5 shows all adverse events within 21 days in both arms. Six events in the CPFX arm compared to two in the CFPM arm were associated with the drug. The most common toxicity was liver dysfunction (16.7% in the CPFX arm and 8.0% in the CFPM arm). Two severe adverse events of grade 3 were observed in the CPFX arm (liver dysfunction and skin rash), and one event in the CFPM arm (liver dysfunction). All patients could continue the study medication without cessation of the therapy due to adverse events.
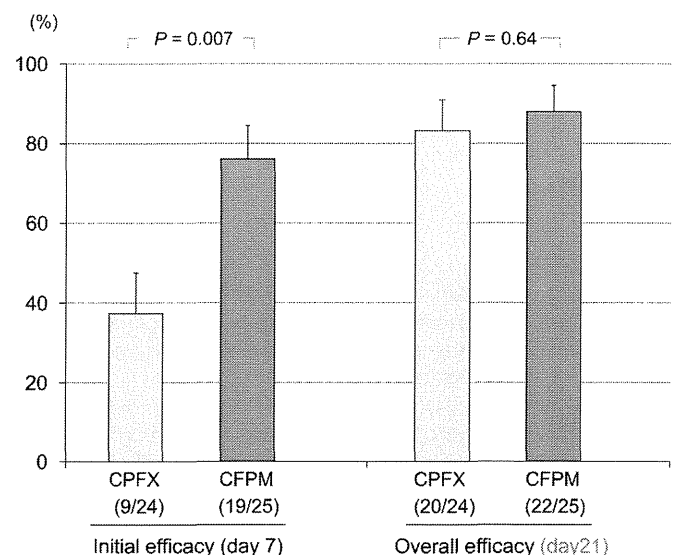

Figure 2. Clinical efficacy of ciprofloxacin and cefepime. Initial treatment evaluation showed a significantly better response in the CFPM arm than in the CPFX arm (76.0% vs. 37.5%, p = 0.007). Overall, treatment evaluation showed almost the same efficacy between the two arms (CPFX, ciprofloxacin; CFPM, cefepime).

Table 4
Response at day 7 by the cause of fever and severity of neutropenia

	CPFX	CFPM	p-Value
Cause of fever			
Microbiologically documented infection	1/5 (20%)	4/6 (67%)	0.12
Sepsis	1/4 (25%)	4/6 (67%)	
Meningitis	0/1	-	
Clinically documented infection (pneumonia and peritonitis)	0/2	-	-
Unknown origin	8/17 (47%)	15/19 (79%)	0.05
Duration of neutropenia[a]			
>7 days	5/16 (31%)	13/17 (77%)	0.02
≤7 days	3/5 (60%)	5/7 (71%)	0.68
Baseline neutrophil count			
≤0.100 × 10^9/l	5/15 (33%)	15/18 (83%)	0.003
>0.100 × 10^9/l	4/9 (44%)	4/7 (57%)	0.61

CPFX, ciprofloxacin; CFPM, cefepime.

[a] Four patients were excluded from the analysis because the neutrophil count did not recover to 0.500 × 10^9/l ($n = 3$), or the neutrophil count did not drop to <0.500 × 10^9/l ($n = 1$).

5. Discussion

The efficacy and safety of CPFX monotherapy for neutropenic patients has not been well investigated. One study showed that patients treated with CPFX had a significantly lower overall success rate than those treated with piperacillin plus amikacin.[20] In contrast, another study comparing CPFX monotherapy with β-lactam plus aminoglycoside showed that the response rate was similar.[19] Furthermore, a prospective randomized study comparing ceftazidime and CPFX as initial therapy also demonstrated that the levels of efficacy were equal.[21] These results imply that the role of CPFX monotherapy for FN has been controversial and needs further assessment because β-lactam-resistant organisms are on the increase.

In this randomized, controlled, open-label trial, we could not prove the non-inferiority of CPFX monotherapy compared with

Table 5
Adverse events within 21 days

Adverse events[b]	Grade	Number of patients in CPFX group ($n = 24$)	Number of patients in CFPM group ($n = 26$)
Cardiovascular	1	0	2
Gastrointestinal	1	2	3
	2	1	1
	3	2	1
	4	1	0
Liver	1	5 (2)[a]	10 (1)[a]
	2	4 (2)[a]	0
	3	1 (1)[a]	1 (1)[a]
Renal	1	1	0
Blood sugar	1	2	3
	2	2	0
Electrolytes	1	3	4
	2	1	1
	3	2	0
Neurological	1	1	1
	2	0	1
	3	0	0
	3	1	0
Cutaneous	1	0	1
	2	2	1
	3	1 (1)[a]	0

CPFX, ciprofloxacin; CFPM, cefepime.

[a] Numbers in the parenthesis indicate the drug-associated events (judged as 'possible' or more).

[b] Adverse events grade is based on the National Cancer Institute (NCI) Common Toxicity Criteria version 2.0.

CFPM. One of the possible reasons for this is that, although CPFX has strong activity against Gram-negative rods, the coverage and activity for Gram-positive cocci including viridans were insufficient.[22,23] In fact, our microbiological data show that the treatment success rate for Gram-positive organisms tended to be inferior in the CPFX arm, and the use of vancomycin was applied only in the CPFX arm.

Another possible reason is that the blood concentration of CPFX might not be adequate because CPFX was administered at a dose of 600 mg/day in this study, a dose that is allowed under the health insurance system in Japan. A recent study demonstrated that only high-dose CPFX (regimens of 400 mg every 8 h or 400 mg every 12 h) can provide good coverage for pathogens with a minimum inhibitory concentration (MIC) of 0.5 μg/ml.[24] This was also confirmed by previous clinical studies, in which monotherapy with CPFX at a low dose (400 mg/day) was not comparable to the standard therapies, but CPFX at a relatively high dose (600 mg/day) was equally effective.[19,20,25] A precise pharmacokinetic study and the provision of an appropriate concentration of CPFX might have led to a better response for FN.[26]

Previous studies have demonstrated that various therapies of CPFX combined with β-lactams such as benzylpenicillin,[27] teicoplanin,[28] and azlocillin,[29] are comparable with the standard therapy for neutropenic patients. In a meta-analysis comparing CPFX plus β-lactam and aminoglycoside plus β-lactam, the former showed better outcomes.[17] Furthermore, CPFX plus β-lactam is reported to be less toxic in terms of nephro- and oto-toxicities.[30] These results suggest that the combination of CPFX with a β-lactam may be a valuable alternative to the more commonly used aminoglycoside plus β-lactam combination in the management of FN.

Assessment of the risk of complications in severe infection is important to determine the type of empiric antibiotic therapy (oral vs. intravenous), the venue for treatment (inpatient vs. outpatient), and the duration of antibiotic therapy.[10] The IDSA guidelines have demonstrated that monotherapy with oral CPFX is acceptable for low-risk patients.[10,31] On the other hand, the guidelines do not recommend monotherapy with CPFX as standard therapy for high-risk patients.[10] We further tried to assess the link between initial treatment response and risk status using both the duration of neutropenia and the neutrophil count as simple biomarkers.[7] Our subgroup analysis showed that among patients at a 'high risk' of neutropenia, those who received CPFX had significantly lower response rates at day 7. In contrast, no significant difference was found for low-risk patients. These results suggest that CPFX monotherapy might be applicable for low-risk FN.

In terms of safety, the two agents appear to have similar safety profiles. The most common adverse event possibly related to the therapies was liver dysfunction, and all the patients could continue therapy.

This trial was prematurely terminated due to slow patient accrual, but not by the predefined early stopping-rule of superiority of CFPM. Prophylactic oral CPFX was not allowed in this study, which might have hindered the accrual. The significance of prophylactic CPFX has been legitimized in recent years.[10] Another possible reason is that this was an open-label randomized controlled trial. Since physicians were able to observe the efficacy of the allocated agents, their impressions might have influenced the slow accrual. Furthermore, the randomization procedure was cumbersome for physicians because fever could occur at any time.

In conclusion, we could not verify the non-inferiority of monotherapy with CPFX to that with CFPM at day 7, although the overall response was similar in both arms. When selecting monotherapy for the treatment of neutropenic patients, CFPM remains the standard initial treatment of choice. CPFX is better for prophylactic than empiric use.

