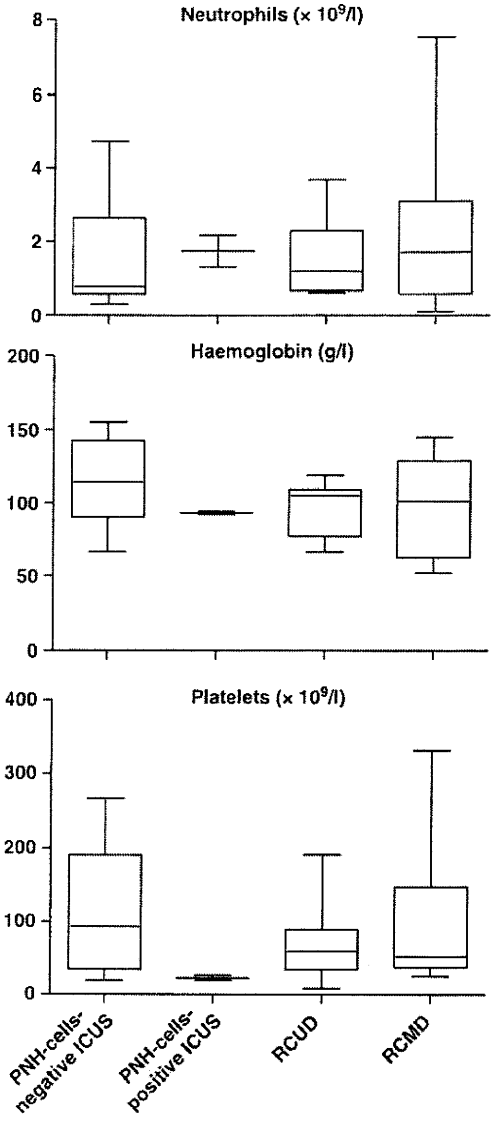

Fig 1. Haematological parameters in patients with idiopathic cytopenia of undetermined significance (ICUS) with or without a minor PNH clone, refractory cytopenia with unilineage dysplasia (RCUD), or refractory cytopenia with multilineage dysplasia (RCMD). Patients with ICUS with a minor PNH clone (n = 2) showed prominent thrombocytopenia. Boxes show 95 percentile confidence intervals; lines indicate the range of haematological parameters.

et al, 2010) and others show predominant thrombocytopenia with increased PNH-phenotype cells.

Another important issue is the limitation of diagnosing ICUS with the current criteria. Patients given diagnoses of ICUS in this study did not show hypoplastic BM suggestive of AA. However, BM sites other than the one examined by biopsy may have been hypocellular (Brunning *et al*, 2008), and the diagnosis of non-severe AA could not be completely excluded, although the degree of their cytopenias did not meet the criteria for AA. Morphological assessment of megakaryocytes is important to diagnose low-grade-dysplasia in MDS patients, although some ICUS patients, for example those with increased PNH-type cells in the present study, do not have enough megakaryocytes to be assessed. These findings indicate that further discussion from both pathophysiological and morphological approaches is necessary to understand overlapping categories of idiopathic cytopenia.

Acknowledgements

Thanks are due to Roderick J. Turner and Professor J. Patrick Barron of the Department of International Medical Communications Centre of Tokyo Medical University, for their review of this manuscript. Thanks are also due to Ms A. Hirota for her assistance. This work was supported in part by a grant-in-aid from the Ministry of Health, Welfare and Labour of Japan (Japanese Research Committee for Intractable Haematopoietic Diseases).

Conflict of interest

No potential conflicts of interest were disclosed.

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Keywords: idiopathic cytopenia of undetermined significance, PNH-phenotype, thrombocytopenia.

Supporting information

Additional Supporting Information may be found in the online version of this article:

Table SI. Frequency of PNH clone in myelodysplastic syndrome with marrow blasts fewer than 5% or idiopathic cytopenia of undetermined significance.

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Bone Marrow Transplantation (2011) 46, 208–216

www.nature.com/bmt

ORIGINAL ARTICLE

Pretransplant serum ferritin and C-reactive protein as predictive factors for early bacterial infection after allogeneic hematopoietic cell transplantation

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Although fluoroquinolones or other antibiotics are commonly used to prevent bacterial infections after hematopoietic cell transplantation (HCT), because of the growing presence of multidrug-resistant microorganisms, it is important to identify patients who are more likely to benefit from antibacterial prophylaxis. To evaluate risk factors for early bacterial infection after allogeneic HCT, we retrospectively analyzed clinical data for 112 consecutive adult patients with hematological malignancies who received transplants without any antibacterial prophylaxis. The cumulative incidence of bacterial infection at 30 days after transplantation was 16%. Among various pre-transplant factors, only high serum ferritin (>700 ng/mL, 47 patients) and high C-reactive protein (CRP) (>0.3 mg/dL, 28 patients) levels were significantly associated with the development of bacterial infection in a multivariate analysis (hazard ratio (95% confidence interval): ferritin, 4.00 (1.32–12.17); CRP, 3.64 (1.44–9.20)). In addition, septic shock and sepsis with organ failure were exclusively observed in patients who had high ferritin and/or high CRP levels. These results suggest that pretransplant serum ferritin and CRP levels can be useful markers for predicting the risk of early bacterial infection after allogeneic HCT. It may be prudent to limit antibacterial prophylaxis to patients with predefined risk factors to ensure the safety of HCT with the use of fewer antibiotics.

Bone Marrow Transplantation (2011) **46,** 208–216; doi:10.1038/bmt.2010.108; published online 3 May 2010
Keywords: antibacterial prophylaxis; fluoroquinolone; bacterial infection; allogeneic hematopoietic cell transplantation

Introduction

Bacterial infection is an important cause of mortality and morbidity after autologous or allogeneic hematopoietic cell transplantation (HCT).[1,2] When neutropenic patients who receive cytotoxic chemotherapy are compared with HCT recipients, the risk of severe bacterial infection appears to be considerably higher in the latter because high-dose chemotherapy and/or TBI may cause severe mucosal damage that facilitates massive bacterial translocation under profound post-transplant immunosuppression. Therefore, the use of oral fluoroquinolones (FQs) or other antibiotics as antibacterial prophylaxis is strongly considered for HCT recipients, although significant variations have been reported among transplant centers and countries.[3–5]

Recently, the widespread emergence of FQ-resistant or multidrug-resistant microorganisms in hematology–oncology units has been suggested to compromise the effectiveness of routine antibacterial prophylaxis with FQs in patients undergoing cytotoxic chemotherapy or HCT.[6–14] In our center, the isolation rate of FQ-resistant Gram-negative bacilli was high (57.1%) during a period when FQs were routinely administered as antibacterial prophylactic agents; in particular, among isolated *Enterobacteriaceae* strains, 66.7, 33.3 and 22.2% were resistant to levofloxacin, piperacillin and ceftazidime, respectively.[8] In an attempt to reduce the emergence of antibiotic-resistant microorganisms, we stopped using any antibacterial prophylaxis in both autologous and allogeneic HCT recipients in 2004,[8] and found that this discontinuation of FQ prophylaxis, even in the setting of myeloablative allogeneic HCT did not significantly affect early mortality after transplantation.[15]

Another approach to balance the safety of HCT with judicious antibiotic use would be to limit the use of antibacterial prophylaxis to HCT recipients who are at high risk of bacterial infection, because a delay in antibiotic treatment may lead to serious complications after infectious episodes in such patients if prophylactic antibiotics are not administered. To identify the pretransplant characteristics of patients who are more likely to be susceptible to bacterial infection after allogeneic HCT, we conducted a single-center retrospective study with the clinical data

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Received 13 January 2010; revised 8 March 2010; accepted 24 March 2010; published online 3 May 2010

of 112 consecutive allogeneic transplants for hematologic malignancies that were performed without antibacterial prophylaxis. As a potential biomarker for predicting bacterial infection, high levels of pretransplant ferritin levels have recently been shown to be associated with an increased incidence of bloodstream infection, as well as decreased overall survival and increased early mortality.[16] In addition, elevated pretransplant serum C-reactive protein (CRP) levels have been shown to be associated with an increased incidence of bacterial infection in the setting of chemotherapy for acute myeloid leukemia[17] and allogeneic transplantation.[18] Sato *et al.*[17] reported that pretreatment serum CRP levels of greater than 0.26 mg/dL were useful for predicting the incidence of documented infection in patients who received their first consolidation chemotherapy for acute myeloid leukemia. As these biomarkers are easy to measure and may be useful in clinical practice, in this study we explored the association between bacterial infection and these biomarkers as well as various patient characteristics.

Subjects and methods

Study population

The medical records of 137 consecutive adult patients with hematological malignancies who underwent T-cell replete allogeneic HCT from September 2004 to March 2009 at Kyoto University Hospital were reviewed. Patients who had active infections before the transplantation procedure ($n=23$) and those who had a recent history of autologous or allogeneic HCT within 1 year ($n=2$) were excluded; thus, a total of 112 patients were included in the analysis, without any duplication of subjects. Patients were considered to have standard-risk disease if they received a transplant without prior chemotherapy or in CR, whereas those who received a transplant in any other status were considered to have high-risk disease. This study was approved by the Ethics Committee of Kyoto University Graduate School and the Faculty of Medicine. Written informed consent for the transplantation protocol was obtained from all of the patients.

Prophylaxis monitoring and diagnosis of infection

A central venous catheter was inserted in the subclavian vein before the beginning of the conditioning regimens for all patients. Each patient was isolated in a single room equipped with a HEPA system from a day before the transplantation until at least 4 weeks after transplantation. Each patient was then maintained on a low-microbial diet and asked to take strict control measures under the supervision of the ward staff to prevent the acquisition of nosocomial pathogens. No bacterial prophylaxis was prescribed for these patients according to our institutional protocols.[8] Intravenous antibiotics with anti-pseudomonal activity were promptly administered in response to episodes of febrile neutropenia or suspected bacterial infections. Trimethoprim-sulfamethoxazole (trimethoprim; 160 mg/day, three times a week) was administered as prophylactic therapy for *Pneumocystis jiroveci* pneumonia from the day of admission until the day of transplantation, and this prophylaxis was reinitiated after the day of neutrophil engraftment. All patients received 200 or 400 mg of fluconazole and 400–1000 mg of acyclovir per day as prophylactic agents from the conditioning period until 30 days after transplantation. Prophylactic fluconazole and acyclovir were usually continued when patients were receiving steroid therapy for acute or chronic GVHD. For each febrile episode, 1 or 2 sets of blood samples were cultured, and cultures of specimens other than blood specimens and imaging examinations to search for the focus of infection were performed according to the clinician's judgment. Microbiologically documented infections included the presence of bloodstream bacterial infection or any other bacterial infection. Bloodstream bacterial infection was diagnosed when at least 1 of the following criteria was met: (1) the culture of blood obtained during a febrile episode tested positive at least once for bacterial organisms other than common skin contaminants, (2) the culture of blood obtained during a febrile episode tested positive for the same common skin contaminant in independent analysis conducted within an interval of 72 h, and (3) the blood culture tested positive at least once for a common skin contaminant, and the patient was diagnosed with septicemia with hypotension (systolic blood pressure, <90 mm Hg) and disseminated intravascular coagulation. Infections other than bloodstream infection were diagnosed when the following criteria were met: (1) sputum, urine or stool samples were found to contain pathogenic bacteria on at least two occasions, and (2) the patient showed symptoms of infection corresponding to those specimens. Septic shock and sepsis with organ failure were defined as severe infection.

