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			
$\leq 0.100 \times 10^9$/l	5/15 (33%)	15/18 (83%)	0.003
$>0.100 \times 10^9$/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 $<0.500 \times 10^9$/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 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.

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.

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Identification of a novel HLA-A*24:02-restricted adenovirus serotype 11-specific CD8+ T-cell epitope for adoptive immunotherapy

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ABSTRACT

Subgroup B adenovirus serotype 11 (Ad11) occasionally causes fatal infections in immunocompromised patients. The present study describes a novel Ad11 epitope presented by HLA-A*24:02 that could be used for adoptive immunotherapy. Ten synthetic Ad11 hexon protein-derived nonamer peptides that bound to HLA-A*24:02 were selected by a computer algorithm and MHC stabilization assay. Stimulation of peripheral blood mononuclear cells from HLA-A*24:02+ donors with each of these synthetic peptides induced peptide-specific CD8+ T-cells for three peptides. Testing the reactivity of these peptide-specific CD8+ T-cells against various target cells confirmed that peptide TYFNLGNKF is naturally processed in Ad11-infected cells and is presented by HLA-A*24:02. Emergence of TYFNLGNKF-specific CD8+ T-cells coincided with the clearance of adenoviruses in a patient with Ad11 disease. Importantly, TYFNLGNKF-specific CD8+ T-cells were suggested to be not serotype cross-reactive. The novel HLA-A*24:02-restricted Ad11 epitope could be used for anti-Ad11 adoptive immunotherapy and to monitor immunity to Ad11 using MHC tetramers.

1. Introduction

Adenoviruses (AdV) cause lethal infections in immunocompromised hosts such as hematopoietic stem cell transplantation (HSCT) and chemotherapy recipients (Chakrabarti et al., 2002; Leen et al., 2006a; Yokose et al., 2009). Although antiviral agents, such as ribavirin and cidofovir, have been used for the treatment of AdV infection, their efficacy is limited by weak intrinsic activity against viruses and by toxicity (Ison, 2006; Lindemans et al., 2010). Furthermore, reconstitution of AdV-specific T-cells is required for the control of AdV infection (Feuchtinger et al., 2005; Heemskerk et al., 2005). These observations have led to the development of adoptive T-cell therapy for the management of AdV infection (Feuchtinger et al., 2006; Leen et al., 2006b).

There are several different approaches to generate virus-specific T-cells for adoptive therapy. In previous reports, peripheral blood mononuclear cells (PBMCs) were stimulated with the lysate of AdV-infected cells or with adenoviral vector-transduced cells to generate AdV-specific T-cells (Feuchtinger et al., 2004; Leen et al., 2004a). However, the clinical use of these strategies is complicated by the concerns associated with transferring live viral particles to patients who are immunocompromised. Another method to generate virus-specific T-cells is to stimulate PBMCs with immunogenic peptides derived from viral proteins. This method is advantageous in that synthetic peptides can be readily produced under good manufacturing practice conditions. Furthermore, the feasibility of this approach has been documented in clinical studies in which cytomegalovirus (CMV)-pp65 peptide-specific cytotoxic

Abbreviations: AdV, adenoviruses; Ad11, adenovirus serotype 11; BIMAS, Bioinformatics and Molecular Analysis Section; CM, culture medium; CMV, cytomegalovirus; CTLs, cytotoxic T-cells; EBV, Epstein-Barr virus; ELISA, enzyme-linked immunosorbent assay; E:T, effector to target; FBS, fetal bovine serum; HC, hemorrhagic cystitis; HSCT, hematopoietic stem cell transplantation; IFN-γ, interferon-γ; IL-2, interleukin-2; K562/A*24:02, K562 cells transduced with HLA-A*24:02; LCLs, Epstein-Barr virus-transformed B-lymphoblastoid cell lines; LYA, LYANSAHAL; mAb, monoclonal antibody; MFI, mean fluorescence intensity; PBMCs, peripheral blood mononuclear cells; TCRs, T cell receptors; TYF, TYFNLGNKF; VYS, VYSGSIPYL.

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T-cells (CTLs) were given therapeutically or prophylactically to HSCT recipients (Meij et al., 2012; Micklethwaite et al., 2007).