Acknowledgements

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ORIGINAL ARTICLE

Unrelated cord blood transplantation vs related transplantation with HLA 1-antigen mismatch in the graft-versus-host direction

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Little information is available regarding whether an unrelated cord blood (UCB) unit or a related donor with a 1-antigen mismatch at the HLA-A, HLA-B or HLA-DR locus in the graft-versus-host direction (RD/1AG-MM-GVH) should be selected as an alternative donor for patients without an HLA-matched related/unrelated donor. Therefore, we conducted a retrospective study using national registry data on patients with leukemia or myelodysplastic syndrome who received transplantation using a single UCB ($n = 2288$) unit or an RD/1AG-MM-GVH ($n = 525$). We found that the survival rate in the UCB group was comparable to that in the RD/1AG-MM-GVH group, although the RD/1AG-MM-GVH group with an HLA-B mismatch showed significantly higher overall and non-relapse mortality. Neutrophil and platelet engraftment were significantly faster, whereas the incidence of acute or chronic graft-versus-host disease (GVHD) was significantly higher in the RD/1AG-MM-GVH group. The incidence of acute or chronic GVHD in the RD/1AG-MM-GVH group with in vivo T-cell depletion was comparable to that in the UCB group, which translated into a trend toward better overall survival, regardless of the presence of an HLA-B mismatch. In conclusion, UCB and RD/1AG-MM-GVH are comparable for use as an alternative donor, except for RD/1AG-MM-GVH involving an HLA-B mismatch.

INTRODUCTION

For patients who lack an HLA-identical sibling, an HLA-matched unrelated donor (MUD) is considered to be the preferred alternative donor in allogeneic hematopoietic cell transplantation (HCT).[1–5] However, it is difficult to find an MUD for patients with rare HLA haplotypes. Furthermore, it takes at least a few months from the start of an unrelated donor search to actually receive a graft. Therefore, there is a large demand for an alternative source to an HLA-identical sibling or MUD, particularly for patients who have a rare haplotype or who need immediate transplantation.

Unrelated cord blood (UCB) has emerged as a promising alternative source for pediatric and adult patients.[6–17] In UCB transplantation, up to two antigen/allele mismatches between a recipient and cord blood unit are acceptable without an increased risk of acute graft-versus-host disease (GVHD). The clinical outcome in UCB transplantation is improving, and is almost comparable to that in HLA 8/8 allele MUD transplantation, although a high risk of graft failure and early treatment-related complications are still major issues.[15–17]

Another alternative source is an HLA-mismatched related donor, particularly when a related donor with a 1-antigen mismatch at the HLA-A, HLA-B, or HLA-DR locus in the graft-versus-host (GVH)

direction (RD/1AG-MM-GVH) is available. HCT from an RD/1AG-MM-GVH results in a higher but acceptable incidence of acute GVHD.[18–20] In previous studies, HLA mismatches in the host-versus-graft (HVG) direction were associated with a higher incidence of graft failure and lower overall survival (OS).[18,19,21] However, the risk of graft failure might have been improved by the use of conditioning regimens that strongly suppress the recipient's immune system.[22] Therefore, in current clinical practice in Japan, stem cell transplantation from an RD/1AG-MM-GVH is being performed while accepting multiple antigen mismatches in the HVG direction without specific ex vivo stem cell manipulation.[18,19,23] We have recently reported that OS in transplantation from an RD/1AG-MM-GVH involving an HLA-B antigen mismatch was inferior, whereas that from an RD/1AG-MM-GVH involving an HLA-A or -DR antigen mismatch was comparable to that from an 8/8-MUD in standard-risk diseases.[23]

Unlike transplantation from an MUD, transplantation using a UCB unit or an RD/1AG-MM-GVH can be performed immediately when necessary. However, little information is available regarding the priority in selecting these alternative donors. Therefore, we conducted a retrospective study using national registry data on 2813 patients with leukemia or myelodysplastic syndrome (MDS)

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who received transplantation using a single UCB or an RD/1AG-MM-GVH.

MATERIALS AND METHODS

Data collection
Data for patients (age: $\geqslant 16$ years) with acute myeloid leukemia, acute lymphoblastic leukemia, MDS and chronic myelogenous leukemia who received a first HCT using a single HLA 0–2 antigen-mismatched UCB unit or an RD/1AG-MM-GVH between 1 January 1998 and 31 December 2009 were obtained from the Transplant Registry Unified Management Program (TRUMP),[24] which includes data from the Japan Cord Blood Bank Network (JCBBN) and the Japan Society for Hematopoietic Cell Transplantation (JSHCT). Our analysis included 2306 patients who received a single UCB graft (UCB group) and 541 patients who received a graft from an RD/1AG-MM-GVH (RD/1AG-MM-GVH group). As of January 2012, double UCB grafts for HCT are not available in Japan. The following patients were excluded: 26 patients who lacked data on survival status, survival date, sex of recipient, or GVHD prophylaxis and 8 patients who received stem cells that had been manipulated by ex vivo T-cell depletion or CD34 selection. Overall, 2288 patients who received a UCB unit and 525 who received a graft from an RD/1AG-MM-GVH fulfilled the criteria. The study was approved by the data management committees of TRUMP and by the institutional review boards of Japanese Red Cross Nagoya First Hospital and Saitama Medical Center, Jichi Medical University, where this study was organized.

Histocompatibility
Histocompatibility data for the HLA-A, HLA-B and HLA-DR loci were obtained from reports from the institution where the transplantation was performed or from cord blood banks. To reflect current practice in Japan, HLA matching in UCB or RD/1AG-MM-GVH transplantation was assessed by serological data for HLA-A, HLA-B, and HLA-DR loci. An HLA mismatch in the GVH direction was defined as when the recipient's antigens or alleles were not shared by the donor, whereas a mismatch in the HVG direction was defined as when the donor's antigens or alleles were not shared by the recipient.

End points
The primary end point of the study was to compare OS rates between the UCB and RD/1AG-MM-GVH groups. Other end points were the cumulative incidences of neutrophil and platelet engraftment, acute and chronic GVHD, relapse, and non-relapse mortality (NRM). Neutrophil recovery was considered to have occurred when the absolute neutrophil count exceeded $0.5 \times 10^9/l$ for 3 consecutive days following transplantation. Platelet recovery was considered to have occurred when the absolute platelet count exceeded $50 \times 10^9/l$ without platelet transfusion. The physicians who performed transplantation at each center diagnosed and graded acute and chronic GVHD according to the traditional criteria.[25,26] The incidence of chronic GVHD was evaluated in patients who survived for at least 100 days.

Statistical analysis
Descriptive statistics were used to summarize variables related to the patient characteristics. Comparisons between groups were performed with the χ^2-test or extended Fisher's exact test as appropriate for categorical variables and the Mann–Whitney U-test for continuous variables. The probability of OS was estimated according to the Kaplan–Meier method, and the groups were compared with the log-rank test. The adjusted probability of OS was estimated according to the Cox proportional-hazards model, with other significant variables considered in the final multivariate model. The probabilities of neutrophil and platelet engraftment, acute and chronic GVHD, NRM, and relapse were estimated on the basis of cumulative incidence methods, and the groups were compared with the Gray test;[27,28] competing events were death without engraftment for neutrophil and platelet engraftment, death or relapse without GVHD for acute and chronic GVHD, death without relapse for relapse, and relapse for NRM. The Cox proportional-hazards model was used to evaluate variables that may affect OS, whereas the Fine and Gray proportional-hazards model was used to evaluate variables that may affect engraftment, GVHD, NRM and relapse.[29] We classified the conditioning regimen as myeloablative if either total body irradiation > 8 Gy, oral busulfan $\geqslant 9$ mg/kg,

