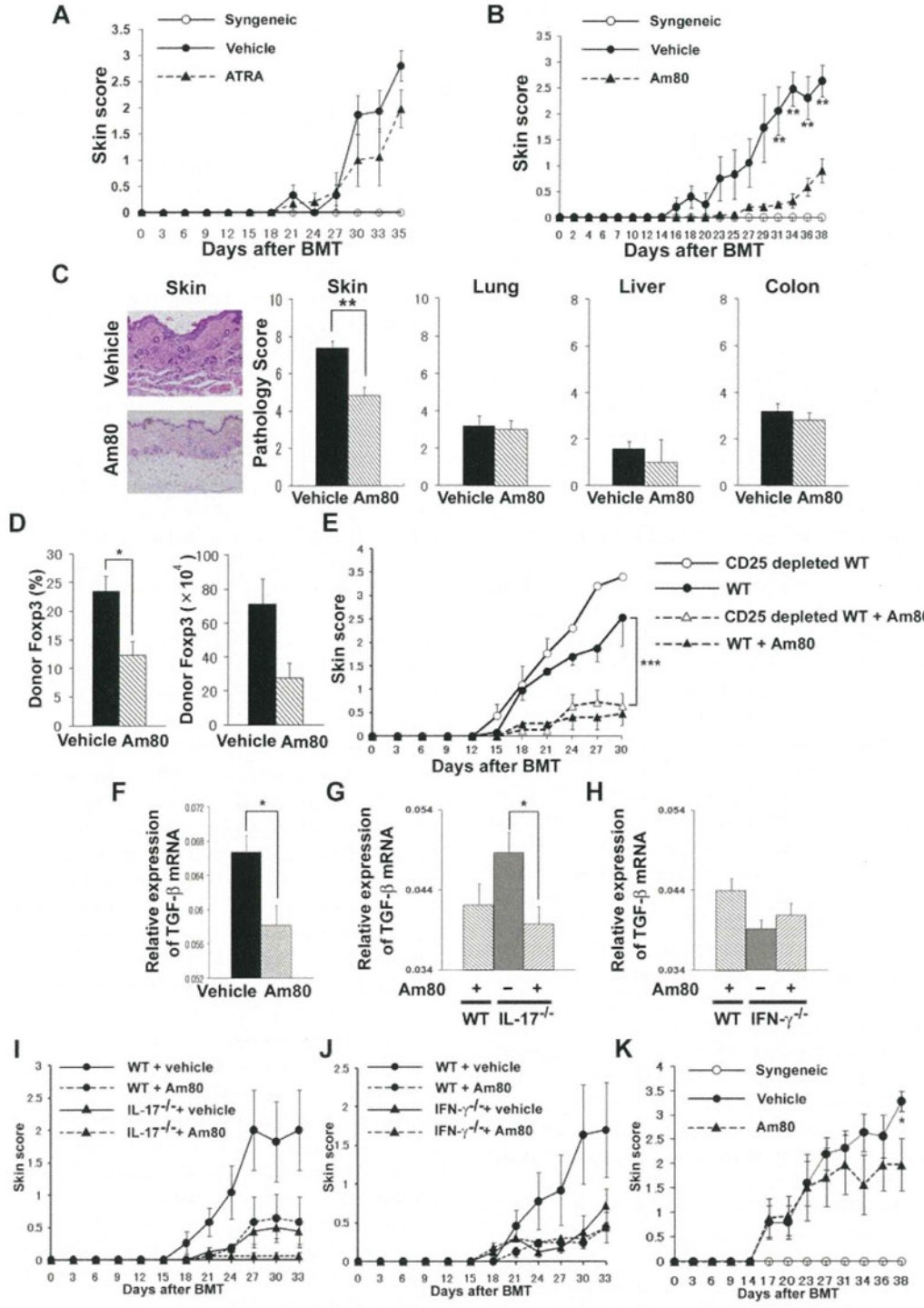

Figure 5. Administration of Am80 ameliorates cGVHD. (A-D) Sublethally irradiated BALB/c recipients were transplanted from WT B10.D2 donors. The recipients received daily administration of ATRA (200 μg/mouse; A), Am80 (1.0 mg/kg of body weight; B), or vehicle solution orally after BMT and were assessed for clinical signs of cGVHD every 3 days. The skin cGVHD scores are shown. (C) Representative histopathology of skin and pathology score of skin, lung, liver, and colon in each group (n = 5-6 per group) on day 16 after BMT are shown. (D) PLN cells of the recipients on day 16 were stained for intracellular Foxp3. The percentages and absolute numbers of $CD4^+Foxp3^+$ Treg cells are shown. Data are from 1 representative of ≥ 2 independent experiments. (E) Sublethally irradiated BALB/c recipients were transplanted with 8×10^6 TCD-BM cells plus 2×10^6 total spleen T cells or CD25-depleted T cells from WT or IFN-$\gamma^{-/-}$ B10.D2 donors. After BMT, recipients were given Am80 or vehicle solution. The skin cGVHD scores are shown. There were 6 recipients in each group; the data are from 1 representative of ≥ 2 independent experiments. (F-K) Sublethally irradiated BALB/c recipients were transplanted from WT (F), IL-$17^{-/-}$ (G), and IFN-$\gamma^{-/-}$ (H) donors. After BMT, recipients were given Am80 or vehicle solution. TGF-β mRNA expression in the ears on day 35 after BMT (F-H) and skin cGVHD scores (I-J) are shown. Data are from 1 representative of ≥ 2 independent experiments (n = 5 per group). (K) The skin cGVHD scores of BMT recipients treated with Am80 or vehicle solution orally daily after day 21 of BMT; data from 3 independent experiments were combined (n = 12-14 per group). $^*P < .05$, $^{**}P < .01$, and $^{***}P < .005$.

TGF-β is a critical mediator of fibrosis in cGVHD skin lesions.[30] TGF-β mRNA expression was decreased in the ear of the Am80 recipients (day 17, $P = .02$; Figure 5F). We then assessed TGF-β mRNA expression in recipients of IL-$17^{-/-}$ or IFN-$\gamma^{-/-}$

donors treated with Am80. Am80 further reduced skin scores and TGF-β expression in recipients of IL-17$^{-/-}$ donors (Figure 5G-I) but not in recipients of IFN-γ$^{-/-}$ donors (Figure 5H,J). These results suggest that the effects of Am80 are more dependent on IFN-γ than on IL-17.

Finally, we examined whether Am80 could be used for the treatment of cGVHD. Am80 was orally administered to mice from day 21 of BMT, when mice had developed clinical signs of cGVHD. Am80 significantly improved clinical scores ($P = .016$; Figure 5K).

Discussion

The results of the present study showed that Th1 and Th17 cells contribute to cGVHD with the use of a MHC-compatible, miHA-incompatible model of cGVHD. In addition, we demonstrated that Am80 down-regulates both Th1 and Th17 cells in vitro and in vivo, resulting in attenuation of cGVHD.

For many years, the best defined subsets of effector T cells of the CD4$^+$ Th lineage were the Th1 and Th2 cells. A third subset of CD4$^+$ effector cells was identified and named Th17 cells, because the signature cytokine that they produce is IL-17.[31] Although the role of Th17 in acute GVHD has been evaluated by several groups with inconsistent results,[32-35] few studies have addressed the role of Th17 in cGVHD. Initially, cGVHD was hypothesized to be a Th2-mediated disease on the basis of the results in a nonirradiated P→F1 model of cGVHD. cGVHD in this model is mediated by host B-cell autoantibody production stimulated by donor Th2 cells. Th1 polarization of donor T cells activates donor CD8$^+$ CTLs to kill host B cells, resulting in amelioration of cGVHD.[36] However, the relevance of this model is unclear in clinical BMT in which host B cells are eliminated by conditioning. Such different effector mechanisms between the models may be associated with distinct requirement of Th subsets for cGVHD between the studies. In the present study, we assessed the kinetics of Th1, Th2, and Th17 cells during the development of cGVHD in the B10.D2→BALB/c model. Th1 and Th2 responses were up-regulated early after BMT, followed by a subsequent up-regulation of Th17 cells. Significantly greater numbers of Th17 cells were detected in the lung and liver from allogeneic recipients than in those from syngeneic recipients. We then evaluated the role of Th17 in cGVHD with the use of IL-17$^{-/-}$ mice as several groups had used,[32-34,37,38] although interpretation of the results deserves caution because the Th17 lineage is uniquely regulated by RORγt,[13,14] and other cytokines such as IL-21 and IL-22 produced by Th17 cells may also contribute to Th17-mediated GVHD. On transfer of IL-17$^{-/-}$ B10.D2 donor T cells, cGVHD was significantly ameliorated compared with that in recipients of WT T cells, suggesting that Th17 contributes to cGVHD in this model. In particular, Th17 plays a significant role in skin cGVHD. This agrees with the recent observation by Hill et al[37] that donor pretreatment with G-CSF induces Th17 differentiation of donor T cells and induces skin GVHD after peripheral blood stem cell transplantation. In an adoptive transfer model of autoimmune cGVHD, Th17 cells infiltrated target tissues.[39] However, a subsequent study showed the absence of donor Th17 cells did not abrogate GVHD pathology,[38] in contrast to our results. In the absence of donor IL-17, Th1 responses were preserved in that study but were reduced in our study. Such difference in Th1 responses may produce different outcomes between the studies. In mouse models of acute GVHD, Yi et al showed enhanced Th1 differentiation of donor T cells by increased production of IL-12 from dendritic cells in the absence of IL-17.[33] By contrast, Kappel et al showed reduced numbers of IFN-γ–positive CD4$^+$ T cells and IFN-γ secretion in culture in the absence of IL-17.[34] These results together with our results suggest that IL-17 may induce IFN-γ, although such a hierarchy of Th1/Th17 pathways may be context or model dependent or both and will need to be studied in the future. Nonetheless, it should be noted that cGVHD still developed in the absence of donor IL-17 cells in our study. Taken together, it is probable that Th17 is not an absolute requirement for cGVHD, and either Th1 or Th17 is sufficient to cause cGVHD.