The wide clinical application of AdV-specific T-cells generated by stimulating PBMCs with immunogenic peptides requires knowledge of T-cell epitopes restricted by prevalent MHC molecules. Previous studies that have identified T-cell epitopes of AdV have focused exclusively on the subgroup C AdV (Leen et al., 2004b, 2008; Tang et al., 2006; Zandvliet et al., 2010). Subgroup B AdV serotype 11 (Ad11) is a major pathogen for hemorrhagic cystitis (HC) (Akiyama et al., 2001; Miyamura et al., 1989; Mori et al., 2012) and occasionally causes disseminated infection with fatal outcomes in immunocompromised patients (Taniguchi et al., 2012). However, T-cell epitopes of Ad11 have not been identified. Therefore, the goal of the following study was to identify a novel T-cell epitope of Ad11 presented by HLA-A*24:02, which is one of the most common HLA class I molecule in many ethnic groups (60% in Japanese population, 20% in Caucasians, and 12% in Africans) (Gomi et al., 1999).

2. Materials and methods

2.1. Donor and patient specimens

PBMCs and serum from HLA-A*24:02+ healthy volunteer donors and a patient with Ad11-associated HC were obtained after informed consent. In addition, urine was collected from a patient with Ad11-associated HC. Measurement of AdV DNA in the patients' serum and urine was performed by real-time polymerase chain reaction, as previously described (Funahashi et al., 2010).

2.2. Cell lines

Epstein-Barr virus (EBV)-transformed B-lymphoblastoid cell lines (LCLs) were generated by infection of PBMCs from healthy donors with concentrated EBV-containing supernatants of cultured B95-8 cells (Leen et al., 2004b). T2-A24 cells, the transporter associated with antigen processing-deficient B and T hybrid cell line T2 transfected with the HLA-A*24:02 gene, were kindly supplied by Dr. Y. Akatsuka (Aichi Cancer Center Research Institute, Nagoya, Japan). K562 cells were transduced with retroviruses that encode CD80 and CD86 and were selected to >90% purity by cell sorting for expression of these co-stimulatory ligands. CD80 and CD86$^+$ K562 were then transduced with retroviruses that encode a full-length HLA-A*24:02 (Phoenix-Ampho System; Orbigen) and sorted twice to obtain cells of >95% purity that expressed HLA-A*24:02 (named K562/A*24:02). Cell lines were cultured in RPMI-1640 medium (Sigma, St. Louis, MO) containing 10% fetal bovine serum (FBS). For preparation of peptide-pulsed LCLs, K562, and K562/A*24:02, the cells were washed once, resuspended in RPMI-1640 medium, and pulsed with the corresponding synthetic peptide at 5 μg/ml at room temperature for 2 h. The cells were then washed once and used in stimulation assays.

2.3. Infection of K562 and K562/A*24:02 with AdV

Ad11 isolated from patients was used in the experiments. K562/A*24:02 were infected with Ad11 at a multiplicity of infection of 100 and used for enzyme-linked immunosorbent assay (ELISA) 72 h after infection. Infection was confirmed by documentation of AdV hexon antigen expression by flow cytometry.

2.4. Peptides

A computer-based program (Bioinformatics and Molecular Analysis Section (BIMAS), HLA peptide binding predictions; http://www-bimas.cit.nih.gov/molbio/hla_bind/) was used to identify potential HLA-A24-binding peptides within the Ad11 hexon protein. The nonamer peptides with a score exceeding 100 were selected and synthesized. HLA-A24-binding peptide, QYDPVAALF, derived from the human CMV-pp65 protein (Kuzushima et al., 2001), and TYFSLNNKF, derived from the human AdV serotype 5 hexon protein (Leen et al., 2004b), were also synthesized. All peptides were synthesized by Medical & Biological Laboratories (Nagoya, Japan).

2.5. MHC stabilization assay

All candidate peptides were tested for their capacity to bind to HLA-A24 molecules on the surface of T2-A24 cells as described previously (Kuzushima et al., 2001). Briefly, T2-A24 cells (3×10^5 cells) were incubated with 200 μL RPMI-1640 medium containing 0.1% FBS, 5×10^{-5} M β-mercaptoethanol (Sigma), 3 μg/ml human β2-microglobulin (Sigma), and each of the peptides at a concentration of 10 μM at 37 °C for 16 h. Following the incubation, surface HLA-A24 molecules were stained with the anti HLA-A23/A24 monoclonal antibody (mAb) and anti-mouse FITC-labeled antibodies. Expression was measured by flow cytometry, and mean fluorescence intensity (MFI) was recorded. Percent MFI increase was calculated as follows: percent MFI increase = (MFI with the given peptide – MFI without peptide)/(MFI without peptide) × 100.