intravenous busulfan $\geqslant 7.2$ mg/kg, or melphalan > 140 mg/m^2 was used in the conditioning regimen, and otherwise classified it as reduced intensity, based on the report by the Center for International Blood and Marrow Transplant Research.[30] For patients for whom the doses of agents used in the conditioning regimen were not available, we used the information on conditioning intensity (myeloablative or reduced intensity) reported by the treating clinicians. Acute leukemia in the first or second remission, chronic myelogenous leukemia in the first or second chronic phase or accelerated phase, and MDS with refractory anemia or refractory anemia with ringed sideroblasts were defined as standard-risk diseases, and other conditions were defined as high-risk diseases. The following variables were considered when comparing the UCB and RD/1AG-MM-GVH groups: the recipient's age group ($\leqslant 50$ years or > 50 years at transplantation), sex of recipient, disease (acute myeloid leukemia, acute lymphoblastic leukemia, chronic myelogenous leukemia or MDS), disease status before transplantation (standard- or high-risk), type of conditioning regimen (myeloablative or reduced intensity), type of GVHD prophylaxis (calcineurin inhibitor and methotrexate, calcineurin inhibitor only, or other), year of transplantation (1998–2004, 2005–2009), and the time from diagnosis to transplantation (< 6 months or $\geqslant 6$ months). In the analysis within the RD/1AG-MM-GVH group, the use of in vivo T cell depletion (no vs yes), stem cell source (peripheral blood (PB) stem cells vs bone marrow (BM)), and the number of HLA mismatches in the HVG direction (0–1 vs 2–3) were also considered. Factors without a variable of main interest were selected in a stepwise manner from the model with a variable retention criterion of $P < 0.05$. We then added a variable of main interest to the final model. All tests were two-sided, and $P < 0.05$ was considered to indicate statistical significance. All statistical analyses were performed with Stata version 12 (Stata Corp., College Station, TX, USA) and EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan).[31] EZR is a graphical user interface for R (The R Foundation for Statistical Computing, version 2.13.0, Vienna, Austria). More precisely, it is a modified version of R commander (version 1.6–3) that was designed to add statistical functions that are frequently used in biostatistics.

RESULTS

Characteristics of patients and transplants
Table 1 shows the patient and transplant characteristics. Recipients of an RD/1AG-MM-GVH were younger than recipients of a UCB unit. Approximately half of the recipients in the RD/1AG-MM-GVH group received PB. The number of HLA mismatches in the GVH direction between a UCB unit and recipient was 0 in 10%, 1 in 33% and 2 in 57%. In the RD/1AG-MM-GVH group, the number of antigen mismatches in the HVG direction was 0 in 12%, 1 in 68%, 2 in 18% and 3 in 3%. Most of the recipients of an RD/1AG-MM-GVH received a calcineurin inhibitor with methotrexate for GVHD prophylaxis, whereas 25% of UCB recipients received only calcineurin inhibitor. In vivo T-cell depletion including antithymocyte globulin (ATG) or alemtuzumab was used in 10% of the RD/1AG-MM-GVH group, but in only 1% of the UCB group. Alemtuzumab was used in only one patient, who received transplantation from an RD/1AG-MM-GVH. Information regarding the dose and type of ATG was missing in two-third of the patients who received ATG. Available data showed that the median dose of thymoglobulin was 2.5 (range 2.5–9.0, $n = 9$) and 2.5 (range 1.25–5.0, $n = 10$) mg/kg and the median dose of ATG-Fresenius was 8.0 (range 5.0–10.0, $n = 3$) and 8.0 (range 5.0–10.0, $n = 7$) mg/kg, in the UCB and RD/1AG-MM-GVH groups, respectively. Two-third of UCB transplantations were performed between 2005 and 2009. The median duration of follow-up for survivors was 2 and 4 years in the UCB and RD/1AG-MM-GVH groups, respectively.

Neutrophil and platelet engraftment
The incidence of neutrophil engraftment at day 50 in the RD/1AG-MM-GVH group was higher than that in the UCB group (UCB group, 73%, 95% confidence interval (CI), 71–75%; RD/1AG-MM-GVH group, 93%, 95% CI, 91–95%; Gray test, $P < 0.001$; Figure 1a). The incidence of platelet engraftment at day 150 in the

Table 1. Patient characteristics

Variable	UCB (n = 2288)	RD/1AG-MM-GVH (n = 525)	P
Age at transplant, median (range)	49 (16–82)	43 (16–74)	<0.001
Recipient sex			
Female	1004 (44%)	239 (46%)	0.494
Male	1284 (56%)	286 (54%)	
Disease			
Acute myelogenous leukemia	1365 (60%)	269 (51%)	0.003
Acute lymphoblastic leukemia	498 (22%)	137 (26%)	
Chronic myelogenous leukemia	124 (5%)	42 (8%)	
Myelodysplastic syndrome	301 (13%)	77 (15%)	
Duration from diagnosis to transplant			
Median time (range), months	7.9 (0.2–768.5)	7.6 (0–251.7)	0.233
Disease risk			
Standard	959 (42%)	249 (47%)	0.050
High	1217 (53%)	257 (49%)	
Unknown	112 (5%)	19 (4%)	
Source of stem cells			
Bone marrow	—	251 (48%)	—
Peripheral blood	—	274 (52%)	
Cord blood	2288 (100%)	—	
HLA compatibility in the graft-versus-host direction			
Matched	225 (10%)	—	<0.001
One-antigen mismatch	753 (33%)	525 (100%)	
Two-antigen mismatch	1310 (57%)	—	
HLA compatibility in the host-versus-graft direction			
Matched	233 (10%)	62 (12%)	<0.001
One-antigen mismatch	716 (31%)	355 (68%)	
Two-antigen mismatch	1339 (59%)	94 (18%)	
Three-antigen mismatch	—	14 (3%)	
Conditioning regimen			
Myeloablative	1390 (61%)	253 (48%)	<0.001
CY + TBI ±	1062	164	
Other TBI regimen	130	20	
BU + CY ±	88	45	
Other non-TBI regimen	110	24	
Reduced intensity	894 (39%)	162 (31%)	
FLU ± TBI ±	840	138	
Other regimen	54	24	
Unclassifiable	4 (0.2%)	110 (21%)	
GVHD prophylaxis			
CSA/TAC + MTX	1410 (62%)	448 (85%)	<0.001
CSA/TAC + MMF	246 (11%)	12 (2%)	
CSA/TAC + Steroid	28 (1%)	13 (2%)	
CSA/TAC only	571 (25%)	45 (9%)	
Unknown	33 (1%)	7 (1%)	
Use of in vivo T-cell depletion			
No	2258 (99%)	472 (90%)	<0.001
Yes	30 (1%)	53 (10%)	
Year at transplant			
1998–2004	760 (33%)	260 (50%)	<0.001
2005–2009	1528 (67%)	265 (50%)	
Follow-up of survivors			
Median time (range), years	2.1 (0.0–10.0)	4.0 (0.1–12.2)	<0.001

Abbreviations: BU, busulfan; CSA, cyclosporine; CY, cyclophosphamide; FLU, fludarabine; MMF, mycophenolate mofetil; MTX, methotrexate; TAC, tacrolimus; TBI, total body irradiation; UCB, unrelated cord blood.

RD/1AG-MM-GVH group was also higher than that in the UCB group (UCB group, 53%, 95% CI, 51–55%; RD/1AG-MM-GVH group, 70%, 95% CI, 66–74%; Gray test, P<0.001; Figure 1b). The use of RD/1AG-MM-GVH was significantly associated with a higher incidence of neutrophil and platelet engraftment in the multivariate analysis (neutrophil engraftment, hazard ratio (HR), 3.46,

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95% CI, 3.00–3.98, $P < 0.001$; platelet engraftment, HR 2.20, 95% CI, 1.89–2.57, $P < 0.001$; Supplementary Table 1). As our previous study revealed that an HLA-B mismatch had an adverse effect on OS in transplantation from an RD/1AG-MM-GVH, patients in the RD/1AG-MM-GVH group with an HLA-A, -B, or -DR mismatch were

Figure 1. Neutrophil (**a**) and platelet engraftment (**b**).

separately compared with the UCB group. We consistently observed superior neutrophil and platelet engraftment in each RD/1AG-MM-GVH group as compared with the UCB group (Supplementary Table 1).