Measurement of serum biomarkers (serum ferritin and C-reactive proteins)

Both serum ferritin and CRP levels were measured using peripheral blood samples obtained just before the start of the conditioning regimen. The serum ferritin concentration was measured by an immunoenzymometric assay (AIA-PACK FER, Tosoh Corporation, Tokyo, Japan) (normal range; $\leqslant 150$ ng/mL), and the serum CRP concentration was measured by a latex agglutination assay (N-Assay LA CRP-S, Nittobo, Tokyo, Japan) (normal range; $\leqslant 0.2$ mg/dL), according to the respective manufacturer's instructions.

Statistical analysis

The primary endpoint was the cumulative incidence of documented bacterial infections during the first 30 days after transplantation. Overall survival and treatment-related mortality were also analyzed as secondary endpoints. To eliminate the effect of a competing risk, the cumulative incidence was assessed using methods described elsewhere.[19] In the analysis of the cumulative incidence of bacterial infections and treatment-related mortality, a competing event was defined as death without an event of interest. The overall survival was estimated using the Kaplan–Meier method. We applied Fine and Gray's proportional hazards model for the sub-distribution of a competing risk to analyze the cumulative incidence of

bacterial infection and treatment-related mortality, and the Cox proportional hazards model for that of overall survival.[20] Factors with *P*-values of less than 0.10 in the univariate analysis were included in the multivariate analysis. Factors evaluated in the analysis included the recipient's age (≤50 or >50), recipient's sex (female or male), diagnosis (myeloid or lymphoid malignancies), disease status at transplant (standard risk or high risk), duration from diagnosis to transplant (≤1 or >1 year), duration from the last pretransplant cytotoxic chemotherapy to conditioning of transplant (no history of prior chemotherapy or >2, or ≤2 months), number of courses of prior cytotoxic chemotherapy (≤5 or >5), source of stem cells (related BM or peripheral blood, unrelated BM, or unrelated cord blood), conditioning regimen (conventional or reduced-intensity regimen), use of granulocyte CSF (G-CSF) (yes or no), serum ferritin levels (≤700 ng/mL, >700 ng/mL, or unknown), and serum CRP levels (≤0.3 or >0.3 mg/dL). We assessed the interaction between ferritin and CRP levels, using interaction terms between a ferritin category with scores of 0 (ferritin ≤700 ng/mL) and 1 (ferritin >700 ng/mL) and a CRP category with scores of 0 (CRP ≤0.3 mg/dL) and 1 (CRP >0.3 mg/dL). The cutoff point for the ferritin levels was the median value and that for the CRP levels was the higher tertile value. The correlation between ferritin and CRP levels was also tested by Pearson's correlation coefficient. *P*-values of less than 0.05 were considered statistically significant. All analyses were conducted using Stata software version 11 (StataCorp., College Station, TX, USA).

Results

Patient characteristics

The patient characteristics are shown in Table 1. The median age of the patients was 47 years (range, 18–66 years). The primary diseases in these patients were as follows: acute myeloid leukemia in 46 patients, acute lymphoblastic leukemia in 11, myelodysplastic syndrome in 16, chronic myelogenous leukemia in four, non-Hodgkin's lymphoma in 19, adult T-cell leukemia/lymphoma in 10, myeloproliferative disorder in four, and plasma-cell myeloma in two. Sixty-six patients (58.9%) had standard-risk disease. The source of stem cells used for HCT was related BM or peripheral blood in 40 patients (35.7%), unrelated BM in 52 (46.4%), and unrelated cord blood in 20 (17.9%). A conventional myeloablative regimen was used in 54 patients (48.2%), and G-CSF was used after HCT in 57 patients (50.9%). The number of patients with pretransplant serum ferritin levels of ≤700, >700 ng/mL, and unavailable were 49, 47 and 16, respectively, and the number of those with pretransplant serum CRP levels of ≤0.3 and >0.3 mg/dL were 84 and 28, respectively.

Table 1 Patient characteristics

Category	*Patients (n = 112)*
Age, years	
Median (range)	47 (18–66)
Sex, n (%)	
Male	49 (43.8)
Female	63 (56.3)
Diagnosis, n (%)	
Acute myeloid leukemia	46 (41.1)
Acute lymphoblastic leukemia	11 (9.8)
Myelodysplastic syndrome	16 (14.3)
Chronic myelogenous leukemia	4 (3.6)
Non-Hodgkin's lymphoma	19 (17.0)
Adult T-cell leukemia/lymphoma	10 (8.9)
Myeloproliferative disorder	4 (3.6)
Plasma-cell myeloma	2 (1.8)
Disease status at transplant, n (%)	
Standard risk	66 (58.9)
High risk	46 (41.1)
Source of stem cells, n (%)	
Related bone marrow or peripheral blood	40 (35.7)
Unrelated bone marrow	52 (46.4)
Unrelated cord blood	20 (17.9)
Conditioning regimen, n (%)	
Conventional-intensity regimen	54 (48.2)
BU/CY	10
TBI/CY-based regimen	44
Reduced-intensity regimen	58 (51.8)
Flu/BU ± TBI	23
Flu/Mel ± TBI	34
Flu/TT	1
Use of G-CSF, n (%)	
Yes	57 (50.9)
No	55 (49.1)
Duration from diagnosis to transplant, n (%)	
≤1 year	57 (50.9)
>1 year	55 (49.1)
Duration from the last pretransplant cytotoxic chemotherapy to conditioning of transplant, n (%)	
No history of previous cytotoxic chemotherapy or >2 months	68 (60.7)
≤2 months	44 (39.3)
Number of courses of previous cytotoxic chemotherapy, n (%)	
≤5 courses	60 (53.6)
>5 courses	52 (46.4)
Pretransplant serum ferritin level (ng/mL)	
Median (range)	694.6 (34.7–12079.1)
Pretransplant serum CRP level (mg/dL)	
Median (range)	0.1 (0.0–4.6)

Abbreviations: Flu = fludarabine; Mel = melphalan; TT = thiotepa, G-CSF = granulocyte CSF.

Documented bacterial infections

A total of 19 episodes of bacterial infections were documented during the first 30 days after HCT; these included 18 episodes of bloodstream infections and 1 of pneumonia. No patient had more than 1 episode of bacterial infection within 30 days after HCT. The bacterial organisms associated with the documented infections are listed in Table 2. The detected bacterial organisms were mainly Gram-negative bacilli (*n* = 16, 84.2%), 15 of which (93.6%) were sensitive to FQs.

Table 2 Documented bacterial organisms within 30 days after transplantation

Category	*Bacterial isolates*
Gram-positive cocci (*n*)	*Enterococcus faecium* (2) *Streptococcus epidermidis* (1)
Gram-negative bacilli (*n*)	*Klebsiella pneumoniae* (5) *Escherichia coli* (4) *Pseudomonas aeruginosa* (2) *Klebsiella oxytoca* (1) *Enterobacter cloacae* (1) *Capnocytophaga species* (1) *Prevotella intermedia* (1) *Bacteroides thetaiotaomicron* (1)

P. aeruginosa was detected in the sputum of one patient with pneumonia. Other organisms were detected in blood culture bottles.

The cumulative incidence of bacterial infections was 16% (95% confidence interval (CI), 10–24%). Among confounding factors that were potentially associated with bacterial infection, only high pretransplant serum ferritin (>700 vs ⩽700 ng/mL) and high CRP (>0.3 vs ⩽0.3 mg/dL) levels were significantly associated with the development of bacterial infection in the multivariate analysis (hazard ratio (95% CI): ferritin, 3.97 (1.35–11.69), $P=0.012$; CRP, 3.63 (1.45–9.10), $P=0.006$) (Table 3). Even when serum ferritin and CRP levels were treated as continuous variables, their impact remained significant. Although there was no correlation between ferritin and CRP levels ($P=0.062$), we analyzed the impact of high ferritin levels in subgroups of patients with either high (>0.3 mg/dL) or low CRP levels (⩽0.3 mg/dL), to exclude the effect of inflammation on ferritin levels. We obtained almost consistent results in both groups (hazard ratio (95% CI): CRP >0.3 mg/dL, 3.67 (0.87–15.63), $P=0.078$; CRP ⩽0.3 mg/dL, 4.12 (0.86–19.64), $P=0.076$). Furthermore, no interaction was observed between the ferritin and CRP categories ($P=0.949$). Next, we re-evaluated the risk of bacterial infection with the combination of these two risk factors (ferritin and CRP levels). Figure 1 shows the cumulative incidence of bacterial infection for patients divided into three risk groups according to this model. The cumulative incidences of bacterial infections were 5.3% (95% CI; 1.0–15.7%) in patients without any risk factors ($n=39$), 20.5% (95% CI; 10.1–33.3%) in those with 1 factor ($n=44$), and 53.8% (95% CI; 24.8–76.0%) in those with two factors ($n=13$). The hazard ratios for 1 and 2 risk factors relative to no risk factors in the multivariate analysis were 4.04 (95% CI, 0.88–18.62) and 14.68 (3.02–71.30), respectively. Among patients with bacterial infections, septic shock or organ failure was observed in one patient with two risk factors and four patients with one risk factor, but not in any patients with no risk factors.

Overall survival and treatment-related mortality

Next, we evaluated the impact of the ferritin and CRP levels on other endpoints in 96 patients for whom data on ferritin levels were available (Figures 2 and 3). The median duration of follow-up was 23 months (range, 2.2–54.9). With regard to overall survival, only a high ferritin level (hazard ratio (95% CI): 2.47 (1.19–5.11), $P=0.015$) and a duration of less than 2 months from the last cytotoxic chemotherapy to the conditioning for transplant (hazard ratio (95% CI): 2.16 (1.10–4.26), $P=0.026$) were significant variables in the multivariate analysis. The causes of death are shown in Table 4. Interestingly, seven patients among those with high ferritin levels died within 100 days (causes of death: acute GVHD, $n=2$; infection, $n=3$; hepatic veno-occlusive disease, $n=1$; organ failure, $n=1$), whereas none of the patients with low ferritin levels died. With regard to treatment-related mortality, only ferritin and CRP levels were adversely associated with higher treatment-related mortality in the multivariate analysis (hazard ratio (95% CI): ferritin, 5.21 (1.41–19.30), $P=0.013$; CRP, 5.76 (1.70–19.48), $P=0.005$).