2.6. Generation and expansion of peptide-specific CD8$^+$ T-cells

PBMCs obtained from healthy volunteers were placed at a concentration of 2×10^6 cells per tube in a 14 ml polypropylene tube with 1 ml of RPMI-1640 medium with 10% human serum [referred to as culture medium (CM)] and directly stimulated with peptides at a concentration of 1 μg/ml. At day 3, CM was added to a final volume of 2 ml and supplemented with 25 IU/ml recombinant human interleukin-2 (IL-2) (R&D Systems, Minneapolis, MN). Cells were transferred to a 24-well plate at day 7, re-stimulated with peptides every 7 days, cultured until day 21 or 28, and tested by interferon-γ (IFN-γ) secretion assay for the presence of peptide-specific CD8$^+$ T-cells. To expand peptide-specific CD8$^+$ T-cells, CD8$^+$ cells producing IFN-γ in the presence of peptides were isolated using IFN-γ secretion assay, followed by expansion in the presence of OKT3 mAb (Janssen Pharmaceutical), IL-2, and feeder cells, as described previously (Sugimoto et al., 2009).

2.7. Antibodies and flow cytometric analysis

All antibodies were purchased from BD Biosciences (San Jose, CA) unless otherwise noted. The following anti-human antibodies for staining of cell surface markers and intracellular molecules were used: IFN-γ-FITC, CD3-PE-Cy5.5 (Invitrogen, Carlsbad, CA), CD8-PE, -APC, or -PerCP-Cy5.5, and HLA-class I-PE (eBioscience, San Diego, CA). PE-conjugated HLA class I tetramers folded with AdV peptides were used to stain virus-specific T cell receptors (TCRs) (Medical & Biological Laboratories). In addition, mouse anti HLA-A23/A24 (One Lambda, Canoga Park, CA) and mouse anti-AdV hexon protein (Abcam, Cambridge, UK) antibodies were used in combination with anti-mouse IgG/IgM-FITC.

The IFN-γ secretion assay was performed using the IFN-γ Secretion Assay – Cell Enrichment and Detection Kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions. Briefly, cells were re-stimulated for 4 h at 37 °C in the presence or absence of peptides (1 μg/ml). Cells were incubated with IFN-γ catch reagent and cultured for 45 min at 37 °C to allow for IFN-γ secretion, followed by staining with anti-IFN-γ, CD3, and CD8 antibodies. Intracellular cytokine staining assay for IFN-γ was performed as previously described with some modifications (Terakura et al., 2012). In brief, cells were re-stimulated with peptide-pulsed

or peptide-unpulsed autologous LCLs and incubated at 37 °C for 4 h. Brefeldin A (Golgiplug, BD Biosciences) was added during the last 2.5 h of incubation to block secretion of cytokines. Subsequently, the cells were fixed, permeabilized, and stained with anti-IFN-γ and CD8 antibodies, using FIX/PERM and PERM/Wash solution (BD Biosciences). Data acquisition was performed with FACSAria or FACSCanto flow cytometer (BD Biosciences), and data were analyzed with FlowJo software (TreeStar, Inc., Ashland, OR).

2.8. ELISA

K562/A*24:02, those loaded with peptides, and those infected with Ad11 were dispensed at 3×10^4 cells/well into triplicate cultures in 96-well, round-bottom plates. Then, 3×10^4 of peptide-specific CD8+ T-cells were added to each well, and after a 24 h of co-culture at 37 °C, IFN-γ in the supernatant was measured using an ELISA method (Endogen).

2.9. Cytotoxicity assay

K562 and K562/A*24:02 loaded with or without peptides were used as target cells in cytotoxicity assay. Target cells were labeled for 2 h with ^{51}Cr, washed twice, dispensed at 1×10^3 cells/well into triplicate cultures in 96-well, round-bottom plates, and incubated for 4 h at 37 °C with peptide-specific CD8+ T-cells at various effector to target (E:T) ratios. Percent-specific lysis was calculated as follows: percent-specific lysis = (experimental cpm − spontaneous cpm) × 100/(maximum cpm − spontaneous cpm).

3. Results

3.1. Selection of potential HLA-A24-binding peptides within Ad11 hexon protein

To identify potential HLA-A24-binding peptides within amino acid sequences of the Ad11 hexon protein, the amino acid sequence of the protein was analyzed by a web-based algorithm designed to predict HLA-binding peptides, based on estimation of the half-time dissociation of the HLA-peptide complex (http://www-bimas.cit.nih.gov/molbio/hla_bind/). Ten peptides with estimated half-time dissociation scores above 100 were selected and synthesized (Table 1). Next, the binding capacities of these peptides to HLA-A*24:02 molecules were tested in MHC stabilization assays using T2-A24 cells. All 10 peptides increased the HLA-A24 expression on the cells, indicating that these peptides bound and stabilized the HLA complex on the cell surface (Table 1). Thus, all 10 peptides were included in the subsequent experiments.