Acute and chronic GVHD

The incidence of grade II–IV or grade III–IV acute GVHD in the RD/1AG-MM-GVH group was significantly higher than that in the UCB group (grade II–IV acute GVHD at day 100: UCB group, 34%, 95% CI, 32–36%; RD/1AG-MM-GVH group, 50%, 95% CI, 45–54%; Gray test, $P < 0.001$; grade III–IV acute GVHD at day 100: UCB group, 11%, 95% CI, 10–13%; RD/1AG-MM-GVH group, 21%, 95% CI, 17–24%; Gray test, $P < 0.001$; Figures 2a and b). The incidence of chronic GVHD or extensive type of chronic GVHD in the RD/1AG-MM-GVH group was also significantly higher than that in the UCB group (chronic GVHD at 3 years: UCB group, 25%, 95% CI, 23–27%; RD/1AG-MM-GVH group, 42%, 95% CI, 38–47%; Gray test, $P < 0.001$; extensive chronic GVHD at 3 years: UCB group, 11%, 95% CI, 10–13%; RD/1AG-MM-GVH group, 29%, 95% CI, 25–34%; Gray test, $P < 0.001$; Figures 2c and d). A multivariate analysis confirmed a higher risk of grade II–IV or grade III–IV acute GHVD, chronic or extensive chronic GVHD in the RD/1AG-MM-GVH group than in the UCB group (grade II–IV acute GVHD; HR 1.64, 95% CI, 1.43–1.90, grade III–IV acute GVHD; HR 2.28, 95% CI, 1.80–2.88, chronic GVHD; HR 1.47, 95% CI, 1.24–1.73, extensive chronic GVHD; HR 2.35, 95% CI, 1.90–2.91, Supplementary Table 2).

OS

The 3-year unadjusted OS rates in the UCB and RD/1AG-MM-GVH groups were 38% (36–41%) and 39% (34–43%), respectively ($P = 0.115$). The use of either UCB or RD/1AG-MM-GVH was not associated with OS rates in the multivariate analysis (UCB vs RD/1AG-MM-GVH, HR, 0.99, 95% CI, 0.87–1.12, $P = 0.833$) in all-risk patients, or either standard-risk ($P = 0.588$) or high-risk patients ($P = 0.639$; Table 2), after adjusting for the following significant risk factors: age > 50 years, male recipient, acute myeloid leukemia vs MDS, high-risk disease, GVHD prophylaxis using only calcineurin inhibitor vs calcineurin inhibitor + methotrexate, and earlier year
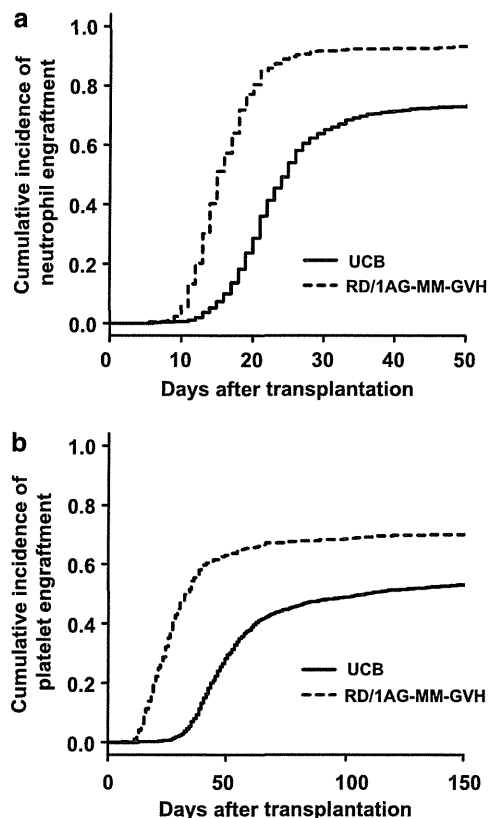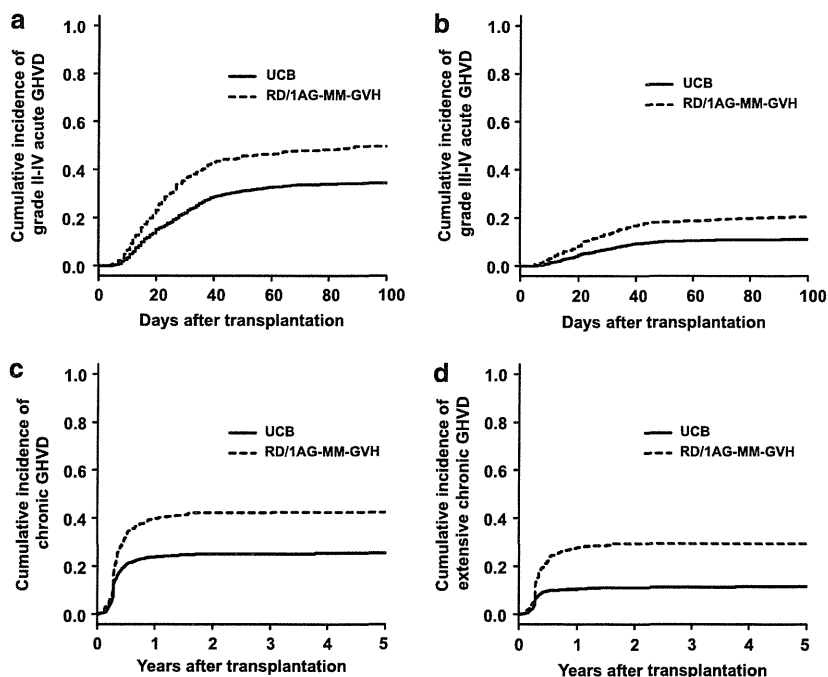
Figure 2. Acute and chronic GVHD. Cumulative incidences of grade II–IV (**a**) and grade III–IV acute GVHD (**b**) and chronic (**c**) and extensive chronic GVHD (**d**) are shown.

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Table 2. Multivariate analysis of overall mortality

Variable	Total[a]		Standard risk[b]		High risk[c]	
	HR (95% CI)	P value	HR (95% CI)	P value	HR (95% CI)	P value
(A)						
UCB	1.00	reference	1.00	reference	1.00	reference
RD/1AG-MM-GVH	0.99 (0.87–1.12)	0.833	1.06 (0.86–1.31)	0.588	0.96 (0.81–1.13)	0.639
(B)						
UCB	1.00	reference	1.00	reference	1.00	reference
RD/HLA-A-MM-GVH	0.92 (0.72–1.18)	0.519	0.99 (0.66–1.48)	0.959	0.90 (0.64–1.26)	0.551
RD/HLA-B-MM-GVH	1.20 (1.01–1.44)	0.043	1.44 (1.05–1.96)	0.023	1.12 (0.89–1.41)	0.326
RD/HLA-DR-MM-GVH	0.85 (0.70–1.02)	0.084	0.88 (0.66–1.19)	0.411	0.84 (0.65–1.08)	0.170

Abbreviations: AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CI, confidence interval; CML, chronic myelogenous leukemia; CSA, cyclosporine; HR, hazard ratio; MDS, myelodysplastic syndrome; MMF, mycophenolate mofetil; MTX, methotrexate; TAC, tacrolimus. [a]Other significant variables in model A were; patient age, 16–49 (reference, 1.00), 50–(HR, 1.50, 95% CI, 1.35–1.66, $P<0.001$); sex of recipient, female (reference, 1.00), male (HR, 1.12; 95% CI, 1.02–1.24; $P=0.023$); diagnosis, AML (reference, 1.00),ALL (HR, 1.11, 95% CI, 0.98–1.26, $P=0.112$), CML (HR, 0.90, 95% CI, 0.72–1.13, $P=0.374$), MDS (HR, 0.81, 95% CI, 0.68–0.95, $P=0.001$); disease risk, standard risk (reference, 1.00), high risk (HR, 2.24; 95% CI, 2.00–2.50; $P<0.001$), status not known, (HR, 1.59; 95% CI, 1.21–2.09; $P=0.001$); GVHD prophylaxis, CSA/TAC + MTX (reference, 1.00),CSA/TAC only (HR, 1.23; 95% CI, 1.09–1.39; $P=0.001$), CSA/TAC + steroid/MMF (HR, 1.02; 95% CI, 0.86–1.21; $P=0.820$), other/missing (HR, 1.21; 95% CI, 0.82–1.78; $P=0.342$); year of transplantation, 1998–2004 (reference, 1.00), 2005–2009 (HR, 0.89; 95% CI, 0.80–0.99; $P=0.038$). [b]Other significant variables in model A were; patient age, 16–49 (reference, 1.00), 50–(HR, 1.72, 95% CI, 1.42–2.07, $P<0.001$); GVHD prophylaxis, CSA/TAC + MTX (reference, 1.00), CSA/TAC only (HR, 1.43; 95% CI, 1.14–1.78; $P=0.002$), CSA/TAC + steroid/MMF (HR, 1.00; 95% CI, 0.73–1.37; $P=0.995$), other/missing (HR, 1.51; 95% CI, 0.67–3.39; $P=0.319$). [c]Other significant variables were; patient age, 16–49 (reference, 1.00), 50–(HR, 1.41, 95% CI, 1.23–1.61, $P<0.001$); diagnosis, AML (reference, 1.00), ALL (HR, 1.13, 95% CI, 0.95–1.34, $P=0.183$), CML (HR, 0.94, 95% CI, 0.70–1.27, $P=0.704$), MDS (HR, 0.73, 95% CI, 0.60–0.89, $P=0.002$).
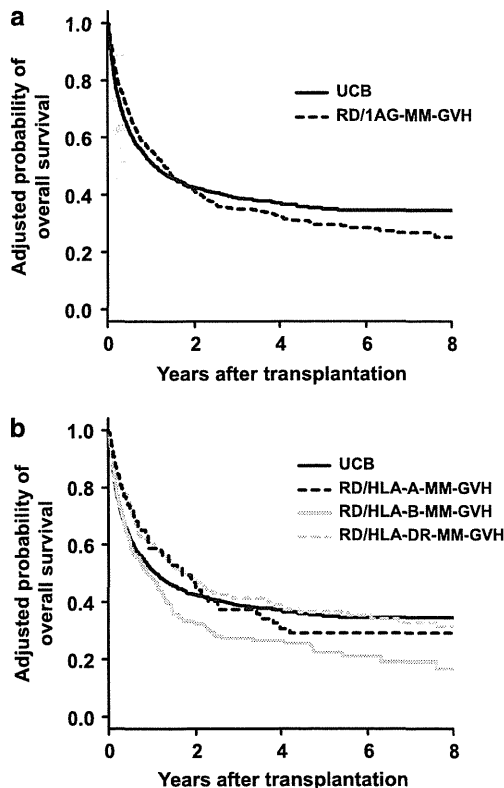