We demonstrated that IFN-γ$^{-/-}$ donor mice and injecting anti–IFN-γ mAb ameliorated cGVHD. Thus, Th1 and Th17 responses play a pathogenic role in cGVHD in this model. These results were consistent with a recent study reporting that cGVHD is mediated by Th1 and Th17 responses because of the progressive loss of CD4$^+$CD25$^+$Foxp3$^+$ T cells during acute GVHD in mice.[39] These results were also consistent with clinical studies showing that Th1 cells and Th17 cells increased in patients with active cGVHD.[40-43] Increased transcription of IFN-γ has also been detected in the affected skin and oral mucosa of patients with cGVHD.[41,44] In this study, we found no differences in Th17 cells between IFN-γ$^{-/-}$ and WT recipients, although significantly greater numbers of Treg cells were detected in IFN-γ$^{-/-}$ recipients. CD25-depleted T cells from IFN-γ$^{-/-}$ mice induced more severe skin cGVHD compared with CD25-replete IFN-γ$^{-/-}$ T cells, but still less severe cGVHD compared with CD25-depleted T cells from WT mice (Figure 3E), suggesting that IFN-γ contributes to the pathogenesis of cGVHD by both Treg-independent and -dependent pathways. Neutralization of IFN-γ ameliorated cGVHD in the absence of donor IL-17 (Figure 3H), suggesting again that both Th1 and Th17 responses contribute to the pathogenesis of cGVHD.

We found that donor-derived Th17 cells were generated in recipients of syngeneic transplantation in addition to allogeneic transplantation. However, the kinetics of Th17 development differed between the syngeneic and allogeneic settings; Th17 cells developed in the early phase after syngeneic transplantation. Kappel et al speculated that Th17 development may be the result of increased immune reconstitution of syngeneic hosts compared with allogeneic hosts with GVHD.[34] We additionally identified a population of donor-derived IFN-γ$^+$IL-17$^+$ cells after allogeneic BMT. It has been shown that a subset of IL-17–producing cells can also produce IFN-γ in vivo.[34,45] Such CD4$^+$IFN-γ$^+$IL-17$^+$ T cells have been postulated to play a causative role in the pathogenesis of experimental autoimmune encephalomyelitis (EAE).[46] IFN-γ$^+$IL-17$^+$ cells were only detected after allogeneic BMT, but not after syngeneic BMT, suggesting that this population is generated by allogeneic stimulation, but not because of lymphopenia-induced proliferation. Further investigations are required to clarify the difference in function between IL-17 single-positive and IFN-γ/IL-17 double-positive cells.

ATRA suppresses Th17 differentiation and effector function by RARα signaling,[18] but ATRA can also bind to RARβ and RARγ, which can form a variety of homodimers and heterodimers with 3 retinoid X receptors.[15] Nonselective receptor binding is thought to be a main cause of the side effects associated with the administration of ATRA and other pan-RAR agonists. Am80 is a synthetic RAR agonist that shows high affinity to RARα/β. In addition to a greater specificity for RARα, Am80 offers several other advantages over ATRA as a therapeutic agent, including less toxicity, greater stability, fewer potential side effects, and superior bioavailability. Am80 is effective in autoimmune disease models of collagen-induced arthritis,[20,47] EAE,[21,29] 2,4-dinitrofluorobenzene–

induced contact dermatitis,[22] and atherosclerosis.[23] Because retinoids can down-regulate Th1 and Th17 cells and can ameliorate autoimmune diseases, we hypothesized that these retinoids would attenuate cGVHD. We demonstrated that Am80 down-regulated Th1 and Th17 differentiation of donor T cells in BALB/c recipients of B10.D2 donors, resulting in reduced cGVHD. Our results suggest that combined blockade of Th1 and Th17 responses may represent a promising strategy to prevent or treat cGVHD, as has been suggested for acute and chronic GVHD.[32,39,48] Most recently, Yu et al used mice deficient for both T-bet and RORγt as T-cell donors and clearly showed that blockade of both Th1 and Th17 differentiation is required to prevent acute GVHD.[14] In addition, TGF-β mRNA expression in the skin decreased in the Am80 recipients of WT and IL-17$^{-/-}$ but not IFN-γ$^{-/-}$ donors. These results suggest that Am80 down-regulates TGF-β and that this effect is more dependent on IFN-γ than on IL-17. Unexpectedly, those recipients administered Am80 had a significantly lower frequency of Foxp3^{+} cells. These results differ from those of in vitro studies performed by Mucida et al,[28] in which retinoic acids were shown to be capable of inhibiting the IL-6–driven induction of Th17 cells and to promote Treg cell differentiation. Thus, retinoic acids enhance Treg differentiation and inhibit both Th17 and Th1 in vitro; however, the effects of retinoids may be more complex in vivo, because retinoids can affect not only T cells but also other immunoregulatory cells. For example, previous in vivo studies reported that Am80 suppressed Treg cells in experimental models of EAE[29] and collagen-induced arthritis,[47] similar to our study. In our study, Am80 suppressed TGF-β expression, a key cytokine in Treg development, which may have resulted in the suppression of Treg.

In conclusion, both Th1 and Th17 contribute to the development of cGVHD. Am80 down-regulates TGF-β and also regulates both Th1 and Th17 cells in vitro and in vivo, resulting in attenuation of cGVHD. Thus, administration of Am80, which is currently available as medication for acute promyelocytic leukemia in Japan,[49] may represent effective strategy for prevention and treatment of cGVHD.

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Authorship

Contribution: H.N. conducted the experiments, analyzed the data, and wrote the manuscript; Y.M. designed the experiments, supervised the research, and wrote the manuscript; H.S., K.K., Y.Y., S.K., and H.U. performed the research; K.T., T. Tanaka, and T.Y. performed histopathologic analyses of the organs; Y.I. provided vital new reagents for the study; and T. Teshima and M.T. supervised the research.

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ORIGINAL REPORT

Phase II Study of SMILE Chemotherapy for Newly Diagnosed Stage IV, Relapsed, or Refractory Extranodal Natural Killer (NK)/T-Cell Lymphoma, Nasal Type: The NK-Cell Tumor Study Group Study

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ABSTRACT

Purpose
To explore a more effective treatment for newly diagnosed stage IV, relapsed, or refractory extranodal natural killer/T-cell lymphoma, nasal type (ENKL), we conducted a phase II study of the steroid (dexamethasone), methotrexate, ifosfamide, L-asparaginase, and etoposide (SMILE) regimen.

Patients and Methods
Patients with newly diagnosed stage IV, relapsed, or refractory disease and a performance status of 0 to 2 were eligible. Two cycles of SMILE chemotherapy were administered as the protocol treatment. The primary end point was the overall response rate (ORR) after the protocol treatment.

Results
A total of 38 eligible patients were enrolled. The median age was 47 years (range, 16 to 67 years), and the male:female ratio was 21:17. The disease status was newly diagnosed stage IV in 20 patients, first relapse in 14 patients, and primary refractory in four patients. The eligibility was revised to include lymphocyte counts of 500/μL or more because the first two patients died from infections. No treatment-related deaths were observed after the revision. The ORR and complete response rate after two cycles of SMILE chemotherapy were 79% (90% CI, 65% to 89%) and 45%, respectively. In the 28 patients who completed the protocol treatment, 19 underwent hematopoietic stem-cell transplantation. The 1-year overall survival rate was 55% (95% CI, 38% to 69%). Grade 4 neutropenia was observed in 92% of the patients. The most common grade 3 or 4 nonhematologic complication was infection (61%).

Conclusion
SMILE chemotherapy is an effective treatment for newly diagnosed stage IV, relapsed or refractory ENKL. Myelosuppression and infection during the treatment should be carefully managed.