Table 1
Ad11 hexon protein derived peptides predicted to bind to HLA-A24, and the results of the MHC stabilization assays.

Amino acid sequence	Start position	Score[a]	% MFI increase[b]
KYTPSNVTL	482	480	141
DYLSAANML	641	360	77
SYQLLLDSL	366	360	57
LYSNVALYL	469	280	186
VYSGSIPYL	696	200	583
LYANSAHAL	889	200	37
NYNIGYQGF	769	180	142
TYFNLGNKF	37	158	82
NYIGFRDNF	322	150	89
GYKDRMYSF	782	120	27

Ad11, adenovirus serotype 11; MFI, mean fluorescence intensity.
[a] Estimated half-time of dissociation from HLA-A24 (min).
[b] Percent MFI increase of HLA-A*24:02 molecules on T2-A24 cells.

3.2. Induction and expansion of peptide-specific CD8+ T-cells from healthy donor PBMCs

To investigate the immunogenic potential of the 10 candidate peptides, HLA-A*24:02+ PBMCs from five healthy donors, of whom three were seropositive and two were seronegative for Ad11, were stimulated in vitro with each of these peptides. After 3-4 weekly stimulations, peptide-specific CD8+ T-cell frequencies were determined by IFN-γ secretion assays. Of the 10 peptides tested, INF-γ-producing CD8+ T-cells specific for the respective peptides were induced in the 3 peptides, TYFNLGNKF (TYF), VYS-

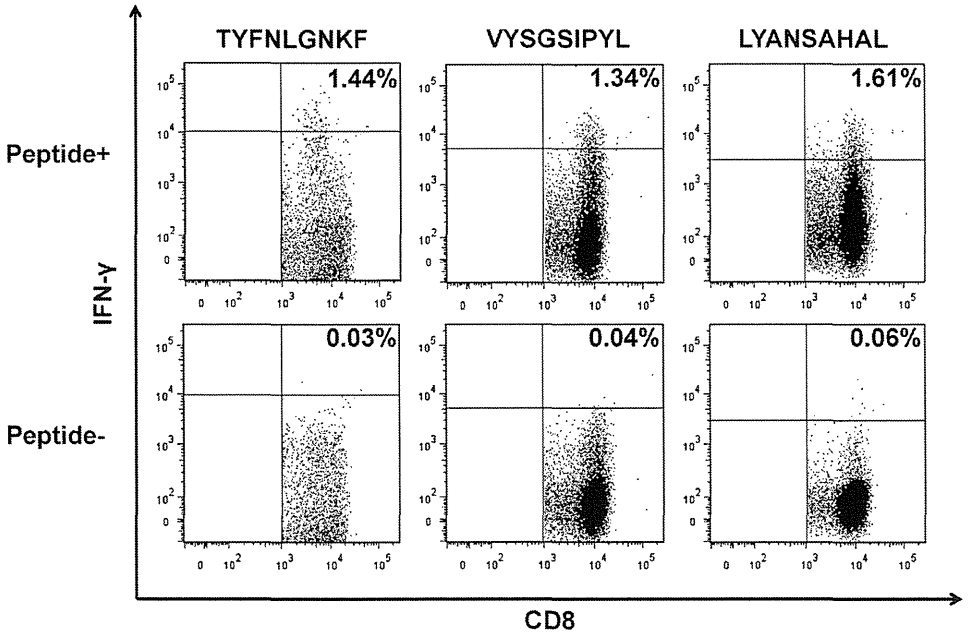

Fig. 1. Induction of peptide-specific CD8+ T-cells from PBMCs of HLA-A*24:02+ healthy donors. PBMCs from five HLA-A*24:02+ healthy donors were stimulated with each of the 10 epitope candidate peptides, and frequencies of IFN-γ-producing CD8+ cells were determined by IFN-γ secretion assays. The numbers in the upper right quadrants are the percentage of IFN-γ-producing cells among CD8+ cells in the presence (upper row) or absence (lower row) of the peptide. Among the 10 peptides tested, 3 induced IFN-γ-producing CD8+ cells.