Figure 3. Overall survival. Overall survival rates in the transplantation using an unrelated cord blood vs a related donor with a 1-antigen mismatch at the HLA-A, HLA-B or HLA-DR locus in the GVH direction (a) or with an HLA-A, -B, or -DR antigen mismatch in the GVH direction (b) are shown.

of transplantation (1998–2004). Figure 3a shows the adjusted survival curves of the two groups. Next, the HLA-A, HLA-B and HLA-DR mismatched groups in transplantation from an RD/1AG-MM-GVH were compared with the UCB group. The OS rate of

patients who received transplantation from an RD/1AG-MM-GVH involving an HLA-B mismatch was significantly lower than that in the UCB group ($P=0.043$; Figure 3b and Table 2), and a subgroup analysis revealed that the adverse effect of an HLA-B mismatch was significant only in standard-risk patients (standard-risk, $P=0.023$; high-risk, $P=0.326$; Table 2).

Relapse and NRM

The 3-year relapse rates in the UCB and RD/1AG-MM-GVH groups were 35% (95%CI, 33–37%) and 32% (95% CI, 28–36%), respectively (Gray test; $P=0.041$; Figure 4a), and a significant decrease in the incidence of relapse was found in the RD/1AG-MM-GVH group in the multivariate analysis (RD/1AG-MM-GVH vs UCB, HR, 0.78, 95%CI, 0.64–0.95, $P=0.012$; Table 3). The impact of reducing the incidence of relapse did not differ according to the HLA mismatch antigen in the RD/1AG-MM-GVH group (Table 3 and Figure 4b). The 3-year NRM rates in the UCB and RD/1AG-MM-GVH groups were 30% (95% CI, 28–32%) and 32% (95% CI, 28–36%), respectively (Gray test; $P=0.474$; Figure 4c), and a significant increase in the NRM rate was observed in the RD/1AG-MM-GVH group in the multivariate analysis (RD/1AG-MM-GVH vs UCB, HR, 1.24, 95% CI, 1.04–1.47, $P=0.016$; Table 3). In particular, the NRM rate of patients who received transplantation from an RD/1AG-MM-GVH with an HLA-B mismatch was significantly higher than that in the UCB group (RD/1AG-MM-GVH vs UCB, HR, 1.50, 95% CI, 1.17–1.92, $P=0.001$; Figure 4d and Table 3).

The causes of death in patients who died without relapse are shown in Supplementary Table 3. The rates of GVHD and organ failure in the RD/1AG-MM-GVH group were higher than those in the UCB group (GVHD, 18 vs 10%, organ failure, 28 vs 19%), whereas the rates of graft failure and infection were lower in the RD/1AG-MM-GVH group (graft failure, 1 vs 5%; infection, 26 vs 38%).

The impact of the use of *in vivo* T-cell depletion in the RD/1AG-MM-GVH group

Based on the fact that the leading causes of death in the RD/1AG-MM-GVH group were GVHD and organ failure, we analyzed the risk factors for the development of acute GVHD in this group.
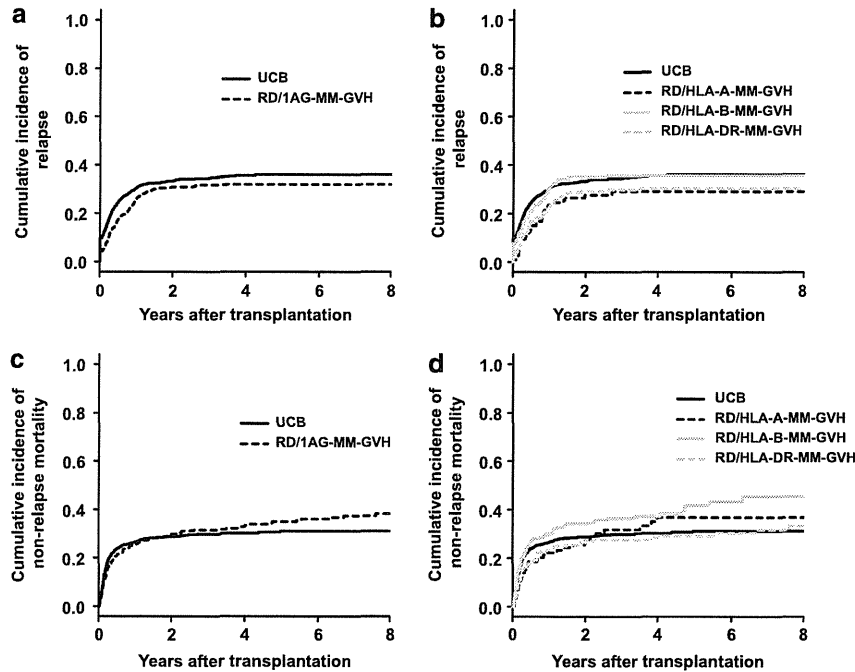
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Figure 4. Relapse and non-relapse mortality. Cumulative incidence of relapse and non-relapse mortality after transplantation using an unrelated cord blood vs a related donor with a 1-antigen mismatch at the HLA-A, HLA-B or HLA-DR locus in the GVH direction (**a**, **c**) or with an HLA-A, -B, or -DR antigen mismatch in the GVH direction (**b**, **d**) are shown.