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INTRODUCTION

Extranodal natural killer (NK)/T-cell lymphoma, nasal type (ENKL), is a lymphoma associated with the Epstein-Barr virus (EBV), which is much more common in Asia and Latin America than in Western countries.[1,2] More than two thirds of patients with ENKL have stage I or II disease in the upper aerodigestive tract.[3-6] The prognosis for localized ENKL has been improving as a result of the use of either concurrent chemoradiotherapy[7,8] or chemotherapy with sandwiched radiotherapy.[9] In contrast, most patients with newly diagnosed stage IV, relapsed, or refractory ENKL treated with conventional chemotherapy designed for aggressive lymphomas, such as cyclophosphamide, doxorubicin, vincristine, and prednisone, survive for less than a year.[6] The poor outcome is partly because ENKL tumor cells express P-glycoprotein, which results in tumor multidrug resistance.[10-12] There are a number of long-term survivors among patients with advanced-stage, relapsed, or refractory ENKL who have undergone hematopoietic stem-cell transplantation (HSCT).[13-15] However, patients who received HSCT in complete response (CR) showed better prognosis than those who received HSCT during non CR. Therefore, the development of an effective chemotherapy for these patients is an important initial step in improving treatment outcomes.

Table 1. SMILE Chemotherapy

Agent	Dose/d	Route	Day
Methotrexate	2 g/m^2*	IV (6 hours)	1
Leucovorin	15 mg × 4	IV or PO	2, 3, 4
Ifosfamide	1,500 mg/m^2	IV	2, 3, 4
Mesna	300 mg/m^2 × 3	IV	2, 3, 4
Dexamethasone	40 mg/d	IV or PO	2, 3, 4
Etoposide	100 mg/m^2*	IV	2, 3, 4
L-asparaginase (*Escherichia coli*)	6,000 U/m^2	IV	8, 10, 12, 14, 16, 18, 20
G-CSF		SC or IV	Day 6 to WBC > 5,000/μL

NOTE. Cycles were repeated every 28 days. Two courses were planned as the protocol treatment.
Abbreviations: G-CSF, granulocyte-colony stimulating factor; IV, intravenously; PO, orally; SC, subcutaneous injection; SMILE, steroid (dexamethasone), methotrexate, ifosfamide, L-asparaginase, and etoposide.
*The recommended dose was determined in the preceding phase I study.

To explore the possibility of more effective induction chemotherapy for NK-cell neoplasms, the NK-Cell Tumor Study Group, comprising Japanese and Asian hematologists, has formulated a novel chemotherapeutic regimen: steroid (dexamethasone), methotrexate, ifosfamide, L-asparaginase, and etoposide (SMILE). These agents are multidrug resistance independent and may be key drugs for NK-cell neoplasms or for EBV-associated disease. From the phase I trial of SMILE, the recommended doses of methotrexate and etoposide were determined.[16] The CR rate in the phase I trial was 50% (three of six eligible patients), and the overall response rate (ORR) was 67% (four of six patients). To further evaluate the efficacy of SMILE chemotherapy, we conducted a subsequent phase II study.

PATIENTS AND METHODS

Eligibility Criteria

Patients with newly diagnosed stage IV, relapsed, or refractory disease who had undergone first-line chemotherapy were eligible. Those with aggressive NK-cell leukemia were excluded because no patients with aggressive NK-cell leukemia had been enrolled in the prior phase I study.[16] Patients who had received autologous HSCT more than 12 months before registration were also eligible. The other inclusion and exclusion criteria for the study were the same as those for the prior phase I study.[16] Briefly, patients from 15 to 69 years of age with a performance status of 0 to 2, based on the Eastern Cooperative Oncology Group scale, and preserved organ functions were included. Neither chemotherapy nor radiotherapy was administered within 21 days before registration. Patients who had clinical symptoms of CNS involvement were excluded.

The pretreatment staging procedures included a physical examination, a bone marrow aspiration and/or biopsy, a chest radiograph, and a computed tomography scan of the nasal cavity, neck, chest, abdomen, and pelvis. An endoscopy of the upper gastrointestinal tract and a positron emission tomography scan were recommended but not mandatory.

After patient enrollment, hematoxylin-eosin–stained sections were histologically reviewed by the Central Pathology Review Board based on the WHO classification.[1] Immunohistochemical staining was performed at the central pathology office using formalin-fixed, paraffin-embedded sections with antibodies against CD3, CD20, CD56, perforin, and granzyme B. In addition, in situ hybridization for EBV-encoded small RNA-1 was performed.

Registration of patients was conducted by facsimile between the participating physicians and the Center for Supporting Hematology-Oncology Trials Data Center (Nagoya, Japan). The study was approved by both the protocol review committee and the institutional review board of each institution. Written informed consent was obtained from all of the patients. The study was registered to the University Hospital Medical Information Network Clinical Trials Registry.

Treatment

SMILE chemotherapy was administrated as indicated in Table 1. On the basis of the results of the phase I trial,[16] administration of granulocyte colony-stimulating factor was mandatory from day 6 and discontinued if the leukocyte count exceeded 5,000/μL after the nadir phase. Antibiotic prophylaxis of sulfamethoxazole-trimethoprim was recommended. The criteria for the initiation of a second course of SMILE were as follows (1): a total of 4 weeks or more had passed since the prior course; (2) all of the following were achieved at least 1 day before the second course of SMILE: a leukocyte count of $\geq$ 2,000/μL, a platelet count of $\geq$ 100,000/μL, AST and ALT levels $\leq$ 5$\times$ the upper limit of normal, total bilirubin of $\leq$ 2.0 mg/dL, or serum creatinine of $\leq$ 1.5 mg/dL; and (3) there were no other symptoms or complications that were not suitable for the initiation of a second course. If there was no recovery 4 weeks after the day of the scheduled second course, the protocol treatment was terminated. Two courses of SMILE chemotherapy were planned for the protocol treatment. After the planned two courses, patients could undergo additional courses of SMILE and/or other chemotherapy, with or without autologous/allogeneic HSCT. The decision was made according to the discretion of treating physicians mainly on the basis of the patient's age, conditions, and the availability of HSC donors.

Response and Toxicity Criteria

The responses were assessed by the Central Imaging Review Board according to criteria modified from the WHO response criteria[17] that were also used in the prior phase I study of SMILE chemotherapy.[16] All of the examinations for restaging were done within 4 to 6 weeks (from day 22 to day 42) of the second course of SMILE. Because ENKL frequently occurs in the nasal/paranasal sites and leaves scar or necrotic tissue, it is sometimes difficult to determine whether or not a patient strictly attains CR using the WHO response criteria[17] or the International Workshop criteria.[18] Therefore, in this trial, CR was defined as the complete disappearance of all objective signs of disease, including enlarged lymph nodes or hepatomegaly and splenomegaly at the restaging. Partial response (PR) was defined as at least a 50% reduction of tumor volume without the occurrence of new lesions at the restaging. Progressive disease was defined as a greater than 25% increase in the sum of tumor lesions or the emergence of one or more new lesion(s) or clinical symptoms that indicate disease progression, such as "B" symptoms or elevated serum lactate dehydrogenase levels. No response was defined as any response that did not fall into the other defined categories. If a patient died before day 42 of the second course of SMILE and could not undergo the defined restaging procedure, the patient's response was recorded as early death. The ORR rate was defined as the proportion of all patients who were able to be evaluated for response who experienced CR or PR.

Toxicity was graded according to the Common Terminology Criteria for Adverse Events (CTCAE) version 3.0. In cases of grade 4 thrombocytopenia,

doses of methotrexate, ifosfamide, and etoposide were reduced to two thirds of their previous levels in the second course. L-asparaginase was discontinued if it induced grades 3 or 4 allergic reactions/hypersensitivity, pancreatitis, or hypotension. If L-asparaginase induced grades 1 or 2 allergic reactions/hypersensitivity, the dose of L-asparaginase was reduced by half. In this case, the use of prednisone at a dose of 1 mg/kg/d was permitted. L-asparaginase was stopped if grade 4 thrombocytopenia or grade 3 nonhematologic toxicity was observed. In the cases for which the first course of L-asparaginase was discontinued, L-asparaginase was readministered if the patient recovered from grade 4 thrombocytopenia or grade 3 nonhematologic toxicity. If the concentration of methotrexate exceeded 1×10^{-7} mol/L 72 hours after the administration during the first course, the dose of methotrexate in the second course was reduced to two thirds.

Statistical Analysis

The primary end point was an ORR after two courses of SMILE chemotherapy. The secondary end points were CR rate after two courses of SMILE chemotherapy, 1-year overall survival (OS), response of the subgroup, or toxicity. The expected ORR was estimated to be 60%, and the threshold ORR was estimated to be 35%, on the basis of our previous observations.[6,19] With a statistical power of 80% and a one-sided, type I error of 5%, the number of eligible patients required for this study was calculated to be 25 using a binomial analysis method. The projected sample size was 28 patients, with an accrual of 3 years and the expectation that 10% of patients would be deemed ineligible.

OS was defined as the time from registration until death from any cause or until the date of the last follow-up for the patients who survived. Survival estimates were calculated using the Kaplan-Meier method, and the hazard ratio (HR) was estimated using a Cox regression. All analyses were performed using STATA SE 10 software (STATA, College Station, TX).

RESULTS

Patient Characteristics

As a result of an excellent accrual, the study protocol was revised to increase the statistical power from 80% to 90% in March 2009. The projected number of patients for this study was increased from 28 to 38. Ultimately, 39 patients were enrolled from 19 institutions between July 2007 and October 2009. Histologic diagnosis of all patients except one was confirmed as ENKL by the Central Pathology Review Board. The single patient who was excluded from further analyses was judged to have CD56-positive rhabdomyosarcoma by the Central Pathology Review Board.