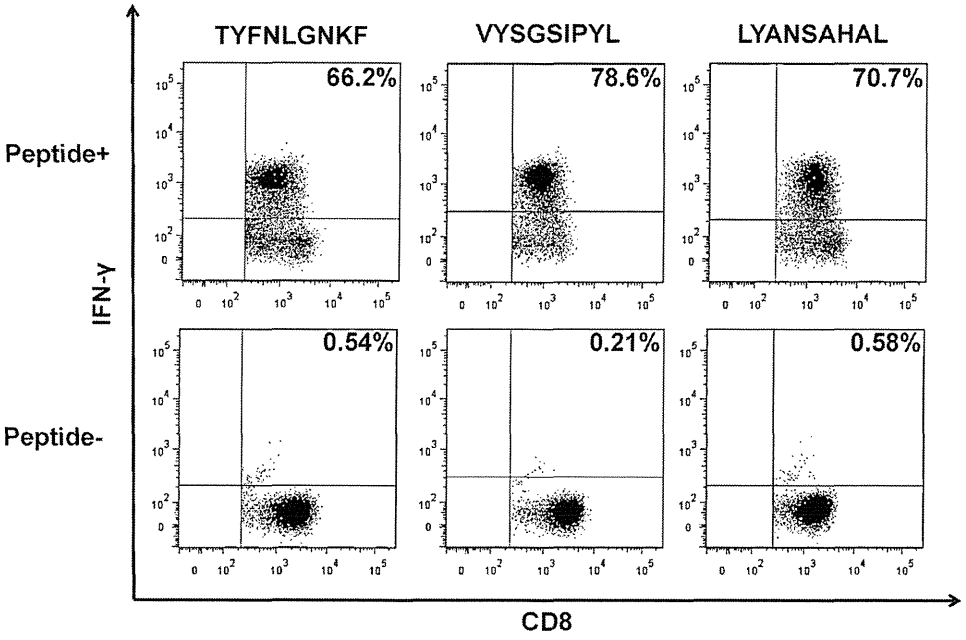

Fig. 2. Enrichment and expansion of peptide-specific CD8+ T-cells. Peptide-specific CD8+ T-cells induced by stimulating PBMCs from HLA-A*24:02+ healthy donors with epitope candidate peptides were isolated using IFN-γ secretion assay and then expanded in the presence of OKT3 mAb, IL-2, and feeder cells. Thereafter, the frequencies of IFN-γ-producing cells among CD8+ cells upon stimulation with autologous LCLs loaded with (upper row) or without (lower row) peptides were determined by intracellular IFN-γ staining assay.

GSIPYL (VYS), and LYANSAHAL (LYA) (Fig. 1). TYF-specific CD8+ T-cells were induced in all three seropositive donors, but not in the two seronegative donors. VYS- and LYA-specific CD8+ T-cells were induced in one of the two seronegative donors. Next, these INF-γ-producing CD8+ T-cells were sorted using an IFN-γ secretion assay, then expanded for subsequent analysis. After sorting and expansion, the frequencies of peptide-specific CD8+ cells among CD8+ cells determined by intracellular cytokine staining assay were above 60% for all three peptides (Fig. 2).

3.3. TYF peptide is naturally processed in Ad11-infected cells and presented in the context of HLA-A*24:02

To determine whether the epitope candidate peptides are naturally processed in Ad11-infected cells and presented by HLA-A*24:02 molecule, the expanded peptide-specific CD8+ T-cells were tested for IFN-γ production against HLA-A*24:02+ cells infected with Ad11. TYF-specific CD8+ T-cells produced significant quantities of IFN-γ against Ad11-infected or TYF peptide-loaded K562/A*24:02, but not against unmanipulated K562/A*24:02 and those loaded with an irrelevant peptide, QYDPVAALF, which is a CMV-pp65 peptide presented by HLA-A*24:02 (Fig. 3A) (Kuzushima et al., 2001). On the other hand, VYS- and LYA-specific CD8+ T-cells did not respond to Ad11-infected K562/A*24:02, indicating that VYS and LYA peptides were not naturally processed and presented on Ad11-infected cells (Fig. 3B, C). Furthermore, TYF-specific CD8+ T-cells effectively lysed TYF peptide-loaded K562/A*24:02 but not TYF peptide-loaded untransfected K562 (Fig. 4). These results indicate that TYF peptide is processed naturally in Ad11-infected cells, presented in the context of HLA-A*24:02, and is an epitope recognized by CD8+ CTLs.

3.4. TYF-specific CTLs do not recognize an HLA-A*24:02 restricted epitope of AdV serotype 5

As the newly identified epitope TYFNLGNKF was similar to the previously identified HLA-A*24:02 restricted epitope of AdV serotype 5 TYFSLNNKF (Leen et al., 2004b), we examined whether