Table 3. Multivariate analysis of relapse and non-relapse mortality

Variable	Relapse[a]		Non-relapse mortality[b]	
	HR (95% CI)	P value	HR (95% CI)	P value
(A)				
UCB	1.00	reference	1.00	reference
RD/1AG-MM-GVH	0.78 (0.64–0.95)	0.012	1.24 (1.04–1.47)	0.016
(B)				
UCB	1.00	reference	1.00	reference
RD/HLA-A-MM-GVH	0.70 (0.49–1.00)	0.050	1.28 (0.93–1.76)	0.130
RD/HLA-B-MM-GVH	0.81 (0.62–1.07)	0.134	1.50 (1.17–1.92)	0.001
RD/HLA-DR-MM-GVH	0.80 (0.61–1.04)	0.096	1.02 (0.78–1.32)	0.901

Abbreviations: AML, acute myeloid leukemia; ALL, acute lymphoblastic leukemia; CI, confidence interval; CML, chronic myelogenous leukemia; CSA, cyclosporine; HR, hazard ratio; MDS, myelodysplastic syndrome; MMF, mycophenolate mofetil; MTX, methotrexate; TAC, tacrolimus. [a]Other significant variables in model A were; diagnosis, AML (reference, 1.00), ALL (HR, 1.09, 95% CI, 0.92–1.29, $P = 0.336$), CML (HR, 1.39, 95% CI, 1.05–1.82, $P = 0.019$), MDS (HR, 0.59, 95% CI, 0.46–0.76, $P < 0.001$); time from diagnosis to transplantation, <6 months (reference, 1.00), ≥6 months (HR, 0.80; 95% CI, 0.70–0.92; $P = 0.002$); disease risk, standard risk (reference, 1.00), high risk (HR, 2.81; 95% CI, 2.41–3.27; $P < 0.001$), status not known, (HR, 2.17; 95% CI, 1.45–3.23; $P < 0.001$); conditioning intensity, myeloablative (reference, 1.00), reduced intensity (HR, 1.22; 95% CI, 1.04–1.44; $P = 0.014$); GVHD prophylaxis, CSA/TAC + MTX (reference, 1.00), CSA/TAC only (HR, 0.65; 95% CI, 0.53–0.78; $P < 0.001$), CSA/TAC + steroid/MMF (HR, 0.75; 95% CI, 0.59–0.96; $P = 0.024$), other/missing (HR, 0.94; 95% CI, 0.55–1.61; $P = 0.825$). [b]Other significant variables in model A were; patient age, 16–49 (reference, 1.00), 50–(HR, 1.70, 95% CI, 1.47–1.98, $P < 0.001$); GVHD prophylaxis, CSA/TAC + MTX (reference, 1.00),CSA/TAC only (HR, 1.70; 95% CI, 1.44–2.01; $P < 0.001$), CSA/TAC + steroid/MMF (HR, 1.18; 95% CI, 0.94–1.49; $P = 0.158$), other/missing (HR, 1.47; 95% CI, 0.86–2.51; $P = 0.154$); year of transplantation, 1998–2004 (reference, 1.00), 2005–2009 (HR, 0.76; 95% CI, 0.66–0.88; $P < 0.001$).

In multivariate analysis, two factors were found to be significantly associated with the risk of developing grade II–IV acute GVHD in the RD/1AG-MM-GVH group: the use of *in vivo* T-cell depletion and source of stem cells (use of *in vivo* T-cell depletion, yes vs no, HR 0.40, $P = 0.002$, PB vs BM, HR 1.61, $P < 0.001$).

Because the use of *in vivo* T-cell depletion significantly lowered the risk of acute GVHD, we re-compared the RD/1AG-MM-GVH group and the UCB group while focusing on the use of *in vivo* T-cell depletion in the RD/1AG-MM-GVH group. The incidence of grade II–IV or grade III–IV acute GVHD or chronic or extensive chronic GVHD in the RD/1AG-MM-GVH group using *in vivo* T-cell depletion was comparable to that in the UCB group

(Supplementary Figure 1 and Supplementary Table 4), whereas the incidences of neutrophil and platelet engraftment were significantly higher in the RD/1AG-MM-GVH group using *in vivo* T-cell depletion than in the UCB group (neutrophil engraftment, HR, 5.52, 95% CI, 3.36–9.05, $P < 0.001$; platelet engraftment, HR 2.01, 95% CI, 1.26–3.21, $P < 0.001$). Compared to the UCB group, the RD/1AG-MM-GVH group with T-cell depletion showed lower overall and NRM, albeit these differences were not significant, which suggests that the use of *in vivo* T-cell depletion may improve the outcome of transplantation from an RD/1AG-MM-GVH (Figure 5, Supplementary Table 5). It is interesting to note that the adverse impact of an HLA-B mismatch vs HLA-A or -DR
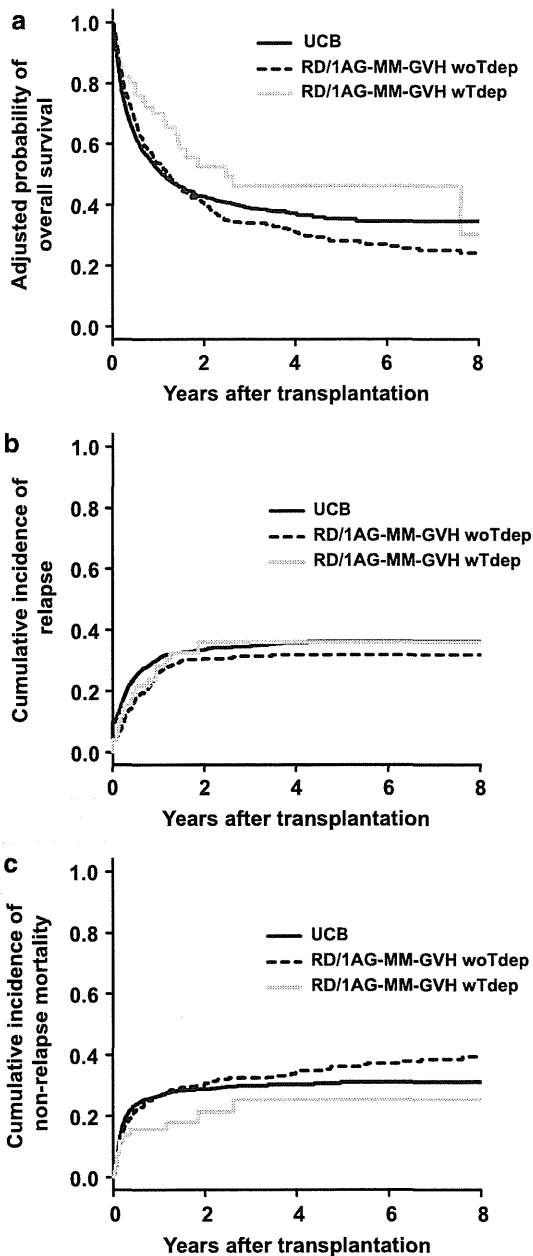
Figure 5. OS (a), relapse (b) and NRM (c) according to the use of *in vivo* T-cell depletion in the RD/1AG-MM-GVH group.

group with an HLA-B mismatch showed significantly higher overall and NRM, whereas the RD/1AG-MM-GVH group with an HLA-A or HLA-DR mismatch showed an OS comparable to that in the UCB group. Neutrophil and platelet engraftment in the RD/1AG-MM-GVH group were significantly faster than those in the UCB group, whereas the incidence of acute or chronic GVHD in the RD/1AG-MM-GVH group was significantly higher. However, the incidence of acute or chronic GVHD in the RD/1AG-MM-GVH group with *in vivo* T-cell depletion was comparable to that in the UCB group, which translated into a better, but not significantly better, OS than that in the UCB group.

In Japan, unrelated BM donor coordination (from donor search to transplantation) takes a median of 4 months, whereas much less time is required for UCB or RD/1AG-MM-GVH transplantation if there is a candidate. This was reflected in the longer duration from diagnosis to transplantation in unrelated BM transplantation.[32] In contrast, UCB and RD/1AG-MM-GVH transplantation show a similar and shorter duration (Table 1 ; 7.9 months vs 7.6 months). Therefore, in cases where both UCB and RD/1AG-MM-GVH are available, donors should be chosen based on their advantages and disadvantages. Compared with UCB, the use of RD/1AG-MM-GVH has a great advantage in neutrophil and platelet engraftment, which is not inconsistent with a previous finding that engraftment in the UCB group was significantly delayed comparing with that in MUD.[33] This translated into a lower rate of death from graft failure or infection in the RD/1AG-MM-GVH group. However, these advantages were offset by a substantial increase in the incidence of acute and chronic GVHD in the RD/1AG-MM-GVH group. The risk of grade III–IV acute GVHD and extensive chronic GVHD in the RD/1AG-MM-GVH group was twice that in the UCB group. If UCB units containing adequate total nucleated cell doses (ex. $>2.5 \times 10^7$/kg) are available,[34] the selection of UCB would be appropriate to avoid the risk of chronic GVHD. In contrast, RD/1AG-MM-GVH would be more appropriate when early neutrophil engraftment should be prioritized, such as for a patient with an active infectious disease at transplantation.