Discussion

In our cohort of 112 patients with hematologic malignancies who underwent allogeneic HCT without antibacterial prophylaxis, we found that only high serum ferritin and high CRP levels before transplantation were significant risk factors for the post-transplant development of bacterial infection; patients with high ferritin levels and those with high CRP levels had an almost 4-fold higher risk of bacterial infection than those with low ferritin levels or those with low CRP levels. In addition, although severe complications associated with bacterial infection were observed in five patients with high ferritin levels and/or high CRP levels, none were seen in patients with low ferritin and low CRP levels. These results suggest that pretransplant serum ferritin and CRP levels may be useful markers for predicting the risk of early bacterial complications after allogeneic HCT.

An association between iron overload and bacterial or fungal infection has been shown in hereditary and secondary hemochromatosis.[21,22] With regard to HCT, Pullarkat *et al.*[16] reported that ferritin levels of ⩾1000 ng/mL were associated with a 2-fold higher risk of bloodstream infection compared with patients with ferritin levels of <1000 ng/mL in myeloablative HCT. In agreement with their finding, in this study, ferritin levels of >700 ng/mL were associated with a 4-fold increased risk compared with the risk in patients with levels of ⩽700 ng/mL. An increase in plasma non-transferrin-bound iron (NTBI) is considered to have an important role in the adverse effect of iron overload on bacterial infection. Under normal conditions, toxic reactions due to the production of NTBI are prevented by circulating transferrin, which forms a compound with Fe^{3+}.[23] However, plasma NTBI increases to a measurable level in patients with iron overload because transferrin is almost saturated with Fe^{3+}.[24] The inhibition of iron utilization in erythrocytes by chemotherapeutic agents and irradiation further increases NTBI levels.[25] Hydroxyl radical reactions by NTBI exacerbate mucosal damage caused by chemotherapeutic agents and irradiation, which allows bacterial organisms to enter through circulation.[26] In addition, iron is an important nutrient for the proliferation of bacteria and fungi.[27] In the HCT

Table 3 Univariate and multivariate analyses of factors that are potentially associated with documented bacterial infection

Category	*Number*	*Univariate analysis*		*Multivariate analysis*	
		Hazard ratio (95% CI)	*P-value*	*Hazard ratio (95% CI)*	*P-value*
Age, years					
⩽50	10/64	1.00	Reference		
>50	9/48	1.27 (0.51 3.17)	0.608		
Sex					
Female	9/49	1.00	Reference		
Male	10/63	0.80 (0.32 2.00)	0.632		
Diagnosis					
Myeloid malignancies	13/72	1.00	Reference		
Lymphoid malignancies	6/40	0.86 (0.33 2.26)	0.766		
Disease status at transplant					
Standard risk	11/66	1.00	Reference		
High risk	8/46	1.13 (0.45 2.84)	0.795		
Source of stem cells					
Related bone marrow or peripheral blood	6/40	1.00	Reference		
Unrelated bone marrow	8/52	0.88 (0.30 2.59)	0.816		
Unrelated cord blood	5/20	1.84 (0.56 6.05)	0.318		
Conditioning regimen					
Conventional-intensity regimen	8/54	1.00	Reference		
Reduced-intensity regimen	11/58	1.48 (0.57 3.79)	0.419		
Use of G-CSF					
No	10/55	1.00	Reference		
Yes	9/57	0.98 (0.39 2.45)	0.966		
Duration from diagnosis to transplant					
⩽1 year	9/57	1.00	Reference		
>1 year	10/55	1.33 (0.53 3.34)	0.550		
Duration from the last pretransplant cytotoxic chemotherapy to conditioning of transplant					
No history of previous cytotoxic chemotherapy or >2 months	13/68	1.00	Reference		
⩽2 months	6/44	0.76 (0.29 2.03)	0.589		
Number of courses of previous cytotoxic chemotherapy					
⩽5 courses	9/60	1.00	Reference		
>5 courses	10/52	1.44 (0.57 3.64)	0.441		
Serum ferritin level					
⩽700 ng/mL	5/49	1.00	Reference	1.00	Reference
>700 ng/mL	14/47	4.04 (1.35 12.05)	0.012	3.97 (1.35 11.69)	0.012
Not available	0/16	—	—	—	—
Serum CRP level					
⩽0.3 mg/dL	10/84	1.00	Reference	1.00	Reference
>0.3 mg/dL	9/28	3.38 (1.36 8.39)	0.009	3.63 (1.45 9.10)	0.006

Abbreviations: CI = confidence intervals; G-CSF = granulocyte CSF.

setting, the ability of NTBI to induce the proliferation of *Staphylococcus epidermidis* has been shown in an *in vitro* study using the serum of patients undergoing HCT.[28]

In addition to the adverse impact of iron overload on early infection-related complications, several studies have suggested that high ferritin levels are adversely associated with overall survival and treatment-related mortality.[16,29 31] In agreement with these studies, our results showed that high ferritin levels are associated with a 2.5-fold increased risk of overall mortality and a 5-fold increased risk of higher treatment-related mortality, compared with low ferritin levels. These studies collectively suggest that iron overload is an important and strong prognostic factor in various clinical outcomes of allogeneic HCT.

Recently, an association between iron chelation therapy and longer overall survival was shown in patients with MDS or severe anemia requiring multiple blood transfusions,[32,33] and adequate iron chelation therapy is recommended for such patients.[34] The administration of oral iron-chelating agents such as deferasirox may be an attractive treatment for iron-overloaded patients compared with deferoxamine, which requires s.c. or i.v.

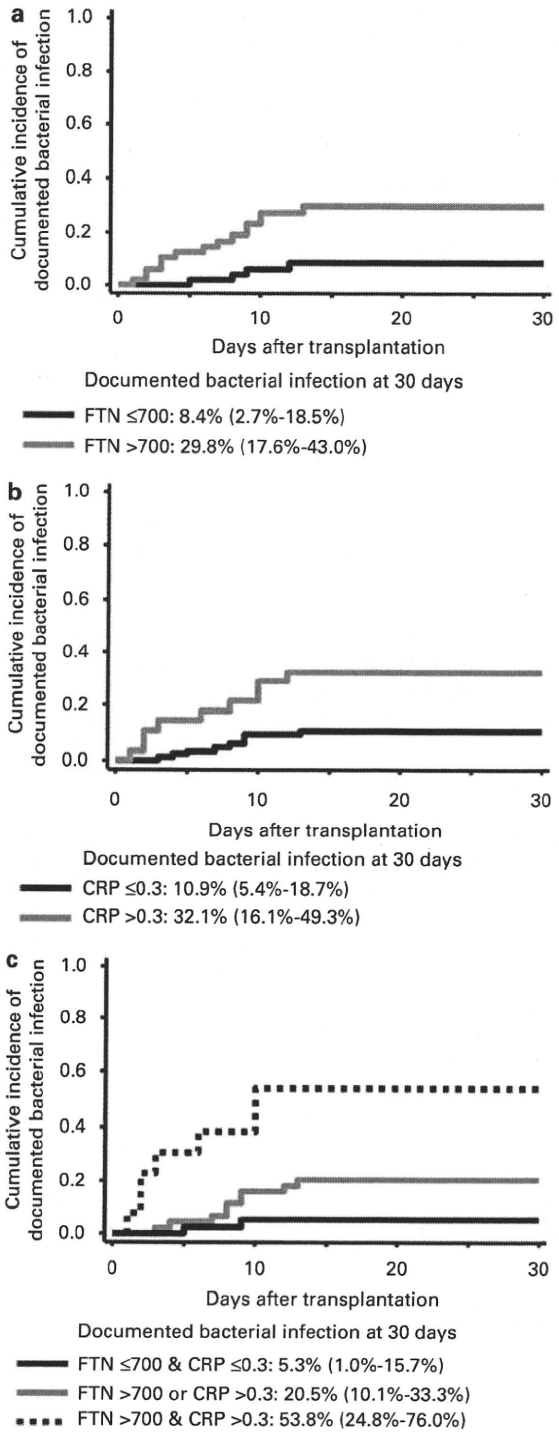

Figure 1 Cumulative incidence of documented bacterial infection within 30 days after transplantation. (**a**) Solid black line, patients with low ferritin levels ($\leqslant$700 ng/mL) ($n=49$); gray line, patients with high ferritin levels ($>$700 ng/mL) ($n=47$), (**b**) Solid black line, patients with low CRP levels ($\leqslant$0.3 mg/dL) ($n=84$); gray line, patients with high CRP levels ($>$0.3 mg/dL) ($n=28$), (**c**) Solid black line, patients with low ferritin ($\leqslant$700 ng/mL) and low CRP levels ($\leqslant$0.3 mg/dL) ($n=39$); gray line, patients with low ferritin and high CRP levels ($>$0.3 mg/dL) or high ferritin ($>$700 ng/mL) and low CRP levels ($n=44$); dotted black line, patients with high ferritin and high CRP levels ($n=13$).

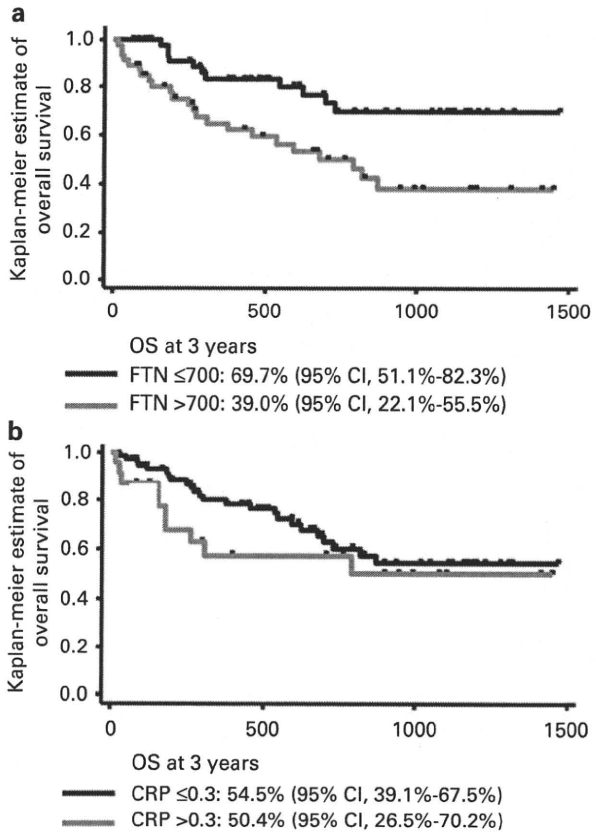

Figure 2 Kaplan–Meier estimate of overall survival after transplantation. (**a**) Solid black line, patients with low ferritin levels ($\leqslant$700 ng/mL) ($n=49$); gray line, patients with high ferritin levels ($>$700 ng/mL) ($n=47$), (**b**) Solid black line, patients with low CRP levels ($\leqslant$0.3 mg/dL) ($n=73$); gray line, patients with high CRP levels ($>$0.3 mg/dL) ($n=23$).

administration. However, the optimal dosage and timing for the administration of deferasirox in allogeneic HCT should be carefully determined in future studies because its renal and gastrointestinal side effects may exacerbate complications of HCT.