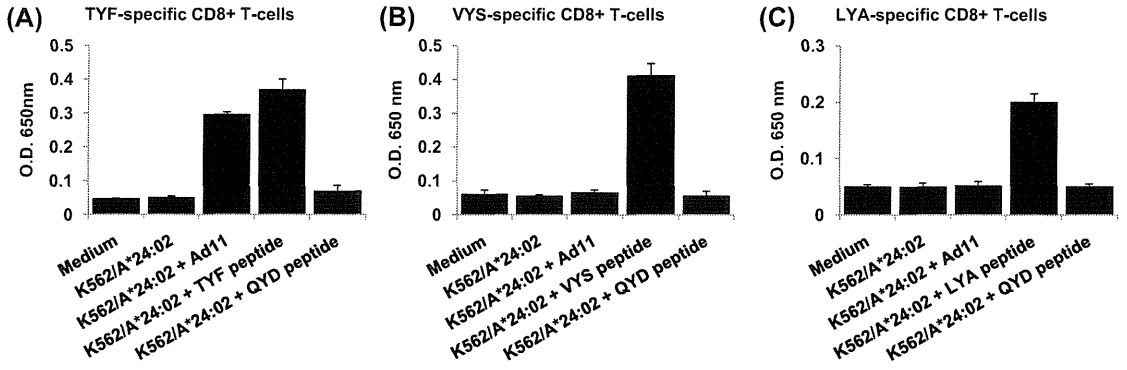

Fig. 3. TYF-specific, but not VYS- and LYA-specific, CD8+ T-cells produce IFN-γ against K562/A*24:02 infected with Ad11. Expanded TYF-specific CD8+ T-cells (A), VYS-specific CD8+ T-cells (B), and LYA-specific CD8+ T-cells (C) were incubated with K562/A*24:02, those loaded with the respective peptides, those infected with Ad11, or those loaded with an irrelevant peptide for 24 h. IFN-γ production in the supernatant was measured by an ELISA. Data are representative of two independent experiments and are the mean ± s.d. of triplicate experiments.

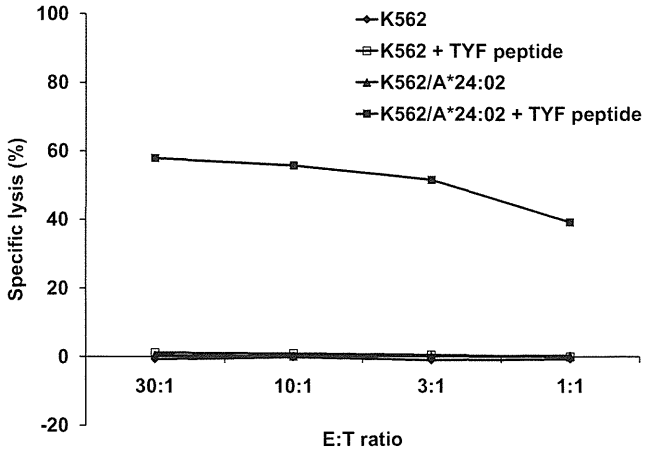

Fig. 4. TYF-specific CD8+ T-cells recognize TYF peptide in the context of HLA-A*24:02 and effectively lyse K562/A*24:02 loaded with TYF peptide. Cytotoxicities of TYF-specific CD8+ T-cells against K562, K562 loaded with TYF peptide, K562/A*24:02, and K562/A*24:02 loaded with TYF peptide were evaluated 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.

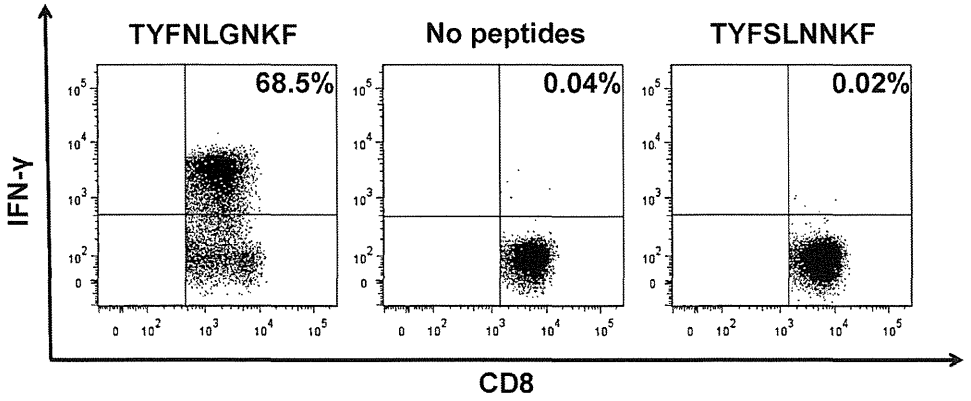

Fig. 5. TYF-specific CD8+ T-cells do not recognize an HLA-A*24:02 restricted epitope of AdV serotype 5. TYF-specific CD8+ T-cells were incubated with autologous LCLs loaded with TYFNLGNKF (a newly identified epitope of AdV serotype 11), those loaded with TYFSLNNKF (a previously identified epitope of 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.