The high incidences of GVHD and GVHD-related death in the RD/1AG-MM-GVH group indicate the need for stronger immunosuppression to improve the clinical outcome. The use of T-cell depletion, mostly by ATG, was significantly associated with a lower incidence of grade III–IV acute GVHD and extensive chronic GVHD in the RD/1AG-MM-GVH group. Although this effect was not statistically significant, the RD/1AG-MM-GVH group with *in vivo* T-cell depletion showed lower overall and treatment-related mortality, which would outweigh a possible increased risk of relapse. These findings in our cohort suggest that ATG may be effective, and the addition of ATG in the RD/1AG-MM-GVH group should be assessed in a prospective study.

As shown in our previous study,[23] overall mortality in the RD/1AG-MM-GVH group involving an HLA-B mismatch was significantly higher than that in the RD/1AG-MM-GVH group with an HLA-A or -DR mismatch, probably because of an additional HLA-C antigen mismatch as expected from linkage disequilibrium between HLA-B and HLA-C and available data on HLA-C antigen.[23,35] The incidence of grade III–IV acute GVHD in the HLA-B mismatch group was higher than that in the HLA-DR mismatch group, but was comparable to that in the HLA-A mismatch group. In addition, the incidence of death from GVHD was similar in the HLA-B and HLA-A/DR mismatch groups (data not shown). Therefore, the reason for the lower overall morality in the RD/1AG-MM-GVH group with an HLA-B mismatch remains unclear. However, the adverse effect of an HLA-B mismatch disappeared when *in vivo* T-cell depletion was used, which suggests that an immunological effect is involved in this mechanism.

This study has several limitations. First, in clinical practice in Japan, matching of HLA-DR is counted at a low resolution, as with HLA-A and HLA-B, whereas it is counted at a high resolution in the

mismatch in the RD/1AG-MM-GVH group disappeared with the use of *in vivo* T-cell depletion (with *in vivo* T-cell depletion; HLA-B vs HLA-A/DR mismatch; HR 1.08, 95% CI, 0.45–2.62, $P = 0.864$, without *in vivo* T-cell depletion; HLA-B vs HLA-A/DR mismatch; HR 1.59, 95% CI, 1.25–2.01, $P < 0.001$).

With regard to the effect of stem cell source, the incidence of acute and chronic GVHD in the RD/1AG-MM-GVH group using BM was lower than that with PB but higher than that with UCB (Supplementary Figure 2). The use of PB or BM did not affect OS, relapse, or NRM (Supplementary Table 5).

DISCUSSION

In this nationwide retrospective study, we found that the survival rate in the UCB group was comparable to that in the RD/1AG-MM-GVH group regardless of the disease risk. The RD/1AG-MM-GVH

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United States and Europe. To evaluate the impact of this difference, we divided patients in the UCB group with two antigen mismatches into two groups by using available HLA-DRB1 allele information: a group with two antigen mismatches with one additional HLA-DRB1 allele mismatch ($n = 609$) and another group with two antigen mismatches without an additional HLA-DRB1 mismatch ($n = 295$). We did not find a significant difference in OS between these two groups ($P = 0.758$), which suggests that HLA-matching using HLA-DR antigen or allele information will not affect OS in the present study. Second, the findings in the present study are based on Asian cohort who received a 'single' UCB or RD/1AG-MM-GVH transplantation. Lighter body weight in Asian population than Caucasian population may make it easy to find a suitable single UCB unit that contains adequate total nucleated cell doses. In addition, as suggested by Oh et al.,[36] limited heterogeneity of Japanese population may affect the outcomes of transplantation. Therefore, the findings should be externally validated in the non-Asian cohort or transplantation using double UCB units. Third, information on the dose and type of ATG was missing in two-third of the patients who received ATG. However, the available data showed that the median dose of thymoglobulin (2.5 mg/kg) or ATG-F (8 mg/kg) was equivalent to the dose that is widely used in our daily practice. Lastly, heterogeneous backgrounds may have resulted in a bias, although we tried to adjust for possible confounders by multivariate analyses. Lastly, the effect of multiple testing should be taken into account for the interpretation of secondary end points.

In conclusion, our findings suggest that both UCB and RD/1AG-MM-GVH are suitable as alternative donors for patients without an HLA-matched sibling or unrelated donor. However, the presence of an HLA-B-antigen mismatch in the GVH direction has an adverse effect on OS because of treatment-related complications. Neutrophil and platelet engraftment in the RD/1AG-MM-GVH group were significantly faster than those in the UCB group, whereas the incidence of acute and chronic GVHD in the RD/1AG-MM-GVH group was significantly higher, which translated into a high incidence of death from GVHD. Donor selection between UCB and RD/1AG-MM-GVH should be determined based on the presence of an HLA-B mismatch in RD/1AG-MM-GVH and from the risks and benefits derived from the risk of graft failure and infection in the UCB group and acute or chronic GVHD in the RD/1AG-MM-GVH group. Additional immune suppression using in vivo T-cell depletion may improve the clinical outcome in the RD/1AG-MM-GVH group by decreasing the incidences of GVHD and NRM and may also overcome the adverse effect of an HLA-B mismatch. This approach should be assessed in a prospective study.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

JK and YK designed the research, organized the project and wrote the manuscript; JK, YA, and YK performed the statistical analysis and analyzed the data; KK and TN-I collected data from JCBBN; and all of the authors interpreted the data and reviewed and approved the final manuscript.

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Phase II study of dose-modified busulfan by real-time targeting in allogeneic hematopoietic stem cell transplantation for myeloid malignancy

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We aimed to evaluate the efficacy and safety of allogeneic hematopoietic stem cell transplantation with targeted oral busulfan (BU) and cyclophosphamide (CY) in a phase II study. Busulfan (1.0 mg/kg) was given initially in six doses. Based on the estimated concentration at steady state after the first dose of BU, subsequent (7th–16th) doses were adjusted to obtain a targeted overall concentration at steady state of 700–900 ng/mL. The primary endpoint was 1-year overall survival (OS). Fifty patients were registered and 46 (median age, 53 years; range, 18–62 years) received planned transplant, including 24 with AML, 16 with myelodysplastic syndrome, and six with CML. Fourteen patients were categorized as standard risk. Nineteen patients received transplant from human leukocyte antigen-identical siblings, 27 from unrelated donors. The BU dose required reduction in 32 patients and escalation in six patients. One-year OS was 65% (95% confidence interval, 50–77%). Cumulative incidence of hepatic sinusoidal obstruction syndrome was 11%. One-year transplant-related mortality was 18%. Both OS and transplant-related mortality were favorable in this study, including patients of older age and with high risk diseases. Individual dose adjustment based on BU pharmacokinetics was feasible and effective in the current phase II study. This trial is registered in the University Hospital Medical Information Network Clinical Trial Registry System (UMIN-CTR, ID:C000000156). (*Cancer Sci* 2012; 103: 1688–1694)

B usulfan is an alkylating agent widely used in high-dose chemotherapy regimens for HSCT.[(1,2)] The BU level in serum has been shown to be an important factor for graft rejection and regimen-related toxicity such as SOS.[(3–5)] Unfavorable profiles of oral BU include delayed and variable absorptive characteristics and high variability in drug metabolism.[(6)] Individualized dose adjustment of BU using the LSM, and its transplantation results, have been investigated widely in Caucasian patients and pediatric populations, but few prospective studies have investigated results in Asian patients.[(7)] Prior to the current study, we carried out a prospective PK study to analyze BU concentration using gas chromatography–mass spectrometry.[(8)] Nine patients were enrolled in the study, and received preparative regimen containing oral BU 1 mg/kg every 6 h for eight or 16 doses. Out of nine patients, only three met the average steady-state plasma concentration levels in the safety range of 650–1000 ng/mL[(4,9)] after the first and 13th dose. From the

results, we developed LSM to estimate the AUC using two different formulas in order to fit even delayed clearance. Subsequently, we carried out a pilot study that used the same targeting method as the current study, and six patients with myeloid malignancy received tBU+CY conditioning with a targeting AUC of Css 700–900 ng/mL. Four patients received dose reduction after the seventh dose of BU, and overall Css of three patients met the safety range of 786–905 ng/mL (Akio Kohno, Mariko Fukumoto, Hiroto Narimatsu, Kazutaka Ozeki, Masashi Sawa, Shuichi Mizuta, Hitoshi Suzuki, Isamu Sugiura, Seitaro Terakura, Kazuko Kudo, and Yoshihisa Morishita, unpublished data, 2003).