At present, only one report has referred to the association between pretransplant CRP levels and transplant outcomes.[18] In that report, pretransplant CRP levels had a marginally significant association with infection within 100 days after reduced-intensity HCT, whereas other confounding factors, including age, disease status, hematopoietic cell transplantation-specific comorbidity index (HCT-CI), and performance status, had no association; this result is consistent with our present findings. One possible explanation of these findings is that the slightly elevated CRP levels might have reflected minute inflammation, which may represent the presence of latent bacterial infection with negative clinical signs and negative results in pretransplant screening tests, such as X-ray or CT scans. Undetectable bacterial organisms colonized under bacteriostatic conditions before transplant might have rapidly proliferated in the post-transplant neutropenic and immunosuppressive state. Therefore, even if no bacterial infection is detected before transplant in screening tests, latent bacterial infection should be considered in patients with high CRP levels. With regard to treatment-related

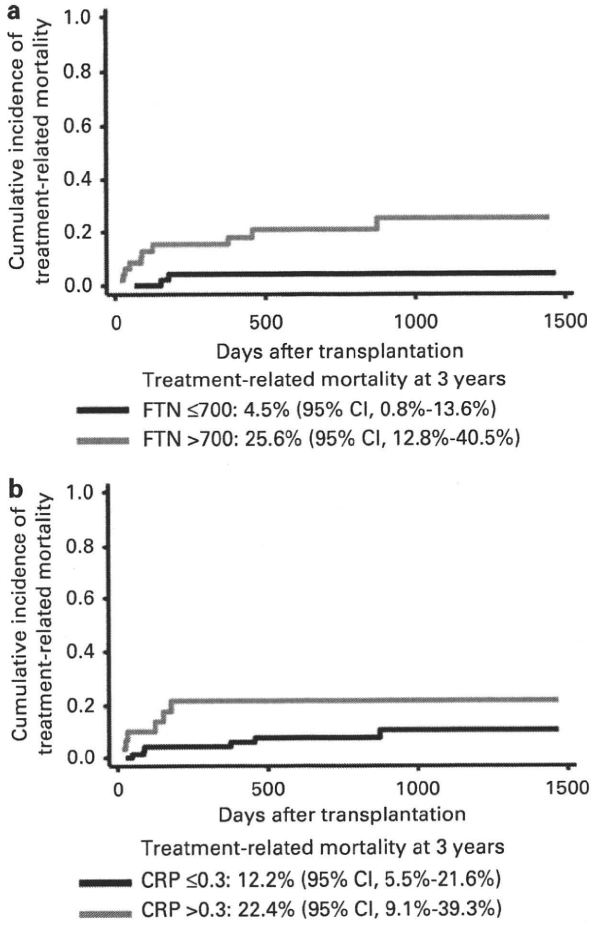

Figure 3 Cumulative incidence of treatment-related mortality after transplantation. **(a)** Solid black line, patients with low ferritin levels (≤700 ng/mL) ($n=49$); gray line, patients with high ferritin levels (>700 ng/mL) ($n=47$), **(b)** Solid black line, patients with low CRP levels (≤0.3 mg/dL) ($n=73$); gray line, patients with high CRP levels (>0.3 mg/dL) ($n=23$).

Table 4 Causes of death

Category	*Low ferritin group (≤700 ng/mL)* (n=49)	*High ferritin group (>700 ng/mL)* (n=47)
Within 100 days after transplant		
Infection	0	2 (29%)
Organ failure	0	2 (29%)
Graft-versus-host disease	0	2 (29%)
Hepatic veno-occlusive disease	0	1 (14%)
Total	0	7
More than 100 days after transplant		
Relapse	9 (82%)	12 (75%)
Infection	1 (9%)	2 (13%)
Organ failure	1 (9%)	0
Idiopathic pneumonia syndrome	0	1 (6%)
Bleeding	0	1 (6%)
Total	11	16

mortality, an elevated pretransplant CRP level was found to be a significant risk factor in our study, consistent with a previous report.[18] The reason for the worse treatment-related mortality in patients with elevated pretransplant CRP levels remains unclear and needs to be clarified in future studies.

To ensure the safety of allogeneic HCT with the limited use of antibacterial agents, the selective prophylactic administration of antibacterial agents such as FQs only to patients at high risk of bacterial infection may be effective. In this study, Gram-negative bacilli that were highly sensitive to FQs (93.6%) were the main bacterial organisms isolated, which suggests that these infections may have been prevented by the prophylactic administration of FQs in our center. However, this approach may be effective only if most of the bacterial isolates at the transplant center were sufficiently sensitive to these prophylactic antibiotics. In future studies, it would be worthwhile evaluating whether the incidence of early bacterial infection can be reduced by the prophylactic administration of antibiotics in patients with predefined risk factors such as high ferritin levels or high CRP levels. Iron chelation therapy before HCT is another intriguing strategy that is worthy of future evaluation.

This study had several limitations. The retrospective study design, small sample size and heterogeneous background of diseases and transplantation procedures may have biased the results. In addition, HCT-CI, including the performance status, was not evaluated in this cohort due to a lack of adequate information. Furthermore, the impact of serum ferritin levels on the outcomes should be interpreted with caution. Although we consistently determined that high ferritin levels have an adverse impact on early bacterial infection regardless of CRP levels, serum ferritin levels can be affected by conditions associated with other diseases.[35] In a future study, it may be worthwhile to quantify iron overload by other methods, such as magnetic resonance imaging of the liver,[36] and to re-analyze the effect of iron content on the outcome.

In conclusion, these results suggest that pretransplant serum ferritin and CRP levels, which can be easily measured in various centers, may be useful markers for predicting the risk of early bacterial complications after allogeneic HCT. However, larger prospective studies are warranted to validate our findings and further research is needed to identify other biomarkers that may be associated with the development of post-transplant bacterial complications.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We are grateful to Rie Goi and Mika Kobayashi for their expert data management and secretarial assistance, and to all of the members of the transplant and infection-control teams at Kyoto University Hospital for their dedicated care of the patients and donors.

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ELSEVIER

Experimental Hematology

Experimental Hematology 2011;39:424–433

Cross-priming of $CD8^+$ T cells in vivo by dendritic cells pulsed with autologous apoptotic leukemic cells in immunotherapy for elderly patients with acute myeloid leukemia

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(Received 11 November 2010; revised 28 December 2010; accepted 1 January 2011)

Objective. **The prognosis for elderly patients with acute myeloid leukemia (AML) remains dismal. To explore the potential of immunotherapy for improving clinical outcomes for these patients, we performed a phase I clinical trial of dendritic cell (DC)–based immunotherapy for elderly patients with AML.**

Materials and Methods. **Autologus monocytes were obtained after reducing tumor burden by chemotherapy. Immature DCs induced with granulocyte-macrophage colony-stimulating factor and interleukin-4 were pulsed with autologous apoptotic leukemic cells as antigens. DCs were administered intradermally to four patients five times at 2-week intervals. To facilitate DC migration to lymph nodes, injection sites were pretreated with killed *Streptococcus pyogenes* OK-432 one day before. DCs were coinjected with OK-432 to induce maturation and interleukin-12 production in vivo.**

Results. **Antileukemic responses were observed by an interferon-γ enzyme-linked immunospot assay or a tetramer assay in two of four patients. In a human leukocyte antigen–A*2402-positive patient, induction of $CD8^+$ T-cell responses to WT1- and human telomerase reverse transcriptase–derived peptides were observed, indicating cross-priming in vivo. The two patients with antileukemic immunity showed longer periods of disease stabilization than the other two patients.**

Conclusions. **This study demonstrates the immunogenicity of autologous DCs that cross-present leukemia-associated antigens from autologous apoptotic leukemic cells in vivo in elderly patients with AML.** **© 2011 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Management of elderly patients with acute myeloid leukemia (AML) remains a challenge because of a high rate of therapy-related mortality and chemotherapy resistance [1]. Antigen-specific immunotherapy, which is less toxic and kills leukemic cells through different mechanisms than chemotherapy, has the potential capacity to improve the clinical outcomes of these patients. Recent identification of several leukemia-associated antigens prompted us to develop immunotherapy for elderly patients with AML [2].

Active immunization by peptide vaccines can induce antileukemic immunity and clinical responses in AML [3–6]. Clinical trials of dendritic cell (DC)–based immunotherapy for AML have also been reported [7–12]. However, the trial using leukemic cell–derived DCs showed that the generation of leukemic cell–derived DCs was feasible in only a limited number of patients, and even in vaccinated patients the treatment could not induce clinical responses [9]. This may be due to lower immunostimulatory activity of leukemic cell–derived DCs than monocyte-derived DCs (MoDCs) [13]. Recently, the efficient generation of MoDCs from patients with AML has been demonstrated in vitro [14], providing a rationale for the use of MoDCs in immunotherapy for AML.

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Supplementary data associated with this article can be found in the online version at doi:10.1016/j.exphem.2011.01.001.

0301-472X/$ - see front matter.
doi: 10.1016/j.exphem.2011.01.001

There are several parameters to enhance the immunogenicity of MoDC vaccines. Whereas monocytes are cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin (IL)-4 conventionally for 5 to 7 days to induce DCs, a shorter period of culture is sufficient to induce equivalently potent DCs [15]. Among DC maturation–inducing factors, microbial components that trigger the production of IL-12 are beneficial to induce effective adaptive immunity [16]. An extended period of stimulation with microbial components results in DC exhaustion in which DCs lose the capacity to produce IL-12 [17]. Thus, a short-term stimulation can generate optimal DCs that retain IL-12 production. Inflammation in the skin before DC injection facilitates DC migration to draining lymph nodes, leading to a stronger immune response [18,19]. Using apoptotic whole tumor cells as antigens may be instrumental in inducing multivalent immune responses [20].

We performed in vitro assays to optimize these parameters. Based on the results of these assays, we conducted a phase I clinical trial of immunotherapy for elderly patients with AML at the second or later remission setting, using DCs loaded with autologous apoptotic leukemic cells. The treatment was well-tolerated and safe and induced antileukemic immunity in two of four patients, which was associated with transient disease stabilization. Importantly, in one patient, cross-priming of leukemia antigen–specific $CD8^+$ T cells in vivo was explicitly demonstrated. This study indicates the safety and immunogenicity of immunotherapy using MoDCs that cross-present leukemic cell antigens in elderly patients with AML.