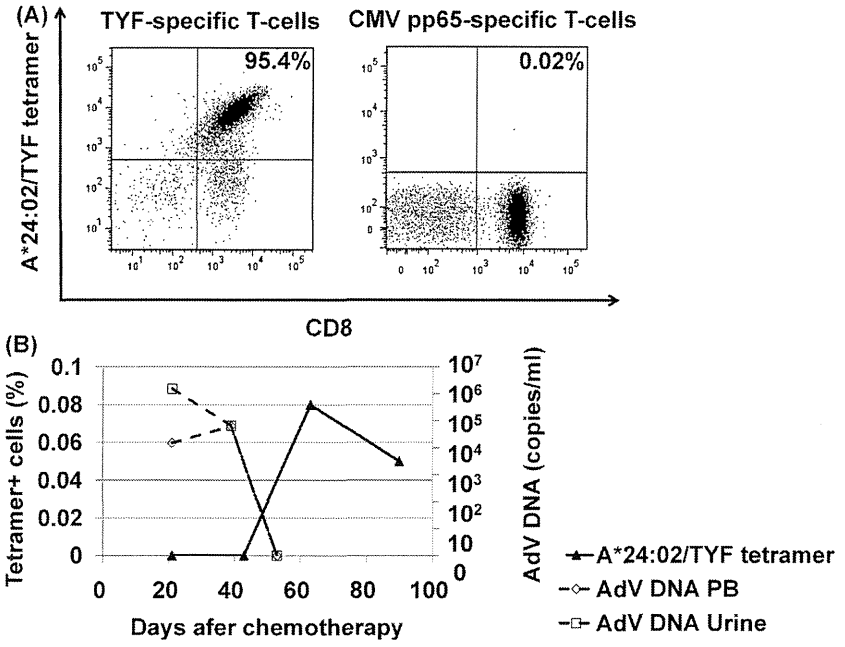

Fig. 6. Frequencies of TYF-specific CD8+ T-cells in a patient with Ad11 disease as determined by staining with HLA-A*24:02/TYF tetramer. (A) TYF-specific CD8+ T-cells and HLA-A*24:02 restricted CD8+ T-cells specific for CMV pp65 peptide QYDPVAALF were stained with PE-labeled HLA-A*24:02/TYF tetramer. 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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Letter to the Editor

Successful unrelated cord blood transplantation for adult acquired aplastic anemia using reduced intensity conditioning without ATG

Acquired aplastic anemia (aAA) patients who are transfusion-dependent and who have failed or relapsed after immunosuppressive therapy need further treatment. In cases in which a human leukocyte antigen (HLA)-identical sibling donor is not available, the use of alternative donor including HLA-matched unrelated donor and unrelated cord blood (CB) are commonly considered, but this strategy is associated with worse outcomes [1]. Because of the abundant availability of acceptable CB units, the use of cord blood transplantation (CBT) has been increasing. Although the use of CBT in patients with aAA has recently been evaluated [2], relatively little information is available on how to achieve proper engraftment with a reduced intensity conditioning (RIC) regimen in aAA patients undergoing CBT. We describe here three adult patients with aAA who underwent transplantation with unrelated CB after a RIC regimen without ATG. The patient characteristics are shown in Table 1. All three patients received single-unit CB containing more than 2.2×10^7/kg of total nucleated cell (TNC) with no more than two of six HLA-mismatches. The conditioning regimen consisted of six doses of fludarabine (Flu) 30 mg/m^2, two doses of cyclophosphamide (CY) 60 mg/kg and total body irradiation (TBI) 2 Gy × 2 with no use of anti-thymocyte globulin (ATG). The graft-versus-host disease (GVHD) prophylaxis regimen was a combination of short-term methotrexate (15, 10, and 10 mg/m^2 on days 1, 3, 6, respectively) and tacrolimus. Because patient #3 had a high titer of anti-HLA antibody, a CB unit that was not cross-reactive with this antibody was chosen.

All three patients rapidly exhibited sustained CB engraftment (Table 2). Chimerism analyses of the CD3$^+$ fraction using various numbers of tandem repeats showed initial full-donor conversion from the first point of analysis in all patients. No secondary graft-failure was observed. Acute GVHD was observed in patient #2 (skin only stage 2, Grade I) and resolved spontaneously. Chronic GVHD was observed in patient #3 (skin, oral involvement) and symptoms resolved quickly after the administration of 0.5 mg/kg oral prednisolone. The regimen was generally well tolerated, and no significant organ damage or severe toxicity occurred. The patients remain alive without transfusion dependence at 68, 44 and 9 months, with Karnofsky scores of 70% (due to postherpetic neuralgia), 100% and 100%, respectively.