The baseline characteristics of 38 eligible patients are listed in Table 2. The median age was 47 years (range, 16 to 67 years), and the male:female ratio was 21:17. Twenty patients (53%) had newly diagnosed stage IV disease, 14 were in first relapse, and four were in primary refractory state. Two patients were treated with radiation alone as the initial therapy. Among the 16 patients who received chemotherapy as their first-line therapy, five patients were treated with anthracycline-containing chemotherapies, and 13 patients were treated with platinum-based regimens. Two patients were treated with chemotherapy containing both anthracycline and platinum.

Table 2. Baseline Patient Characteristics (N = 38)

Characteristic	No. of Patients	%
Age, years		
Median	47	
Range	16 to 67	
Sex		
Male	21	55
Female	17	45
Site(s) of involvement at diagnosis		
Upper aerodigestive tract	35	92
Extra-upper aerodigestive tract only	3	8
Disease state		
Newly diagnosed stage IV	20	53
First relapse	14	37
Refractory to the first-line treatment	4	11
Stage at enrollment		
IE or IIE	11	29
IIIE or IV	27	71
"B" symptoms present	18	47
Elevated serum LDH	16	42
Performance status		
0	21	55
1	12	32
2	5	13
Prior treatment		
None	20	53
Radiotherapy alone	2	5
Chemotherapy alone	3	8
Concurrent chemoradiotherapy	9	24
RT-DeVIC	6	
CCRT-VIPD or VIDL	2	
RT-CHOP	1	
Other combined modality therapies	4	11

Abbreviations: CCRT, concurrent chemoradiotherapy; DeVIC, dexamethasone, etoposide, ifosfamide, and carboplatin; LDH, lactate dehydrogenase; VIDL, etoposide, ifosfamide, dexamethasone, and L-asparaginase; VIPD, etoposide, ifosfamide, cisplatin, and dexamethasone.

Treatment

Twenty-eight patients (74%) completed the planned treatment. In two patients, the treatment was discontinued on day 4 because of methotrexate-associated encephalopathy and intestinal perforation owing to rapid tumor lysis. L-asparaginase was discontinued in four patients due to adverse events (AEs), including two patients with allergy to L-asparaginase (both in the second course), one patient with pancreatitis (grade 2, in the first course), and one patient with liver function derangement (in the first course). In two of these four patients, L-asparaginase was readministered at a 50% dose reduction. One allergic patient received simultaneously prednisolone 1 mg/kg. In another four patients, L-asparaginase was also stopped per protocol, owing to AEs of preceding agents (methotrexate, ifosfamide, and etoposide), including two patients with infections and two patients with thrombocytopenia. The relative dose-intensity of L-asparaginase in the first course of SMILE was 81%. Two of these eight patients who had L-asparaginase discontinued achieved CR. The relative dose-intensity of CR patients was 92%.

Additional courses of SMILE were given for 21 patients (one course, 10 patients; two courses, three patients; three courses, two patients; four courses, six patients). The median number of courses of SMILE administered was three (range, one to six courses). Treatment of the 28 patients who completed two courses of SMILE were as follows: chemotherapy only (n = 7), autologous HSCT (n = 4), or allogeneic HSCT (n = 17; myeloablative, n = 15, nonmyeloablative, n = 2). No difficulties in mobilizing peripheral blood HSC were encountered in the four patients who received autologous HSCT. Among the seven patients who did not complete the protocol treatment, two of them received no additional treatment and died as a

Table 3. Incidence and Maximum Severity of Adverse Events (N = 38)

Adverse Event	Grade 3 No.	Grade 3 %	Grade 4 No.	Grade 4 %
Hematologic				
Leukopenia	9	24	29	76
Neutropenia	3	8	35	92
Anemia	18	47	1	3
Thrombocytopenia	9	24	15	40
Nonhematologic				
Hypofibrinogenemia	4	11	0	0
APTT elongation	4	11	0	0
Hypoalbuminemia	6	16	0	0
Hyperbilirubinemia	3	8	1	3
AST elevation	12	32	0	0
ALT elevation	10	26	2	6
Creatinine	2	5	0	0
Hyponatremia	11	29	1*	3
Hyperglycemia	7	18	0	0
Amylase	6	16	1*	3
Appetite loss	8	21	1*	3
Diarrhea	4	11	0	0
Nausea	5	13	0	0
Mucositis	5	13	0	0
Vomiting	2	5	0	0
Infection	17	45	6†	16
Somnolence	1	3	2	5
Encephalopathy	0	0	1	3

NOTE. Grade 3 hypernatremia, allergic reaction, fever, and dehydration were observed in one patient each.
Abbreviation: APTT, activated partial thromboplastin time.
*Related to grade 2 pancreatitis in one patient.
†Including the two patients who died as a result of infection (two treatment-related deaths).

Table 4. Response After Two Cycles of SMILE Chemotherapy (N = 38)

Response	All Patients (N = 38) No.	All Patients (N = 38) %	Newly Diagnosed Stage IV (n = 20) No.	Newly Diagnosed Stage IV (n = 20) %	First Relapse (n = 14) No.	First Relapse (n = 14) %	Refractory to the First-Line Therapy (n = 4) No.	Refractory to the First-Line Therapy (n = 4) %
CR	17	45	8	40	9	64	0	0
PR	13	34	8	40	4	29	1	25
NR	1	3	1	5	0	0	0	0
PD	4	10	1	5	1	7	2	50
ED	3	8	2	10	0	0	1	25

Abbreviations: CR, complete response; ED, early death; NR, no response; PD, progressive disease; PR, partial response; SMILE, steroid (dexamethasone), methotrexate, ifosfamide, L-asparaginase, and etoposide.

result of disease. Three patients were treated with other chemotherapy, and two of them underwent allogeneic HSCT without response.

Toxicity

Table 3 lists all grade 3 or 4 AEs that occurred in the 38 eligible patients who were enrolled onto this trial. After the death of initial two patients from grade 5 infections (patients 1 and 2; see Appendix, online only), the protocol was revised to include a careful assessment of infection and the incorporation of a lymphocyte count of $\geq 500/\mu L$ into the eligibility criteria. There were no subsequent treatment-related deaths.

Grade 4 neutropenia was common (92%). The nonhematologic grade 4 toxicities included infection (n = 6), hyperbilirubinemia (n = 1), ALT elevation (n = 2), and encephalopathy (n = 1); two patients experienced grade 4 somnolence, which was complicated by a grade 3 infection in one patient and by grade 4 encephalopathy in the other patient. One patient experienced grade 2 pancreatitis and had complications from grade 4 hyponatremia, hyperamylasemia, and appetite loss. The most common grade 3 nonhematologic AE was infection (45%). Allergic reactions due to L-asparaginase of any grade were observed in five patients (three with grade 1, one with grade 2, and one with grade 3). The toxic profiles according to disease status at the time of study entry (newly diagnosed/relapsed/refractory) are shown in Appendix Table A1 (online only). The grade 4 hematologic toxicity rates of newly diagnosed and relapsed patients were 95% and 93%, respectively. The grade 4 nonhematologic toxicity rates of newly diagnosed and relapsed patients were 35% and 14%, respectively. None of these differences were statistically significant ($P = .99$ and $P = .25$). No clinical predictors of toxicity were found. Only hyponatremia was associated with newly diagnosed and refractory diseases.

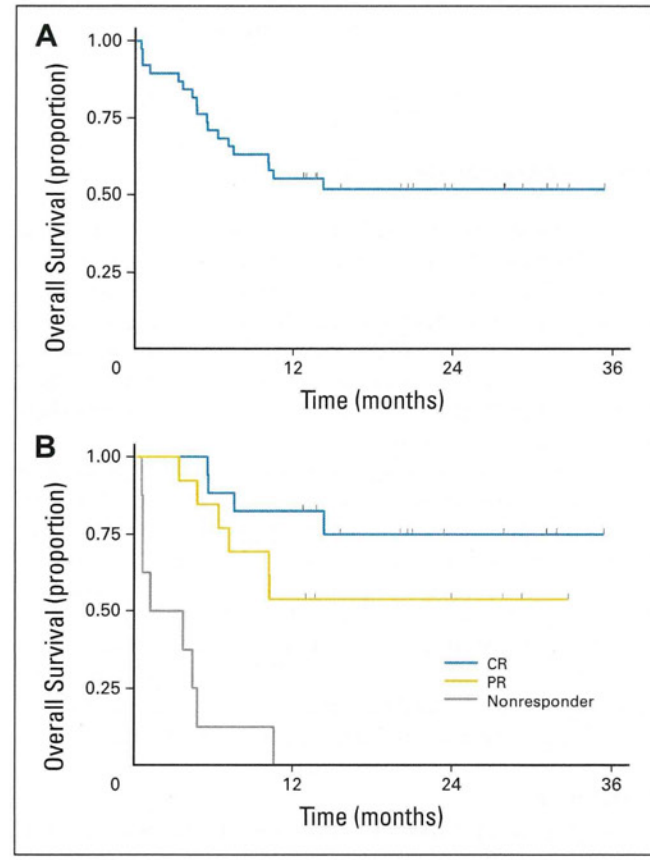

Fig 1. Kaplan-Meier estimates of overall survival (OS) of patients treated with steroid (dexamethasone), methotrexate, ifosfamide, L-asparaginase, and etoposide chemotherapy. (A) The 1-year OS of 38 patients was 55% (95% CI, 38% to 69%). The median follow-up of survivors was 24 months (range, 13 to 35 months). (B) The 1-year OS was 82% (95% CI, 55% to 94%) for patients who attained complete response (CR) and 54% (95% CI, 25% to 76%) for those who attained partial response (PR).