Materials and methods

Generation, maturation, and cryopreservation of DCs for in vitro assays

Peripheral blood mononuclear cells (PBMCs) were obtained from healthy volunteers by density gradient centrifugation using Lympholyte H (Cedarlane, Ontario, Canada). Monocytes were purified using anti–CD14-conjugated microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), or enriched by plastic adherence by incubating PBMCs at 37°C for 2 hours and removing nonadherent cells by pipetting. Monocytes were cultured with 800 IU/mL GM-CSF (Primmune, Kobe, Japan) and 500 IU/mL IL-4 (Primmune) in CellGro DC medium (CellGenix Technologie Transfer, Freiburg, Germany) for 3 days (3d-DCs) or 6 days (6d-DCs). In some experiments, 3d-DCs were frozen in CP-1 freezing medium (Kyokuto Pharmaceutical Industrial, Tokyo, Japan). CP-1 contains 12% hydroxymethyl starch and 10% dimethyl sulfoxide in normal saline and was mixed with 8% human serum albumin before use. DCs were matured with 0.1 KE/mL OK-432 (Picibanil; Chugai Pharmaceuticals, Tokyo, Japan), a penicillin-killed and lyophilized preparation of a low-virulence strain (Su) of *Streptococcus pyogenes* (group A) [21].

In vitro analysis of DC functions

Flow cytometric analysis, measurement of IL-12p70 production, T-cell–stimulatory capacity of DCs for allogeneic naive $CD4^+$ T cells, and the cytokine profile of $CD4^+$ T cells primed with DCs were analyzed as described previously [15,22].

Uptake of apoptotic cells by DCs and the cross-presenting capacity of DCs

Efficiency of uptake of apoptotic cells by DCs was assessed as described previously [23] using myeloid leukemia cell lines K562, OUN-1 [24] (Dr. Yasukawa, Ehime University, Japan), and a T-cell leukemia cell line MT2, which were killed by 120 Gy γ-irradiation and 48-hour serum-free culture in RPMI-1640 (Wako Pure Chemical Industries, Osaka, Japan). To examine the cross-presenting capacity of DCs, human leukocyte antigen (HLA)-A*2402–positive, immature 3d-DCs were pulsed with HLA-A*2402–negative, Epstein-Barr virus–transformed lymphoblastoid cell lines, which were killed as described here. DCs were matured with OK-432 (0.1 KE/mL) and prostaglandin E_2 (1 μg/mL) (MP Biomedicals, Solon, OH, USA) for 6 hours, and cocultured with autologous T cells at a DC-to-T cell ratio of 1:10. IL-2 (50 IU/mL; Teceleukin; Shionogi & Co., Ltd., Osaka, Japan) was added on the next day. For a positive control, DCs pulsed with HLA-A*2402–restricted EBNA3B peptide (TYSAGIVQI; KURABO Industries, Osaka, Japan) were used. Expansion of EBNA3A- and EBNA3B-specific $CD8^+$ T cells were evaluated by HLA tetramer staining [25].

Clinical trial protocol

The protocol was approved by the Ethics Committee, Graduate School and Faculty of Medicine, Kyoto University. Each patient gave written informed consent in accordance with the Declaration of Helsinki. The primary and secondary objectives were the assessment of safety and immunological and clinical responses, respectively.

Autologous leukemic cells were harvested before induction chemotherapy. Patients were required to be between 16 and 79 years of age and have a diagnosis of AML according to World Health Organization criteria [26,27]. Patients were excluded if they had another concurrent malignancy, an active autoimmune disease, positivity for blood-borne infectious agents, or a history of penicillin allergy (because OK-432 contains penicillin). Patients were enrolled if 5×10^7 or more leukemic cells were harvested. Thereafter, patients were treated with chemotherapy. More than 4 weeks after the last chemotherapy, patients proceeded to the DC vaccination if leukemic cells in bone marrow (BM) were $<20\%$. In addition, to assess the clinical efficacy of DC vaccination, the presence of an evaluable lesion in BM, which was defined as 0.1% or more of leukemic cells by flow cytometry, was required. Furthermore, patients should have an Eastern Cooperative Oncology Group performance status of 0 to 2 and adequate vital organ functions. Patients were excluded if they had eligibility for hematopoietic stem cell transplantation or an uncontrollable infection. Concomitant chemotherapy and radiotherapy were prohibited.

DC vaccine generation

DC vaccines were generated from autologous monocytes under current Good Manufacturing Practice conditions. Autologous leukemic cells to be used as antigens were obtained as mononuclear cells (MNCs) by density gradient centrifugation over Ficoll-Hypaque (GE Healthcare, Buckinghamshire, UK) from BM and/or peripheral blood (PB) samples. MNCs were frozen in CP-1 freezing medium and stored at −150°C. Before added to DCs, MNCs were killed by 120 Gy

γ-irradiation and 48 hours serum starvation. Killing of MNCs was confirmed by the percentage of Annexin V–positive cells being 90% or more by flow cytometry and reduced uptake of [^{3}H]-thymidine to the baseline level.

Apheresis products, which were obtained with COBE Spectra (Caridian BCT, Lakewood, CO, USA) from 10 L blood, were processed by elutriation using Elutra (Caridian BCT) to enrich monocytes. At the time of apheresis, no leukemic cells were observed in the PB of the patients, as assessed by a routine clinical laboratory test. Monocytes were cultured with 800 U/mL GM-CSF and 500 U/mL IL-4 in CellGro DC medium in gas-permeable plastic bags (VueLife 118; CellGenix Technologie Transfer) at 37°C, 5% CO_2 to generate immature DCs. After 48 hours, DCs were pulsed with autologous apoptotic leukemic cells and 2 μg/mL keyhole-limpet hemocyanin (KLH; Biosyn Corporation, Carlsbad, CA, USA). The endotoxin level in the KLH preparation examined by the supplier was <0.1 IU/mg. After an additional 24 hours, DCs were frozen as immature DCs in CP-1 freezing medium and stored at −150°C.

Administration of the DC vaccine

A total of 1×10^7 DCs were intradermally injected at four sites in bilateral arms and thighs. Twenty-four hours before DC administration, the injection sites were pretreated by 0.2 KE/site OK-432. At the time of DC administration, DCs were thawed and mixed with 1 KE OK-432. Then, the mixture of DCs and OK-432 was injected. The DC administration was repeated at 2-week intervals for five administrations.

Monitoring of immunological and clinical responses

Antigen-specific immune responses were assessed at indicated time points. Immune responses to KLH and autologous leukemic cells were tested by skin delayed-type hypersensitivity tests and interferon (IFN)-γ enzyme-linked immunospot (ELISPOT) assays. In addition, in a HLA-A*2402–positive patient, immune responses to HLA-A*2402–restricted peptides derived from leukemia-associated antigens were examined by IFN-γ ELISPOT assay and HLA tetramer staining. The peptides used in the assays were the natural $WT1_{235-243}$ peptide (CMTWNQMNL) [24], the modified $WT1_{235-243}$ peptide (CYTWNQMNL) [28], the human telomerase reverse transcriptase $(hTERT)_{461-469}$ peptide (VYGFVRACL) [29], and the lower matrix 65-kd phosphoprotein (pp65) of cytomegalovirus (CMV) (amino acids 328–336; QYDPVAALF) [30]. All peptides were purchased from Multiple Peptide Systems (San Diego, CA, USA). Both PBMCs and BM mononuclear cells (BMMCs) were subjected to assays before and after 1-week in vitro stimulation with antigen- or peptide-pulsed DCs in the presence of 15 U/mL IL-2 (Teceleukin). To evaluate clinical responses, percentages of leukemic cells in BM were monitored by morphology and flow cytometry at indicated time points.

Skin delayed-type hypersensitivity test

The 4×10^5 antigen-pulsed DCs were intradermally injected in the forearm. Sizes of induration and erythema were measured 48 hours later. Erythema that was 1.5-fold or larger in diameter than the antigen-unpulsed control was considered positive.

IFN-γ ELISPOT assay

IFN-γ ELISPOT assays (Mabtech, Nacka Strand, Sweden) were performed using antigen-pulsed DCs and peptide-pulsed C1R-A*2402 (Dr. Masafumi Takiguchi, Kumamoto University, Kumamoto, Japan). Stimulator cells were plated at 2×10^4 cells/well. As responder cells, fresh and in vitro–stimulated MNCs from PB and BM were plated with fresh MNCs at 1 to 2×10^5 cells/well and in vitro–stimulated MNCs at 1 to 2×10^4 cells/well. After overnight incubation, spots were developed using 3-amino-9-ethylcarbazole (Sigma Chemical, St Louis, MO, USA) and counted by KS ELISPOT compact (Carl Zeiss MicroImaging, Tokyo, Japan). Numbers of specific spot-forming cells were calculated by subtracting the number of spots with unpulsed DCs from the number of spots with antigen-pulsed DCs.

HLA tetramer staining

Natural $WT1_{235-243}$ peptide/HLA-A*2402 tetramer was purchased from Medical & Biological Laboratories (Nagoya, Japan). Modified $WT1_{235-243}$ peptide/HLA-A*2402 tetramer and a peptide derived from the HIV envelope (env) protein/HLA-A*2402 tetramer were produced as described previously [30]. Fresh and in vitro–stimulated MNCs were stained with a tetramer and fluorescein isothiocyanate–conjugated anti-CD8 monoclonal antibody (BD Biosciences) and analyzed by flow cytometery (FACSCalibur; BD Biosciences) [30].

Results

In vitro assays to optimize generation of DCs

To optimize generation of DCs, we performed in vitro functional assays. We first compared DCs differentiated from monocytes in the presence of GM-CSF and IL-4 for 3 days with 6-day differentiated DCs conventionally used in clinical trials. After 24-hour exposure to OK-432, both 3d-DCs and 6d-DCs showed similar levels of surface molecule expressions, IL-12p70 production, and T-cell stimulatory capacity for allogeneic naïve $CD4^+$ T cells (Supplementary Figure E1; online only, available at www.exphem.org), indicating that 3d-DCs have functions comparable with 6d-DCs. Next, we examined the capacity of 3d-DCs to cross-present apoptotic cell–associated antigens. At the DC-to-apoptotic cell ratio of 1:1, 11% to 33% of immature 3d-DCs incorporated apoptotic leukemia cell lines (Fig. 1A). Moreover, HLA-A*2402–positive DCs pulsed with killed lymphoblastoid cell lines from an HLA-A*2402–negative donor induced expansion of $CD8^+$ T cells specific for the HLA-A*2402–restricted epitopes of EBNA3A and EBNA3B (Fig. 1B), indicating the capacity of DCs to cross-present apoptotic cell–derived antigens.