Here we report three CBT recipients who received successful single-unit CBT after a RIC regimen. All three patients exhibited sustained full donor-type hematopoiesis without further intervention to increase donor-type chimerism. The conditioning regimen included 180 mg/m^2 Flu and 120 mg/kg CY with 2 Gy × 2 TBI, which may be regarded as a relatively strong regimen in terms of immunosuppressive and cytotoxic ability. Thus, one might think this regimen too potent for the induction of sustained engraftment of CB. However, Liu et al. reported that RIC regimen, consisting of Flu 120 mg/m^2, CY 1200 mg/m^2 (equivalent to 40 mg/kg if the patient's body weight was 50 kg) and rabbit ATG 30 mg/kg, was not sufficiently potent enough to induce engraftment after CBT in patients with aAA. They reported two early deaths and 16 graft-failures among the 18 CBT recipients conditioned with the above regimen [3]. Thus, it is reasonable to use a CY dose >40 mg/kg, and further study to determine the optimal CY dose between 40 and 120 mg/kg is warranted.

To ensure rapid and proper CB engraftment, graft cell contents, such as TNC, CD34$^+$ cell count and CD8$^+$ cell count, are important factors [4]. In Western countries, ATG is commonly used as the conditioning regimen for CBT. Nevertheless, the use of ATG will decrease lymphocytes, including graft-facilitating CD8$^+$ lymphocytes, which may lead to attenuation of total potency for the facilitation of engraftment in exchange for the beneficial effect of reducing the incidence of severe acute GVHD. Indeed, only one of seven CB recipients for aAA who received ATG-containing regimen achieved engraftment in a previous retrospective study in Japan [5]. Thus, we replaced ATG with 4 Gy TBI in our regimen, which may be another reason for successful engraftment.

One of the biggest differences in CBT between Western countries and Japan may be the attitude toward the use of ATG. In the recent protocol of European group, two doses of ATG 2.5 mg/kg and a single agent GVHD prophylaxis are recommended [6]. To reduce the incidence of severe acute GVHD, physicians in Europe and US would be likely to use ATG more frequently, which might result in failure to observe better engraftment. In fact, it is reported that a conditioning regimen without ATG provided a low incidence of graft-failure [7]. Taken together, we believe that ATG should not be included in the conditioning regimen for CBT, not only for a single-unit CBT but also for a double-unit CBT. We also have shown the superiority of two-drug GVHD prophylaxis (including methotrexate) over single-drug prophylaxis in CBT [8]. To compensate prophylactic effect of ATG to control severe GVHD, it would be preferable to develop the GVHD prophylaxis after transplantation without ATG. Further study to determine whether or not ATG should be used in order to achieve prompt engraftment and subsequent higher quality of life and survival after RIC-CBT is warranted.

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Table 1
Patient demographics and CB unit characteristics.

Pt no.	Age/sex	BW (kg)	Disease status at transplant	Interval from diagnosis to CBT (year)	Transfusion dependency	ABO mismatch	HLA serological mismatch	HLA allele mismatch	HLA-antibody	Donor-specific antibody	TNCC (10^7/kg)	$CD34^+$ (10^5/kg)
1	48/M	51	Severe	1.2	RBC	Match	2/6	3/8	–	–	3.67	0.50
2	53/M	65	Severe	22.1	RBC/PC	Major/minor	1/6	4/8	–	–	2.79	0.44
3	37/F	51	Non-severe	26.9	RBC/PC	Major/minor	2/6	3/8	+	–	2.24	0.55

Pt, patient; M, male; F, female; BW, body weight; RBC, red blood cell concentration; PC, platelet concentration; HLA, human leukocyte antigen; TNCC, total nucleated cell count.

Table 2
Engraftment, chimerism and other outcomes.

Pt no.	Days to ANC >500/μl	Days to reticulocyte >1%	Days to plt > 20,000/μl	Days to plt > 50,000/μl	Chimerism after CBT	Acute GVHD	Chronic GVHD	Other complications	Survival, mo	KS (%)
1	19	30	25	191	Day 20, 95% donor	No	No	Postherpetic neuralgia	Alive, 68	70
2	21	28	37	44	Day 19, 100% donor	Grade I (skin 2)	No	Polymyalgia rheumatica	Alive, 44	100
3	22	37	32	43	Day 25, 100% donor	No	Yes (skin, oral)	No	Alive, 9	100

Pt, patient; ANC, absolute neutrophil count;plt, platelet; KS, Karnofsky score.

Conflict of interest

All authors declare that there are no competing financial interests.

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