Efficacy and Survival

Among the 38 eligible patients, the response was CR in 17 patients (45%), PR in 13 patients, no response in one patient, progressive disease in four patients, and early death in three patients (Table 4). The ORR was 79% (90% CI, 65% to 89%). There were no differences in either the ORR or CR rate between patients with newly diagnosed stage IV disease and those with first-relapse disease. With respect to progressive disease in four patients, one occurred during the first course of SMILE, one after the first course, and two after the completion of two courses.

The median follow-up time of the living patients was 24 months, with a range of 13 to 35 months. The OS rate at 1 year, which was one of the secondary end points, was 55% (95% CI, 38% to 69%; Fig 1A). The progression-free survival (PFS) at 1 year was 53% (95% CI, 36% to 67%). The patients who attained response with SMILE chemotherapy had a higher OS (Fig 1B). The OS and PFS by the disease state at entry are shown in Figure 2A and 2B. Patients with relapsed disease showed better 1-year OS (79%) and PFS (71%) as compared with patients with refractory disease (P = .04 and .05, respectively). The survival curves of patients (excluding early deaths; n = 35) according to the type of poststudy therapy (autologous/allogeneic HSCT/chemotherapy) are shown in Figures 2C and 2D. Patients who received autologous HSCT seemed to show better OS and PFS, but the difference was not statistically significant. Univariate analysis for OS showed that presence of B symptoms (HR, 3.1, P = .01), performance status of 1 or 2 (HR, 3.1, P = .002), elevated serum lactate dehydrogenase (HR, 6.1, P = .001), and hemoglobin of less than 12 g/dL (HR, 3.9, P = .007) were significant prognostic factors.

DISCUSSION

Our results indicate that SMILE chemotherapy is effective for the treatment of newly diagnosed stage IV, relapsed, or refractory ENKL. The ORR after two cycles of SMILE (79%; 90% CI, 65% to 89%) clearly exceeded the threshold ORR (35%).[20] The 1-year OS rate (55%) was much improved compared with the previous treatment strategy.

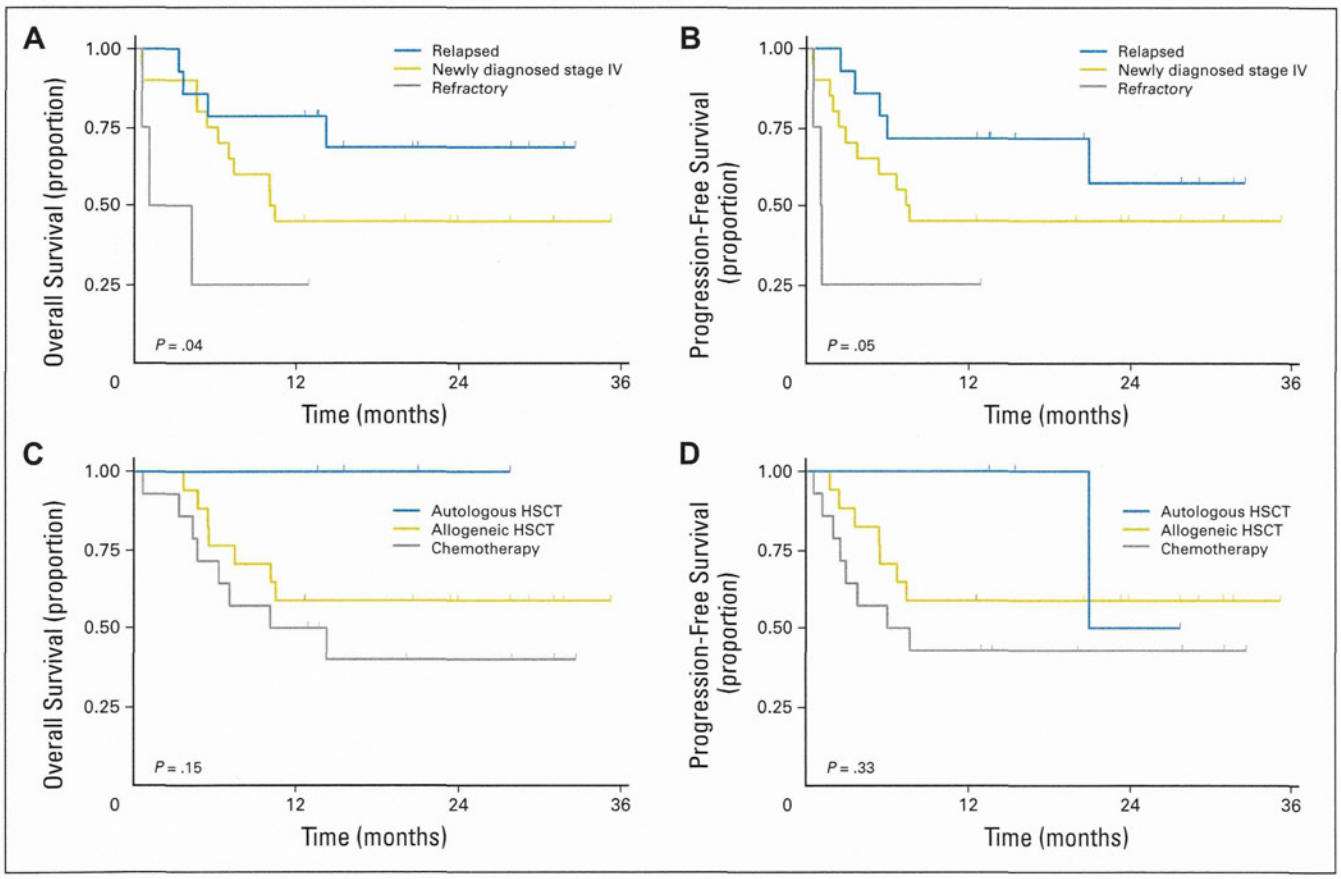

Fig 2. Kaplan-Meier estimates of overall survival (OS) and progression-free survival (PFS) of patients by the subgroup analysis. (A) OS of patients by the disease state at entry. The 1-year OS was 45% (95% CI, 23% to 65%) for patients with newly diagnosed stage IV disease, 79% (95% CI, 47% to 93%) for patients with relapsed disease, and 25% (95% CI, 1% to 67%) for patients with refractory disease. The difference was statistically significant (P = .04). (B) PFS of patients by the disease state at entry. The 1-year PFS was 45% (95% CI, 23% to 65%) for patients with newly diagnosed stage IV disease, 71% (95% CI, 41% to 88%) for patients with relapsed disease, and 25% (95% CI, 1% to 67%) for patients with refractory disease. The difference was statistically significant (P = .05). (C) OS of patients excluding early death (n = 35) by type of poststudy therapy. The 1-year OS was 100% for patients who received autologous hematopoietic stem-cell transplantation (HSCT), 59% (95% CI, 36% to 78%) for patients who received allogeneic HSCT, and 41% (95% CI, 19% to 63%) for patients treated with chemotherapy only. The difference was not statistically significant (P = .15). (D) PFS of patients excluding early death by type of poststudy therapy. The 1-year PFS was 100% for patients who received autologous HSCT, 59% (95% CI, 36% to 78%) for patients who received allogeneic HSCT, and 35% (95% CI, 14% to 57%) for patients treated with chemotherapy only. The difference was not statistically significant (P = .33).

With regard to the safety of SMILE, myelosuppression and infection should be carefully monitored during and after SMILE chemotherapy. To avoid severe AEs, the use of granulocyte colony-stimulating factor is considered mandatory, starting on day 6 and continuing until recovery beyond the nadir. In addition, full-dose administration of SMILE should be avoided for patients who are in poor condition, including those with lymphopenia less than 500/μL or large tumor burden. A lymphocyte count was added to the eligibility criteria because all three of the patients who died of neutropenic infection in the phase I and phase II SMILE studies had low lymphocyte counts before treatment. Decreased-dose SMILE[21] and less-intensive L-asparaginase chemotherapies[22-24] are candidate strategies for those patients with poor pretreatment conditions.