An extended period of exposure of DCs to lipopolysaccharide leads to DC exhaustion [17], as indicated by loss of IL-12–producing capacity by DCs. To examine whether OK-432 induces DC exhaustion, we analyzed the maturation kinetics of OK-432–stimulated 3d-DCs. Upregulation of the surface molecules (Fig. 2A) and IL-12p70 production (Fig. 2B) became evident 4 and 8 hours after OK-432 stimulation, respectively. Maximal levels of surface molecule expressions and IL-12p70 production were observed at 48 hours. Next, we examined how many hours of exposure to OK-432 is sufficient to elicit a maturation signal to DCs, using 3d-DCs that were cultured for a total of 48 hours with different

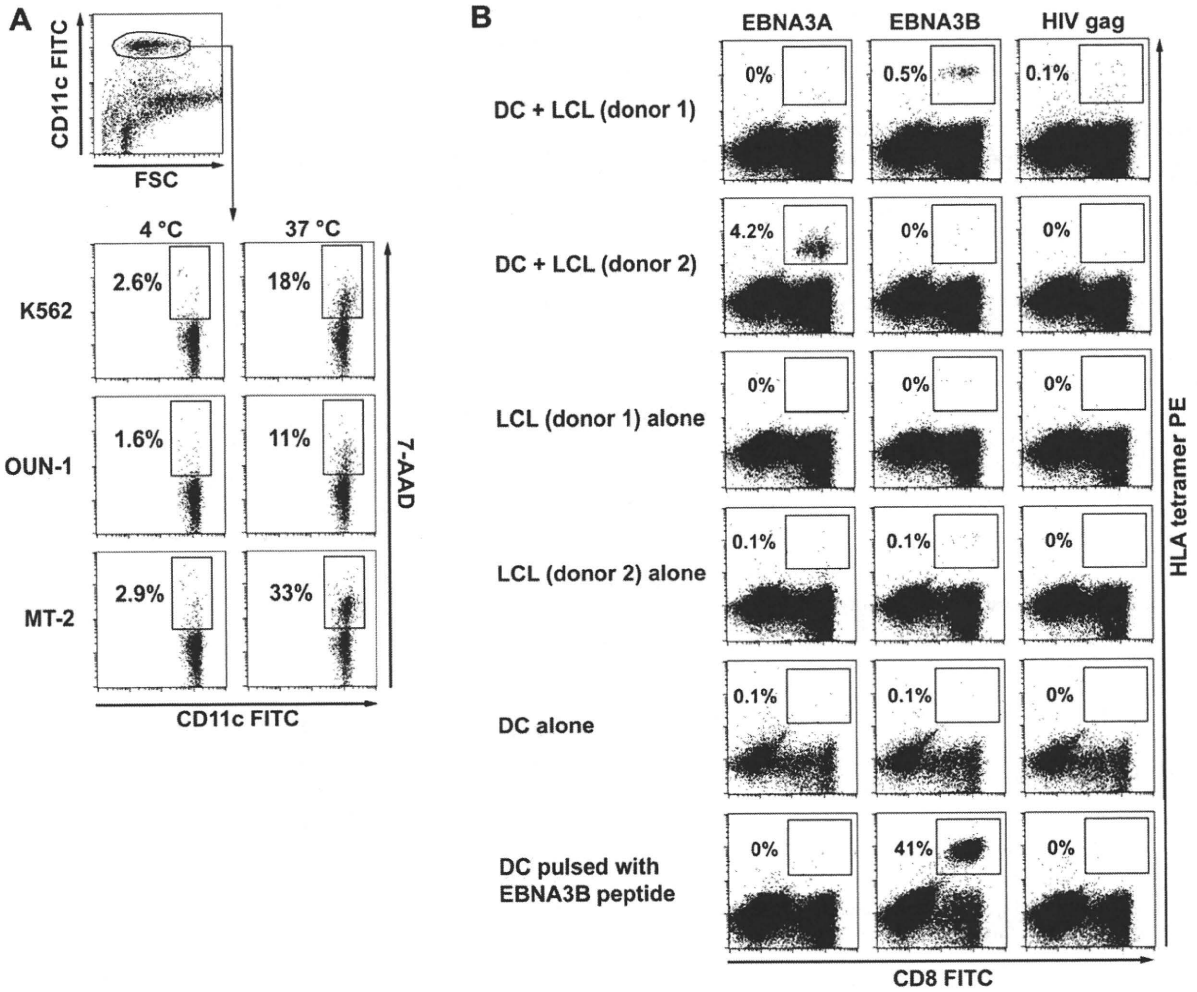

Figure 1. 3d-DCs incorporate apoptotic cells and cross-present cell-associated antigens. (**A**) Uptake of apoptotic cells by 3d-DCs. Apoptotic K562, OUN-1, and MT2 were labeled with 7-aminoactinomycin D (7-AAD) (20 μg/mL), and cocultured with immature 3d-DCs at a DC-to-apoptotic cell ratio of 1:1. After 4 hours of incubation at 4°C or 37°C, cells were stained with fluorescein isothiocyanate–conjugated anti-CD11c monoclonal antibody and analyzed by flow cytometry. Cells positive for both CD11c and 7-AAD were considered to be DCs that had phagocytosed apoptotic cells. (**B**) The cross-presenting capacity of DCs. Immature 3d-DCs from a HLA-A*2402–positive donor were pulsed with apoptotic HLA-A*2402–negative donor-derived lymphoblastoid cell lines (LCLs), matured with OK-432 and prostaglandin E_2, and cocultured with autologous T cells. For a positive control, DCs pulsed with the EBNA3B peptide were used as a stimulator. After 7 days, expansions of EBNA3A- and EBNA3B-specific $CD8^+$ T cells were evaluated by HLA tetramer staining. Dead cells are excluded by staining with propidium iodide. Numbers shown indicate percentages of tetramer-positive cells among $CD8^+$ cells. Representative data from two experiments are shown.

durations of exposure to OK-432 at the start of culture. As short as 2-hour exposure upregulated CD83 and CD86 (Fig. 2C) and induced IL-12p70 production (Fig. 2D) during the subsequent 46-hour culture without OK-432. Although at the time of 8-hour exposure, the induction of CD83, CD86 (Fig. 2A), and IL-12p70 (Fig. 2B) was low, 8-hour exposure was sufficient to induce maximal levels of CD83 and CD86 expression (Fig. 2C) and IL-12p70 production (Fig. 2D). Notably, although initial 24-hour exposure to OK-432 induced the maximal levels of CD83 and CD86 expression (Fig. 2C), DCs did not produce a detectable level of IL-12p70 during the last 24-hour culture (Fig. 2D). These data indicate that, like lipopolysaccharide [17], OK-432–induced IL-12p70 production was limited within the first 24 hours and most active between 8 and 24 hours after OK-432 stimulation. The functional significance of ongoing IL-12p70 production by DCs in priming naïve $CD4^+$ T cells was supported by the data that 3d-DCs matured with OK-432 for 6 hours showed a superior capacity to induce IFN-γ–producing T cells to those matured for 24 hours (Fig. 2E). Thus, extended stimulation with OK-432 induces DC exhaustion. To avoid it, we decided to administer immature DCs together with OK-432 to patients and to induce DC maturation in vivo.

It is convenient to prepare a large number of DCs from a single batch of apheresis and freeze them in aliquots. We assessed the effect of cryopreservation on DCs. Whereas cryopreserved immature 3d-DCs showed somewhat higher percentages of dead cells after 24-hour culture with or

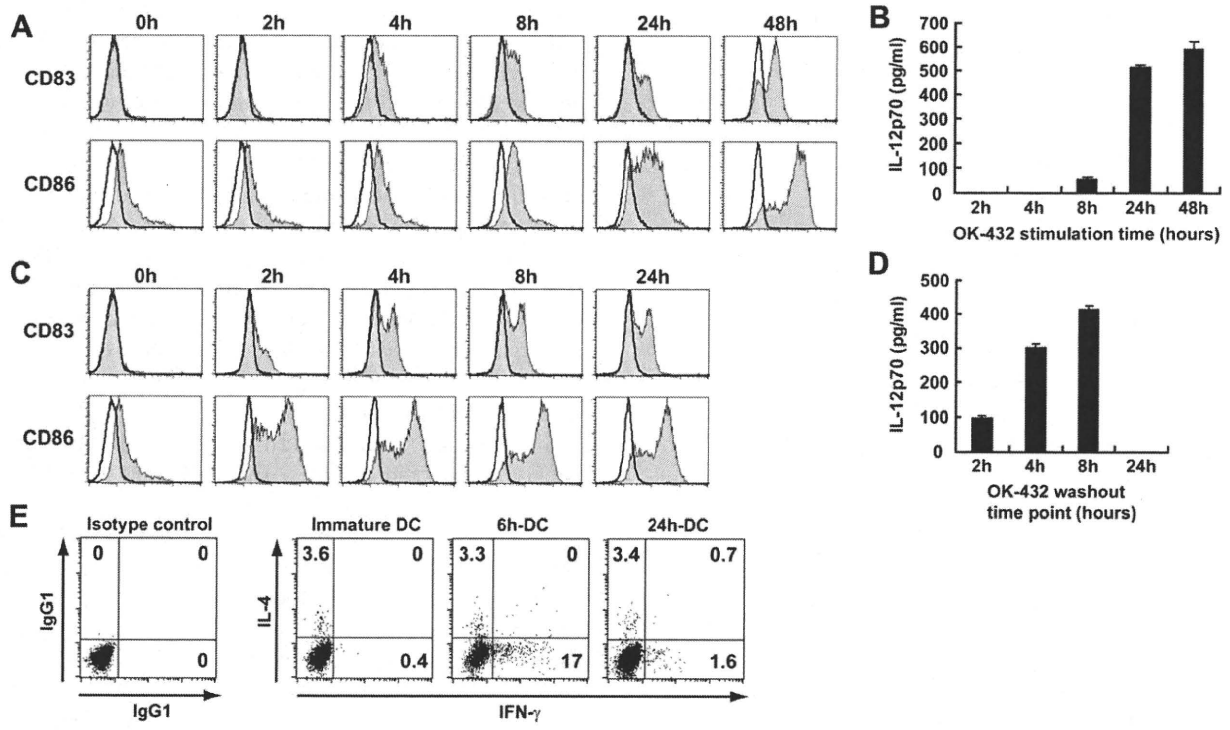

Figure 2. Short-term stimulation with OK-432 is optimal to generate Th1-inducing mature DCs. (**A**, **B**) Immature 3d-DCs were cultured in the presence of OK-432 (0.1 KE/mL) for indicated time periods, then harvested and analyzed. (**C**, **D**) Immature 3d-DCs were cultured in the presence of OK-432 for indicated time periods, washed, replated, and further cultured for a total of 48 hours. Cells and supernatants harvested at 48 hours were analyzed. (**A**, **C**) Expression of CD83 and CD86 was analyzed by flow cytometry. Dead cells were excluded by staining with propidium iodide. Open histograms indicate staining with isotype controls. (**B**, **D**) IL-12p70 production in culture supernatants of DCs (5×10^5 cells/mL) were measured by enzyme-linked immunosorbent assay. Error bars indicate the standard deviation of duplicate measurements. (**E**) Naïve $CD4^+$ T cell differentiation induced by DCs. Immature 3d-DCs were matured with OK-432 (0.1 KE/mL) for 6 or 24 hours and cocultured with allogeneic naïve $CD4^+$ T cells for 7 days. Cytokine profiles of T cells were analyzed by intracellular cytokine staining. Numbers indicate percentages of cells in each quadrant. Representative data from four experiments are shown.

without OK-432, and tended to produce a lower amount of IL-12p70 upon OK-432 stimulation as compared with non-cryopreserved DCs (Supplementary Figure E2A, C; online only, available at www.exphem.org), similar levels of CD83 and CD86 expression were induced by OK-432 in both DCs (Supplementary Figure E2B; online only, available at www.exphem.org). Thus, although cryopreservation of immature DCs impaired their function to some extent, cryopreserved DCs largely retained the viability and expression of immunostimulatory molecules. Considering the practical convenience to prepare a stock of DCs at one time, we decided to freeze DCs as immature DCs. Taken together, these data demonstrate that DCs generated in the present study are capable of inducing $CD8^+$ T-cell responses to apoptotic cell–derived antigens, and that immature DCs can be cryopreserved without critical loss of functions.