From these results, we carried out a prospective phase II trial in Japanese patients with myeloid malignancies to evaluate the clinical results of allogeneic HSCT undergoing individualized high-dose oral BU+CY conditioning.

Materials and Methods

Eligibility criteria. Patients from 16 to 65 years old were eligible if they had a diagnosis of AML, CML, or MDS, with an Eastern Cooperative Oncology Group performance status of 0–2, and no previous history of HSCT. Standard risk was defined as AML in first complete remission, MDS in refractory anemia or refractory anemia with ringed sideroblasts, and CML in chronic phase. High risk was defined as the remaining disease type. Patients receiving T cell depletion, or those with clinically significant infection or severe abnormalities of cardiac, pulmonary, and hepatic functions were excluded. Included patient/donor pairs were either related HLA matched by serological typing of A, B, and DR locus, unrelated HLA matched, or HLA DRB1 one locus mismatched by genotypical typing of A, B, and DRB1 locus. Unrelated donors were chosen by coordination with the Japan Marrow Donor Program. Written informed consent was obtained from each patient according to the Declaration of Helsinki. The study

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protocol was approved by the Institutional Review Board of each center.

Conditioning regimen, GVHD prophylaxis, and supportive care. Patients received a conditioning regimen consisting of BU 1.0 mg/kg given orally four times a day for six doses on two consecutive days (dose 1–6). Six hours after dose 6, patients received an adjusted dose of BU four times a day for 10 doses (dose 7–16) on three consecutive days (Fig. S1). Cyclophosphamide 60 mg/kg was given i.v. on two successive days. Both BU and CY were dosed based on actual body weight if it was <120% of ideal body weight, and adjusted body weight for those exceeding 120%. Sodium valproate was given as seizure prophylaxis before and during BU treatment. Fluconazole was used as fungal prophylaxis.

Either cyclosporine or tacrolimus in combination with methotrexate was used for GVHD prophylaxis. Cyclosporine was given i.v. at a dose of 3 mg/kg per day in two divided doses starting on day −1. Tacrolimus was given i.v. at a dose of 0.025 mg/kg continuously starting on day −1. Methotrexate was given at a dose of 10 mg/m^2 on day 1 and 7 mg/m^2 on days 3 and 6. Oral cyclosporine or tacrolimus was substituted for i.v. administration when tolerated. In the absence of GVHD, the cyclosporine and tacrolimus doses were tapered after day 50. Acute GVHD of grade 2 or more was treated with methylprednisolone 1–2 mg/kg. Chronic GVHD was treated by the protocols of each institute.

Supportive care measures were used according to institutional guidelines. Daily granulocyte colony stimulating factor was started on day 6 and continued until absolute neutrophil count exceeded 500/μL for two consecutive days.

Pharmacokinetic studies of BU. For PK studies of BU, blood samples were obtained 0, 30, 60, 120, 300, and 360 min after the first oral dose. Frozen plasma samples were sent to the laboratory at Kitasato University, and plasma BU concentrations were assayed by gas chromatography–mass spectrometry.[8] The AUC was calculated by LSM using the formulas shown in Table 1.

Average Css levels of BU were determined by the ratio of the BU AUC$_{LSM}$ over the dosing interval to the time between doses. The BU dose after the sixth dose was adjusted when Css after the first dose was not within 700–900 ng/mL. Dose adjustment was not carried out for patients whose Css after the first dose was 700 –900 ng/mL. A targeted dose was calculated to achieve an average Css after all doses of 800 ng/mL. The optimal dose of BU was calculated as follows: optimal single dose of BU (mg/kg) = 800 (ng/mL) × first dose (mg/kg)/Css of first dose (ng/mL).

The dose of the 7th to 16th BU was calculated as follows: revised dose (mg/kg) = [optimal single dose (mg/kg) × 16 (times) − first dose (mg/kg) × 6 (times)]/10 (times).

Definitions of outcomes. The study was designed as a phase II prospective trial. The primary endpoint of the study was 1-year OS after transplantation. The secondary endpoint was DFS, PK of BU, aGVHD, and cGVHD, and the frequency and

Table 1. Formulas for limited sample model (LSM) in patients receiving allogeneic hematopoietic stem cell transplantation treated with targeted oral busulfan and cyclophosphamide

i	In cases C_6/C_2 = or <0.5
	AUC$_{LSM}$ = 0.5$C_{0.5}$ + 0.75C_1 + 2.5C_2 + 2.0C_6 + 4C_6/(LnC_2 − LnC_6)
ii	In cases C_6/C_2 > 0.5
	AUC$_{LSM}$ = 0.5$C_{0.5}$ + 0.75C_1 + 2.5C_2 + 2.0C_6 + 2C_6/(LnC_2 − LnC_6)

In the previous pilot study, formula (i) bore a strong approximation to actual area under the blood concentration time curve (AUC), but not in patients with an elongated absorption or a delayed elimination of busulfan. The formula of the LSM was modified in the case of C_6/C_2 > 0.5 and formula (ii) was used for those patients. C_x, serum busulfan level obtained at x hours after the first dose.

severity of SOS, regimen-related toxicity up to day 28, mortality at day 100, hematological recovery, and DFS and OS of each disease category.

All patients were prospectively monitored for engraftment,[10] post-transplant toxicities, GVHD, hepatic SOS, and infection. Failure to reach an absolute neutrophil count of 0.5 × 10^9 cells/ L by day 28 after transplantation was defined as graft failure, and the patient was withdrawn from the study. The aGVHD was evaluated daily until day 28 and weekly from day 29 to 100 and graded by established criteria.[11] The cGVHD was evaluated up to day 365. Treatment and the outcome of aGVHD and cGVHD were also evaluated. Sinusoidal obstruction syndrome was clinically evaluated before day 28, and diagnosed,[12–14] then graded clinically[12] according to the published criteria. Liver toxicity that occurred after day 21 and fulfilled the above criteria of SOS was defined as late-onset SOS. Clinical data after day 29 until day 100 was additionally surveyed to evaluate late-onset SOS retrospectively.

Disease monitoring was carried out by bone marrow aspiration within 1 week before or after days 30, 60, and 90 after transplantation. Relapse was defined by hematological recurrence for AML,[15,16] and by hematological or cytogenetic relapse for CML. Deaths in the absence of persistent relapse were categorized as non-relapse mortality. Additional surveillance was carried out and the onset of SOS and regimen-related toxicities from days 29 to 100 were collected retrospectively. Long-term survival data and data of relapse after day 365 were also collected retrospectively.

Statistical analysis. The primary endpoint of the study was 1-year OS after transplantation. The expected 1-year OS was estimated to be 60%, and its threshold was estimated to be 40%. With a statistical power of 90% and a one-sided, type I error of 5%, the number of eligible patients required for this study was calculated to be 46 using a binominal analysis method. The projected sample size was 50 patients, with the expectation that 10% of patients would be deemed ineligible.

Disease-free survival was calculated from the date of transplantation until the date of relapse or the date of death in complete remission. This trial has been registered in the University Hospital Medical Information Network Clinical Trial Registry System (UMIN-CTR, ID:C000000156). Data were analyzed with Stata 9.2 statistical software (Stata, College Station, TX, USA).

Results

Patient characteristics. Patients were registered from October 2003 through March 2007. Fifty patients were registered. One patient who developed severe hemorrhagic ulcer of the ileum after registration was considered to be ineligible. One patient developed metastatic breast cancer before receiving the conditioning regimen and was withdrawn. Forty-eight patients received tBU+CY conditioning. One patient developed systemic convulsion on day −6 before transplantation, and the study was discontinued. Another patient received cord blood transplantation due to unexpected emergent unavailability of the unrelated bone marrow and was included only in the PK analysis. The remaining 46 patients who completed tBU+CY conditioning and received the planned transplantation were analyzed in the subsequent outcome study. Characteristics and a transplantation summary of these 46 patients at the time of registration are shown in Tables 2 and 3, respectively.

Treatment-related toxicity and hepatic veno-occlusive disease. Forty-five of 46 patients undergoing tBU+CY conditioning (98%) experienced grade II or higher regimen-related toxicity, and 38 of 48 patients (79%) experienced grade III or more toxicity within 28 days post-transplantation (Table S1). Infection (70%), oral mucositis (52%), nausea and vomiting (30%), and

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