L-asparaginase–based chemotherapy has been highlighted as a promising treatment for ENKL. L-asparaginase was shown to induce apoptosis of ENKL cells in vitro; this result was attributed to low asparagine synthetase expression.[25] In fact, there were several case reports in the early 2000s in which ENKL showed an excellent response to L-asparaginase.[26-30] Recently, a phase II study of L-asparaginase, methotrexate, and dexamethasone (AspaMetDex) for relapsed or refractory ENKL was reported by a French group.[22] Nineteen patients were enrolled, and the CR rate was 61%. The median survival time was 12.2 months, and the 1-year OS was 45%. The AspaMetDex therapy is also promising, but there are several differences from the SMILE study. First, 53% of patients in our SMILE study had newly diagnosed stage IV ENKL which showed poor prognosis with conventional chemotherapy.[6] In contrast, the GELA (Groupe d'Etude des Lymphomes de l'Adulte)/GOELAMS (Groupe Ouest-Est des Leucémies et des Autres Maladies du Sang) study included only patients with relapsed/refractory disease. This resulted in a different ratio of patients with advanced-stage disease between the SMILE study (27 of 38 patients, 71%) and the AspaMetDex study (seven of 19 patients, 37%). Second, 17 of the 19 patients were initially treated with anthracycline-based chemotherapy in the French study. In contrast, 81% of the patients who had prior therapy in our study received platinum-based chemotherapy before SMILE, which suggests that different patient groups were selected in the two studies. Currently, these SMILE and AspaMetDex regimens are both promising for relapsed/refractory ENKL. A comparative study is required for a conclusion, but is not realistic for this type of rare lymphoma.

The optimal course of SMILE chemotherapy and the most appropriate timing of HSCT for patients after two courses of SMILE remain undetermined. In addition, the optimal treatment strategy for patients who cannot undergo HSCT needs further clinical evaluation. It has been speculated that the SMILE regimen may also be effective for T-cell lymphomas because ENKL and mature T-cell lymphomas share several clinical and pathologic features, such as extranodal predilection and expression of cytotoxic molecules. This speculation should be confirmed in further clinical studies.

In conclusion, the results of this phase II study demonstrate that two cycles of SMILE is an effective chemotherapy regimen for patients with newly diagnosed stage IV, relapsed, or refractory ENKL. However, the SMILE regimen is potentially toxic, and careful patient monitoring is needed.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Although all authors completed the disclosure declaration, the following author(s) indicated a financial or other interest that is relevant to the subject matter under consideration in this article. Certain relationships marked with a "U" are those for which no compensation was received; those relationships marked with a "C" were compensated. For a detailed description of the disclosure categories, or for more information about ASCO's conflict of interest policy, please refer to the Author Disclosure Declaration and the Disclosures of Potential Conflicts of Interest section in Information for Contributors.

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CASE REPORT

Perfusion method for bone marrow cell collection in poor mobilizer lymphoma patient

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Abstract We previously described a unique procedure for the collection of bone marrow cells (BMCs) using a perfusion method (PM). In cynomolgus monkeys, this method resulted in lower contamination with T cells (<10%). Here, we performed PM on a poor mobilizer lymphoma patient. We confirmed the safety of the intra-bone marrow injection of saline to collect the BMCs. The collected BMCs showed minimal contamination with T cells (<15%) and red blood cells (RBCs) (<4%) from the peripheral blood. It took a total of only 30 min to collect the BMCs. Moreover, transfusion of RBCs was unnecessary. There were no relevant post-operative side effects except for self-limiting pain at the sites of collection, and the patient was able to walk around the hospital after the operation.

Keywords Bone marrow cell · Perfusion method · Poor mobilizer · Aspiration method · Malignant lymphoma

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1 Introduction

In spite of the enormous advances in medical technology, the procedure for collecting bone marrow cells (BMCs) has not changed in the past 40 years [1]. Therefore, healthy donors have been exposed to anesthetic procedures, blood loss, and multiple needle punctures, resulting in damage to the pelvis. We have carried out extensive experiments on monkeys to establish a novel method for BMC collection that would allow the burden on donors to be reduced [2–4].

The novel method is called the "perfusion method" (PM), while the conventional method is called the "aspiration method" (AM). In the PM, there was minimal contamination of T cells and red blood cells (RBCs) with the peripheral blood (PB) in monkeys. In addition, the PM allowed us to enrich the hemopoietic stem cells (HSCs) [4]. Recently, in China, we demonstrated that BMCs could be successfully collected using the PM even from a healthy relative (donor) of a thalassemia patient (recipient) [5].

In this paper, we discuss the use of the PM for a poor mobilizer patient with recurrent lymphoma.

2 Case presentation

A 58-year-old male patient (60 kg BW) was diagnosed with diffuse large B cell lymphoma in September 2004. He received chemotherapy with 8 cycles of R-CHOP. He achieved his 1 complete remission (CR). However, in February 2006, he relapsed. He received three cycles of R-ESHAP, achieving his 2 CR. Therefore, we attempted to collect peripheral blood stem cells (PBSCs) following R-ESHAP. However, this collection was not successful due to the insufficient number of $CD34^+$ cells ($<0.5 \times 10^6$/kg). After 3 months, we attempted to collect PBSCs using

cyclophosphamide 2 g/m^2 for 2 days. However, we again failed to collect PBSCs. From this result, the patient was diagnosed as a poor mobilizer, and BMC collection was proposed as an alternative source. Our study, including the use of the new PM collection method for poor mobilizer patients, had been approved by our institutional Ethics Committee. The patient gave written informed consent before starting the protocol. We confirmed that there was no bone marrow (BM) involvement before the operation.

Having taken these steps, we used the PM to collect BMCs under general anesthesia in December 2006. As shown in Fig. 1, one BM puncture needle was inserted into the posterior superior iliac spine, and the other needle was inserted into the iliac spine approximately 3–5 cm along the iliac crest from the first needle. The first needle was connected to a syringe containing 30 ml of saline, and the other needle was connected to a syringe containing heparin (0.5 ml, 10 U/ml). The saline was then pushed gently from the syringe into the medullary cavity to collect the BM fluid in the syringe containing heparin. This procedure was repeated 3 times bilaterally and then performed in reverse. Table 1 shows the characterization of BMCs collected using the PM. Of note is that there was minimal contamination of the BMCs with the PB: Ht was approximately 3%, and CD4$^+$ or CD8$^+$ cells were less than 10% each. We collected a total of 390 ml of BM fluids. There were 0.13×10^8/kg total nucleated cells and 0.12×10^6/kg CD34$^+$ cells. These results indicated that BMCs collected using the PM contain about 10% of the threshold number of progenitors for transplantation. It took a total of only 30 min to collect the BMCs. Moreover, there was no requirement for the transfusion of RBCs. After the operation, there were no relevant side effects except for self-limiting pain at the sites of collection, and the patient could walk around the hospital after the operation. However, we failed to collect sufficient numbers of BMCs, and the patient therefore continued to receive the conventional salvage chemotherapy and radiotherapy after our study.

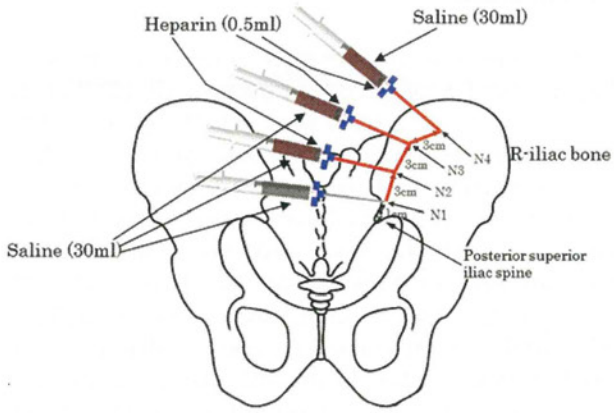

Fig. 1 Perfusion method for BMC collection. One BM puncture needle is inserted into the posterior superior iliac spine, and the other needle is inserted into the iliac spine 3–5 cm from the first needle along the iliac crest. The first needle is connected to a syringe containing 30 ml saline. The second needle is connected to syringe containing heparin (0.5 ml, 10 U/ml). The saline is then pushed gently from the syringe into the medullary cavity to collect the BM fluid in the syringe containing heparin. This procedure is repeated 3 times bilaterally. This procedure is then performed in reverse bilaterally. Thus, the BMCs are collected using the perfusion method for a total of 12 times

3 Discussion

Based on our previous data [2–4] using more than 100 cynomolgus monkeys, we have here provided the first report suggesting the safety and usefulness of the PM for a poor mobilizer lymphoma patient. There were neither accidents nor side effects other than mild self-limiting pain at the site of collection, and the patient returned almost to baseline health status very quickly. We also double-checked the patient's data and physical status before and after the PM operation to confirm that there were no remarkable changes. The results suggested that infusing saline directly into the BM cavity does not affect the cardiovascular system or the environment of the marrow. The procedure also involves minimal blood loss and avoids the multiple needle punctures that can damage the pelvic structure. In particular, our method showed 1/10th the

Table 1 Characterization of BMCs harvested by PM from a "poor mobilizer" patient

Source	Cell count (no.) ($\times 10^8$)	CD34$^+$ (no.) ($\times 10^6$)	CD34$^+$ (%)	Ht (%)	CD4 (%)	CD8 (%)	CD14 (%)	CD19 (%)
PBL (healthy volunteer)					21.3	12.8	6.2	3.6
BM								
Forward	2.8	3.3	1.17	2.5	4.88	9.26	6.43	2.11
Reverse	3.2	3.9	1.22	3.8	5.15	10.56	8.02	2.09
Mean	3	3.6	1.20	3.2	4.99	9.91	7.22	2.1

Total cell counts: 2×10^6/ml $\times$ 400 ml $= 0.8 \times 10^9$

amount of RBC contamination of the collected BMCs than the conventional AM.