Patients, feasibility, and safety

Thirteen patients were recruited to the study for the leukemic-cell harvest at the onset of AML. After chemotherapy, four patients were eligible for DC vaccination (Table 1). In these patients, $>5 \times 10^7$ DCs for five vaccinations could be generated from a single apheresis. Autologous apoptotic leukemic cells were added to DCs as antigens at leukemic cell-to-DC ratios of 1:3.3 to 1:6.5, depending on the numbers of collected leukemic cells (Supplementary Table E1; online only, available at www.exphem.org). Status of PB and BM at the time of apheresis are shown in Supplementary Table E1 (online only, available at www.exphem.org). Representative data of surface molecule expressions on DCs are shown in Supplementary Figure E3 (online only, available at www.exphem.org).

All of the patients completed the five vaccinations safely (Table 1). In all the patients, grade 1 to 2 fever and grade 2 skin reactions at the injection sites were observed. The fever was resolved within 2 days after vaccination and most likely related to administration of OK-432. The skin reactions at the injection sites were transient and characterized by erythema, pruritus, and tenderness. No significant toxicities to vital organs or signs of autoimmunity were observed.

Induction of antigen-specific immune responses to KLH and leukemic cells

Induction of an immune response to KLH was detected by skin delayed-type hypersensitivity tests and/or IFN-γ ELISPOT assays in three patients, with the exception of patient no. 4

Table 1. Patient characteristics and results of the DC vaccination

Patient no.	Age/Sex	Diagnosis	DC vaccination was started		LC in BM at the first vaccination[a] (%)	Adverse effects[b]	Immune response		Clinical response	Died at (days after the last vaccination)
			After the last CT (d)	After diagnosis (d)			KLH	LC		
1	76/F	AML-MRC	82	93	1.8	Fever (1) Injection site reaction (2)	Yes	No	PD Died of sepsis with leukemia	186
2	75/M	AML-MRC	40	155	0.6	Fever (1) Injection site reaction (2)	Yes	Yes	Transient disease stabilization Died of leukemia	391
3	70/M	AML-MRC	44	344	2.9	Fever (2) Injection site reaction (2)	Yes	Yes	Transient disease stabilization Died of sepsis with leukemia	192
4	66/M	AML M2	67	144	0.2	Fever (1) Injection site reaction (2)	No	No	PD Died of leukemia	66

AML-MRC = acute myeloid leukemia with myelodysplasia-related changes; CT = chemotherapy; F = female; LC = leukemic cells; M = male; PD = progressive disease.
[a]Percentages of leukemic cells in bone marrow were determined by flow cytometry.
[b]Numbers in parentheses indicate grade of toxicity according to the National Cancer Institute-Common Terminology Criteria for Adverse Events version 3.0.

(Table 1 and data not shown). Two patients (patient nos. 2 and 3) showed induction of immune responses to leukemia-associated antigens. In patient no. 2, who was HLA-A*2402–negative, IFN-γ ELISPOT assays using autologous leukemic cell–pulsed DCs revealed the induction of antileukemic immunity in PBMCs and BMMCs without in vitro stimulation after the fourth vaccination (Fig. 3A). The antileukemic immune response was still detected 1 month after the fifth vaccination in in vitro–stimulated PBMCs and BMMCs (Fig. 3B), but was no longer detected without in vitro stimulation (Fig. 3A). We could not test antileukemic immunity at subsequent time points in this patient because the patient developed leukocytopenia, probably owing to progression of myelodysplastic syndrome.

In patient no. 3, who was HLA-A*2402–positive, HLA-A*2402–restricted peptides from WT1 and hTERT were used in immunological monitoring. $CMVpp65_{328-336}$ peptide was used as a positive control in ELISPOT assays (Fig. 4B). No responses to the leukemia-associated antigens were observed until the fourth vaccination. However, 2 months after the fifth vaccination, positive responses to the modified $WT1_{235-243}$ and the $hTERT_{461-469}$ peptides were detected in in vitro–stimulated PBMCs by HLA tetramer staining (Fig. 4A) and an IFN-γ ELISPOT assay (Fig. 4B), respectively. The PBMCs binding to the modified $WT1_{235-243}$ peptide/HLA-A*2402 tetramer also bound to the natural $WT1_{235-243}$ peptide/HLA-A*2402 tetramer (Fig. 4A), indicating that these cells were capable of recognizing the natural WT1 peptide presented on leukemic cells. These responses were short-lived and almost completely disappeared 3 months after the fifth vaccination. No responses were detected in PBMCs or BMMCs without in vitro stimulation (data not shown). Thus, the vaccinations induced HLA class I–restricted, antileukemic immunity, indicating that the DCs cross-presented leukemia-associated antigens in vivo. In addition, in patient no. 2, leukemic cell-reactive T cells were detected in BM (Fig. 3), the main tumor site in leukemia.

Clinical outcomes

The two patients with antileukemic immunity had longer periods of disease stabilization than the other two patients without antileukemic immunity (Fig. 5A). Notably, in patient no. 3, the percentages of leukemic cells in BM dropped from 11% to 5.2% during the second month after the fifth vaccination, when a positive antileukemic immunity was observed (Fig. 5B). Thus, these observations suggest that induction of antileukemic immunity was associated with extended the periods of disease stabilization in these patients.

Discussion

Novel therapies with less toxicity are necessary for intractable AML in elderly patients. In this study, we conducted a phase I clinical trial of immunotherapy for such patients using DCs pulsed with autologous apoptotic leukemic cells.

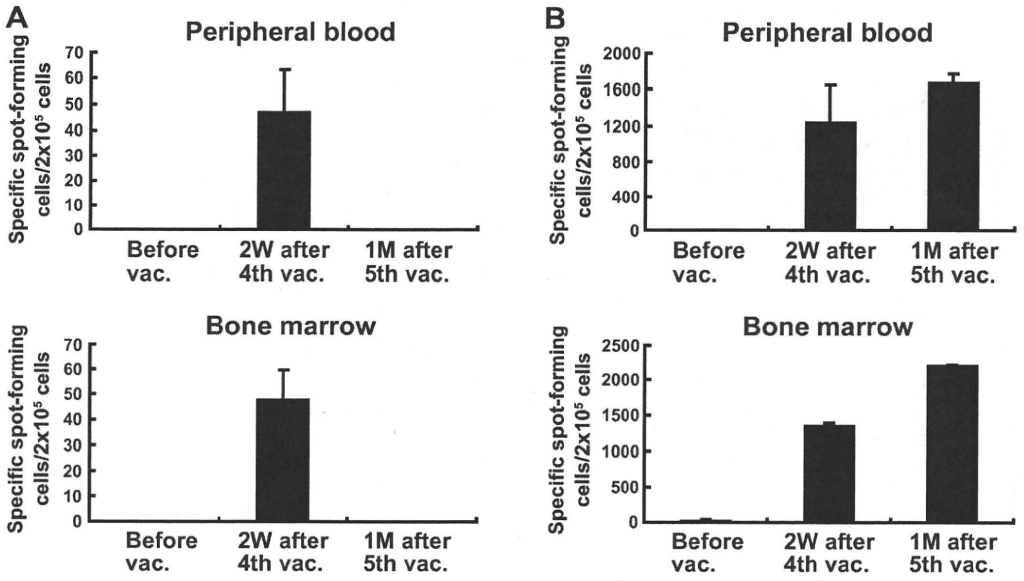

Figure 3. IFN-γ ELISPOT assay in patient no. 2. MNCs from PB and BM were obtained at indicated time points and subjected to IFN-γ ELISPOT assays directly after isolation (**A**) or after 1 week of stimulation with antigen-pulsed DCs (**B**). In IFN-γ ELISPOT assays, 2×10^5 MNCs (**A**) and 1×10^4 MNCs (**B**) were incubated with 1×10^4 leukemic cell–pulsed or unpulsed DCs. Numbers of specific spot-forming cells per 2×10^5 MNCs, calculated by subtracting numbers of spots with unpulsed DCs from numbers of spots with leukemic cell–pulsed DCs. Error bars indicate the standard deviation of duplicate measurements.