Unfortunately, however, this study resulted in a poor cell yield. Little is known about why certain patients are poor PBSC mobilizers. Previous reports have also indicated that conventional BMC collection does not provide any clinical benefit on second-line stem cell collection in poor PBSC mobilizers [6, 7]. Therefore, the low yield of BMCs may be because the BM HSCs had been exhausted due to hematological toxicity from intensive chemotherapy.

We believe that the PM is far superior to the AM for the following reasons:

1. The PM reduces the burden on donors.
   (i) Shortens the time required for the collection of BMCs (4 h → 30 min).
   (ii) Attenuates the back pain in donors because of the reduction in the number of bone holes required (200 vs. 8).
2. The PM allows pure BMCs [including HSCs and mesenchymal stem cells (MSCs)] to be harvested due to the reduced contamination with the PB.
   (i) Reduces T cell percentages (>20 vs. <10%). The incidence of GvHD in the PM is far lower in the AM. We have recently provided evidence that rabbits treated with PM + BMT (IBM-BMT) show significantly longer survival than rabbits treated with AM + BMT [8].
   (ii) Reduces Ht values (35% → 4%). No necessity to remove RBCs.

In conclusion, the PM was well tolerated in this poor mobilizer lymphoma patient, and our novel approach may ameliorate the burden on bone marrow transplantation donors. At present, we are attempting to investigate how the BMC yield can be increased using the PM in healthy donors.

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Escape of leukemia blasts from HLA-specific CTL pressure in a recipient of HLA one locus-mismatched bone marrow transplantation

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ABSTRACT

A case of leukemia escape from an HLA-specific cytotoxic T lymphocyte (CTL) response in a recipient of bone marrow transplantation is presented. Only the expression of HLA-B51, which was a mismatched HLA locus in the graft-versus-host direction, was down-regulated in post-transplant leukemia blasts compared with that in pre-transplant blasts. All CTL clones, that were isolated from the recipient's blood when acute graft-versus-host disease developed, recognized the mismatched B*51:01 molecule in a peptide-dependent manner. The pre-transplant leukemia blasts were lysed by CTL clones, whereas the post-transplant leukemia blasts were not lysed by any CTL clones. The IFN-γ ELISPOT assay revealed that B*51:01-reactive T lymphocytes accounted for the majority of the total alloreactive T lymphocytes in the blood just before leukemia relapse. These data suggest that immune escape of leukemia blasts from CTL pressure toward a certain HLA molecule can lead to clinical relapse after bone marrow transplantation.

1. Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is curative for leukemia by virtue of the immune reaction mediated by donor T lymphocytes, termed the graft-versus-leukemia (GVL) effect [1]. For HSCT recipients from HLA-matched donors, the GVL effect can be triggered by minor histocompatibility antigens [2–4], and several studies using sequential flow cytometric analysis with tetramers have clearly demonstrated that minor histocompatibility antigen-specific T lymphocytes increase in frequency in the recipient's blood before and during clinical regression of leukemia [5–10]. On the other hand, for HLA-mismatched HSCT recipients, extremely limited biological studies have demonstrated that the GVL effect can be mediated by mismatched HLA-specific donor T lymphocytes [11].

Allogeneic HSCT is a well-established immunotherapy for leukemia, but, unfortunately, some recipients relapse after transplantation. It is difficult to evaluate the role of individual factors in relapse. Nevertheless, it is reasonable to assume that the selective pressure exerted by donor T lymphocytes can lead to the outgrowth of pre-existing leukemia variants that have lost expression of gene products such as HLA molecules. Some studies have demonstrated loss of the mismatched HLA haplotype in the leukemia blasts of HSCT recipients as a consequence of loss of heterozygosity in chromosome 6 [12–14]. However, the mechanisms involved in leukemia relapse after HLA locus-mismatched HSCT remain largely uninvestigated.

This paper presents a case of selective HLA down-regulation in post-transplant leukemia blasts but not in pre-transplant blasts of a recipient who received bone marrow transplantation from an HLA one locus-mismatched donor. All cytotoxic T lymphocyte (CTL) clones that were isolated from the recipient's blood during acute graft-versus-host disease (GVHD) demonstrated cytotoxicity specific for the mismatched HLA-B molecule, lysed pre-transplant blasts but not post-transplant blasts, and persisted in the patient's blood until leukemia relapse. These results suggest that immune escape of leukemia blasts from CTL pressure toward a certain HLA allele can lead to clinical relapse.

2. Patient, materials and methods

2.1. Patient

A 24-year-old man with primary refractory T lymphoblastic leukemia/lymphoma received allogeneic bone marrow transplantation without ex vivo T lymphocyte depletion from his mother. Because the patient had neither a sibling nor an HLA-matched unrelated donor, his mother was chosen as an alternative donor. PCR sequencing-based typing for HLA alleles of the patient and mother revealed one HLA-B allele mismatch in

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Table 1
HLA types of the patient and donor.

	A	B	C	DRB1	DQB1	DPB1
Patient	1101/2402	5401/5101	0102/–	0901/–	0303/–	0501/–
Donor	1101/2402	5401/5201	0102/1202	0901/1502	0303/0601	0501/–

The mismatched HLA allele in the graft-versus-host direction is underlined.

the graft-versus-host direction (Table 1). The preparative regimen consisted of 180 mg/m^2 melphalan and 12 Gy total body irradiation. GVHD prophylaxis consisted of 0.03 mg/kg tacrolimus and short-term methotrexate. Neutrophil engraftment (neutrophil count $\geqslant 0.5 \times 10^9$/l) was achieved 14 days after transplantation with full donor-type chimera. The patient developed severe acute GVHD involving the skin, gut, and liver on day 46 (maximum stage: skin 3, gut 2, and liver 1; maximum grade: III on day 53), evaluated according to previously published criteria [15]. Acute GVHD was temporarily controlled by additional immunosuppressants, but it was incurable and transitioned to chronic GVHD. On day 261, the patient relapsed with ascites, a hydrocele, and a subpapillary tumor. Leukemia blasts in the ascites fluid were confirmed by cytological examination. Immunosuppressant therapy was required to control GVHD until his death on day 279.

2.2. Cell culture

CTL clones were isolated from a blood sample as described previously [16]. Briefly, peripheral blood mononuclear cells (PBMCs) obtained from the recipient on day 56, when severe acute GVHD developed, were stimulated in vitro with aliquots of γ-irradiated PBMCs that had been obtained from the recipient pre-transplant and cryopreserved. After three weekly stimulations, the CTL clones were isolated from the polyclonal T lymphocyte culture by limiting dilution. The CTLs were expanded by stimulation every 14 days with 30 ng/ml OKT3 monoclonal antibody (Janssen Pharmaceutical), using unrelated allogeneic γ-irradiated (25 Gy) PBMCs and γ-irradiated (75 Gy) EB virus-transformed lymphoblastoid cells (B-LCL) as feeder cells. The culture medium consisted of RPMI-1640-HEPES (Sigma–Aldrich) containing 10% pooled, heat-inactivated human serum, and recombinant human IL-2 (R&D Systems). The T lymphocytes were used in assays 14 days after stimulation or 1 day after thawing of a frozen aliquot. All samples were collected after written informed consent had been obtained. B-LCLs were maintained in RPMI-1640-HEPES with 10% FBS. COS cells were maintained in DMEM (Sigma–Aldrich) with 10% FBS.

2.3. Flow cytometric analysis

Leukemia blasts were incubated at 37 °C for 30 min with anti-HLA-A24/A23 (One lambda), anti-HLA-A11/A1/A26 (One lambda), and anti-HLA-B51/B52/B49/B56 (One lambda) antibodies to detect A24, A11, and B51, respectively, of patient cells followed by incubation at 37 °C for 15 min with fluorescein isothiocyanate-conjugated antimouse IgM (Beckman Coulter). To detect HLA-DR9 of patient cells, leukemia blasts were incubated at 37 °C for 30 min with fluorescein isothiocyanate-conjugated anti-HLA-DR antibody (BD Pharmingen). Antibody to detect HLA-B54 without cross-reaction to B51 was not available. After washing, the cells were analyzed by a BD FACSAria (BD Biosciences). Leukemia blasts were sorted by BD FACSAria with anti-CD7 (BD Biosciences) and anti-CD10 (eBiosciences) antibodies from pre-transplant bone marrow and post-transplant ascites fluid samples. The purities of pre-transplant and post-transplant blasts were ~62% and ~99%, respectively. CTL clones were analyzed using three-color flow cytometry for expression of CD3, CD4, and CD8 using phycoerythrin-cyanin 5.1-conjugated anti-CD3 (Beckman Coulter), phycoerythrin-conjugated anti-CD4 (BD Biosciences), and fluorescein isothiocyanate-conjugated anti-CD8 (BD Biosciences) antibodies.