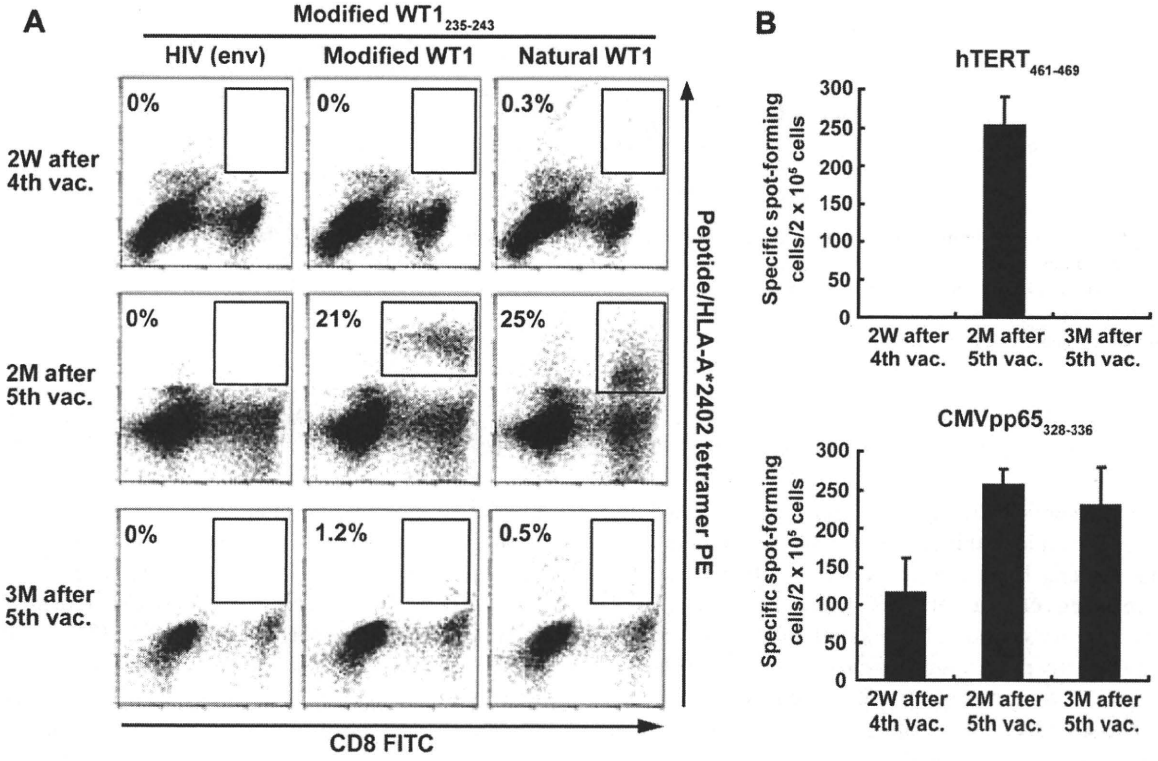

Figure 4. Immune responses in patient no. 3. (**A**) HLA tetramer staining. MNCs from PB were obtained at indicated time points, stimulated for 1 week with DCs pulsed with the modified $WT1_{235-243}$ peptide, stained with phycoerythrin-labeled peptide/HLA-A*2402 tetramers and fluorescein isothiocyanate–labeled anti-CD8 monoclonal antibody, and analyzed by flow cytometry. Dead cells were excluded by staining with propidium iodide. Numbers indicate percentages of tetramer-positive cells among $CD8^+$ cells. (**B**) IFN-γ ELISPOT assay. MNCs were stimulated for 1 week with DCs pulsed with the $hTERT_{461-469}$ or $CMVpp65_{328-336}$ peptide, and subjected to IFN-γ ELISPOT assays. In the assays, 2×10^4 MNCs were incubated with 2×10^4 C1R-A*2402 pulsed with or without the $hTERT_{461-469}$ or $CMVpp65_{328-336}$ peptide. Before vaccination, the assay was performed using DCs as a stimulator, which induced many nonspecific spots. Thus, the data before vaccination are not shown. Numbers of specific spot-forming cells per 2×10^5 MNCs, calculated by subtracting numbers of spots with unpulsed C1R-A*2402 from numbers of spots with antigen-pulsed C1R-A*2402, were depicted. Error bars indicate the standard deviation of duplicate measurements.

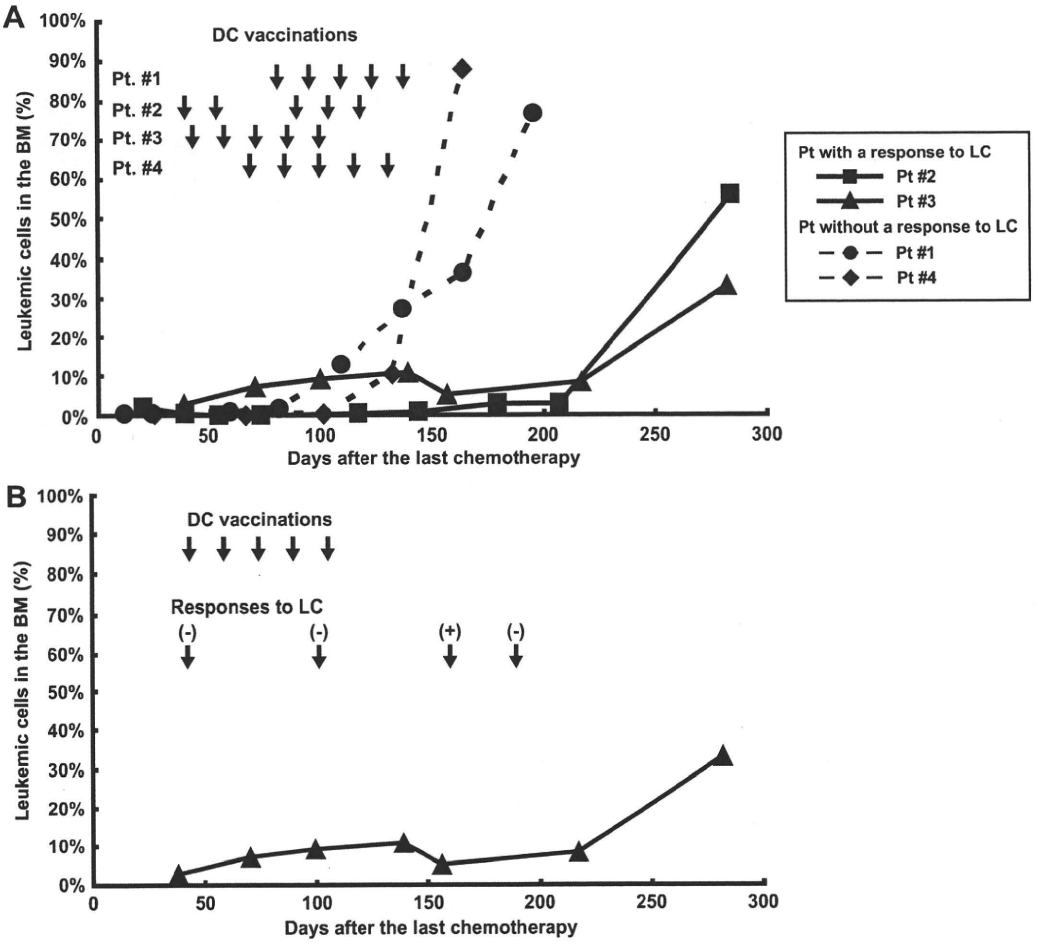

Figure 5. Clinical courses during the DC vaccination. (**A**) Percentages of leukemic cells in BM as determined by flow cytometry in four vaccinated patients are shown. Solid lines indicate patients with immune responses to leukemic cells (LCs) (patients 2 [■] and 3 [▲]). Dashed lines indicate patients without immune responses to LCs (patients 1 [●] and 4 [◆]). Arrows indicate time points when DC vaccines were administered to each patient. (**B**) The clinical course of patient no. 3. Arrows indicate time points when immunological monitoring was performed. Plus (+) or minus (−) signs indicates that immune responses to leukemic cells were detected or not detected at that time point, respectively.

Induction of antileukemic immunity was observed in two of four vaccinated patients. This is the first study that demonstrates cross-priming of $CD8^+$ T cells by DCs pulsed with apoptotic leukemic cells in vivo in humans, thus providing a proof of principle of this approach. The limited number of patients prevented us from drawing any definitive conclusion regarding clinical efficacy from the present trial. However, longer periods of disease stabilization observed in the two patients with antileukemic immunity compared to the other two patients without antileukemic immunity implied that induction of antileukemic immunity might have impacted on the clinical course of these patients.

There are several features in the method of DC vaccination in this trial: short-term 3-day culture to generate DCs in an attempt to reduce labor, cost, and time; use of whole leukemic cells as antigens to induce multivalent immune responses; use of the microbial adjuvant OK-432 as a maturation-inducing factor to generate Th1-inducing DCs; in vivo maturation of DCs to avoid DC exhaustion by extended stimulation in vitro with OK-432; and prior induction of inflammation at the injection sites to facilitate DC migration to draining lymph nodes.

We used autologous apoptotic leukemic cells as antigens because several studies have shown that apoptotic cells are more efficiently cross-presented by DCs to $CD8^+$ T cells than soluble antigens such as tumor lysate [31–34]. Furthermore, MoDCs has been shown to cross-present apoptotic leukemic cells to $CD8^+$ T cells in vitro [35]. Apoptotic cells as antigens also have advantages over peptides, in that the DCs have the ability to process multiple antigens from the apoptotic cells and present those antigens on their own HLA molecules. In this study, we clearly showed that MoDCs cross-presented leukemia-associated antigens WT1 and hTERT from apoptotic leukemic cells. Furthermore, T cells reactive to leukemic cells were detected in BM.

A murine study has shown that DC maturation not by inflammatory cytokines but by pathogen-derived components is crucial for DCs to acquire the capacity to differentiate naïve $CD4^+$ T cells into effector T cells [16]. We used OK-432, a preparation of killed *Streptococcus pyogenes* [21], which strongly triggers DC maturation through Toll-like receptor 4 [36–39]. We showed that, like lipopolysaccharide [17], longer stimulation with OK-432 induces DC exhaustion, resulting in the reduced capacity of DCs to induce Th1 responses. Several preclinical studies have shown that DCs briefly exposed to Toll-like receptor ligands are better inducers of Th1-type and cytotoxic T-cell responses [17,40,41]. Moreover, a clinical trial suggests superiority of briefly matured DCs in pediatric patients with cancer [42]. In this trial, we administered immature DCs together with OK-432 to avoid DC exhaustion before administration. The induction of IFN-γ detected by the ELISPOT assay implied IL-12 production by DCs in vivo.

Only a small proportion of intradermally administered DCs reach draining lymph nodes [43,44]. In a mouse model, pretreatment of administration sites with inflammatory cytokines enhance DC migration to regional lymph nodes [18]. Based on this finding, we pretreated administration sites with a low dose of OK-432. Because of unavailability of a cell-processing facility for cells labeled with indium-111 oxyquinoline [43,44], we could not evaluate the efficiency of DC migration to lymph nodes. Whether this administration procedure is superior to others should be evaluated in future studies.

In this study, multiple vaccinations were required to elicit antileukemic immunity, which rapidly declined after cessation of vaccination. Maintenance of antileukemic immunity might lead to improvement of clinical efficacy, and might be fulfilled by increasing the number of vaccinations, which was, however, impossible in this study because of the limited availability of autologous leukemic cells. Thus, if a peptide is available for the induced antileukemic $CD8^+$ T-cell response, peptide vaccination may be added after DC vaccination. Furthermore, blockade of immunosuppressive mechanisms may be combined.

In conclusion, we demonstrated the feasibility, safety, and immunogenicity of DC-based immunotherapy for elderly patients with AML. Cross-priming of $CD8^+$ T cells by DCs pulsed with autologous apoptotic leukemic cells was provoked in vivo. The results were promising, yet further intensification of vaccine potency is clearly required. This novel therapeutic approach may lead to improvement of clinical outcomes in elderly patients with AML, which has been difficult to achieve with other therapeutic approaches.

Acknowledgments

We thank Satoshi Teramukai, Harue Tada, and Masanori Fukushima (Department of Clinical Trial Design and Management, Translational Research Center, Kyoto University Hospital) for patient enrollment, Maki Utsumi for her excellent technical assistance, and physicians for referral of patients. This study was supported by Coordination, Support and Training Program for Translational Research from Ministry of Education, Culture, Sports, Science, and Technology of Japan, and The Third Term Comprehensive Control Research for Cancer from the Ministry of Health, Labor, and Welfare, Japan.

Conflict of interest disclosure

No financial interest/relationships with financial interest relating to the topic of this article have been declared.

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