2.4. Chromium release assay

Leukemia blasts and B-LCLs were used as target cells in a cytotoxicity assay. Leukemia blasts and B-LCLs were labeled for 2 h with ^{51}Cr. After washing, the cells were dispensed at 2×10^3 cells/well into triplicate cultures in 96-well plates and incubated for 4 h at 37 °C with CTL clones at various E:T ratios. Percent-specific lysis was calculated as [(experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm)] ×100.

2.5. Determination of T cell receptor (TCR)-Vβ gene usage and nucleotide sequences

TCR Vβ usage was assessed by RT-PCR using primers covering the entire families of functional TCR Vβ chains [17–19]. Briefly, total RNA was extracted from individual CTL clones, and cDNA was synthesized using SuperScript III RT (Invitrogen). RT-PCR reactions were carried out with the appropriate Vβ sense primers specific for different Vβ families and a primer specific for the constant region of TCR-β. Subsequently, the complementarity determining region 3(CDR3) of each positive PCR product was sequenced with corresponding antisense primer. TCR Vβ gene usage was determined by the international ImMunoGeneTics information system (IMGT) software, IMGT/V-QUEST (http://www.imgt.org/).

2.6. HLA-B cDNA constructs

Total RNA was extracted from the patient and donor B-LCLs and converted into cDNA. Constructs containing the full-length *HLA-B*51:01*, *B*52:01*, and *B*54:01* cDNA were generated from the cDNA by PCR and cloned into the pEAK10 expression vector (Edge BioSystems). Two mutated *HLA-B*51:01* cDNA constructs, in which amino acid at position 63 or 67 was substituted with the corresponding amino acid in *B*52:01*, and two more mutated *HLA-B*51:01* cDNA constructs, in which the amino acid at position 194 or 199 was substituted with the corresponding amino acid in *B*44:03*, were produced using the QuikChange Site-Directed Mutagenesis Kit (Stratagene).

2.7. Transfection of B-LCLs and COS cells with HLA cDNA

B-LCL (5×10^6) were transfected by electroporation (200 V, 500 μFD) in 200 μl of potassium-PBS with the 15 μg of pEAK10 plasmid encoding *HLA-B*51:01* cDNA and selected with puromycin (Edge BioSystems), beginning 48 h after transfection. Three days after selection, they were used as targets in a chromium release assay. COS cells (5×10^3) were plated in individual wells of 96-well flat-bottom plates and transfected with 100 ng of the pEAK10 plasmid encoding *HLA-B*51:01*, *HLA-B*52:01*, *HLA-B*54:01*, or mutated *HLA-B*51:01* cDNA using the FuGENE 6 Transfection Reagent (Roche).

2.8. CTL stimulation assay

COS transfectants (5×10^3) were cocultured with CTL clones (2×10^4) in individual wells of 96-well flat-bottom plates for 24 h at 37 °C, and IFN-γ production was measured in the supernatant using ELISA (Endogen).

2.9. Enzyme-linked immunospot (ELISPOT) assay

T lymphocytes were isolated from recipient's PBMCs by negative depletion using the Pan T Cell Isolation Kit II (Miltenyi Biotec) and used as responder T cells. Responder T cells at a concentration of 2×10^5 per well were plated in individual wells of the 96-well MultiScreen-IP filter plates (Millipore) coated with anti-human interferon (IFN)-γ antibody (5 μg/ml; Mabtech) and tested in triplicate against a total of 2×10^5 stimulator cells: patient B-LCL, donor B-LCL, and *HLA-B*51:01*-transfected donor B-LCL. The plates were incubated for 24 h at 37°C, washed, and incubated with biotinylated anti-human IFN-γ antibody (1 μg/ml; Mabtech) for 2 h at room temperature. After addition of streptavidin (Fitzgerald Industries International) to the wells, the plates were developed with a 3-amino-9-ethylcarbazol substrate kit (Vector Laboratories). Spots were counted using a microscope, and mean numbers were calculated from triplicate wells after subtraction of the number of spots obtained with medium alone.

3. Results

3.1. Selective down-regulation of HLA-B locus in post-transplant leukemia blasts

To determine whether expressions of some HLA loci in post-transplant relapsed leukemia blasts were down-regulated or lost, flow cytometric analysis was performed for HLA-A*24:02, A*11:01, B*51:01, and DR*09:01 using anti-HLA-A24/A23, -HLA-A11/A1/A26, -HLA-B51/B52/B49/B56, and -pan HLA-DR antibodies, respectively. The expression of B*51:01 was down-regulated in post-transplant leukemia blasts compared with that in pre-transplant blasts, whereas expressions of A*24:02, A*11:01, and DR*09:01 were the same or higher in post-transplant blasts than in pre-transplant blasts (Fig. 1). These data led us to question whether B*51:01-selective pressure mediated by donor T lymphocytes was present in the patient post-transplant.

3.2. Isolation of alloreactive CTL clones

Ten CTL clones, termed TK1 to TK10, were isolated from the peripheral blood of the recipient during acute GVHD. In a cytotoxicity assay, all isolated clones lysed recipient B-LCL but failed to lyse donor B-LCL (Fig. 2), demonstrating that all clones were alloreactive. Flow cytometric analysis revealed that all CTL clones

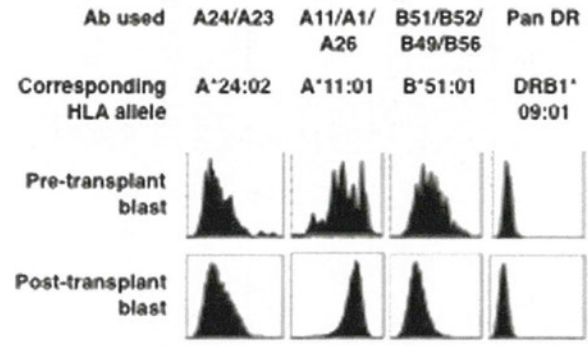

Fig. 1. HLA expression on leukemia blasts. Pre-transplant and post-transplant leukemia blasts were stained with anti-HLA-A24/A23, anti-HLA-A11/A1/A26, anti-HLA-B51/B52/B49/B56, and anti-HLA-pan DR antibodies to detect A*24:02, A*11:01, B*51:01, and DRB1*09:01, respectively. Data are representative of four experiments.

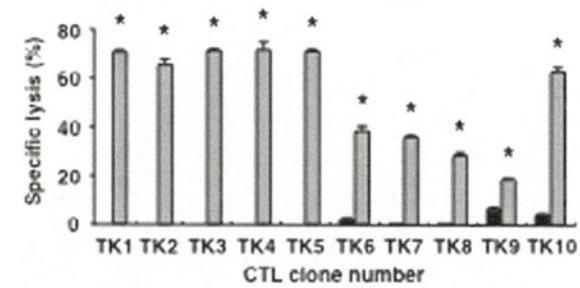

Fig. 2. Cytotoxicities of CTL clones against B-LCLs. B-LCLs that originated from the recipient (gray) and the donor (black) were used as targets for CTL clones. Specific lysis is shown as the mean and SD of triplicate cultures at an E:T ratio of 10:1. *Significant difference ($p < 0.0001$: Student's *t*-test) in the lysis of recipient B-LCL compared with donor B-LCL. Data are representative of three experiments.

Table 2
Clonotypes of isolated CTL clones.

CTL	TCR Vβ	Nucleotide and deduced amino acid sequences of complementarity determining region 3																		
TK1	Vβ6.5	GCC	AGC	AGT	CCC	GGG	ACT	AGC	GGA	ACC	TAC	GAG	CAG	TAC	TTC					
		A	S	S	P	G	T	S	G	T	Y	E	Q	Y	F					
TK2	Vβ20	AGT	CAG	GGG	CCG	GCG	GTT	ACC	GGG	GAG	CTG	TTT	TTT							
		S	Q	G	P	A	V	T	G	E	L	F	F							
TK3	Vβ20	AGT	CAG	GGG	CCG	GCG	GTT	ACC	GGG	GAG	CTG	TTT	TTT							
		S	Q	G	P	A	V	T	G	E	L	F	F							
TK4	Vβ19*1	GCC	AGT	ACT	TGG	GGT	TAC	CCA	CAG	GGG	CCC	GGT	GCG	GAT	ACC	GGG	GAG	CTG	TTT	TTT
		A	S	T	W	G	Y	P	Q	G	P	G	A	D	T	G	E	L	F	F
TK5	Vβ19*1	GCC	AGT	ACT	TGG	GGT	TAC	CCA	CAG	GGG	CCC	GGT	GCG	GAT	ACC	GGG	GAG	CTG	TTT	TTT
		A	S	T	W	G	Y	P	Q	G	P	G	A	D	T	G	E	L	F	F
TK6	Vβ12	GCC	AGC	AGT	TTA	GCT	AGC	GGG	AGG	GCC	TCC	CAT	GAG	CAG	TTC	TTC				
		A	S	S	L	A	S	G	R	A	S	H	E	Q	F	F				
TK7	Vβ12	GCC	AGC	AGT	TTA	GCT	AGC	GGG	AGG	GCC	TCC	CAT	GAG	CAG	TTC	TTC				
		A	S	S	L	A	S	G	R	A	S	H	E	Q	F	F				
TK8	ND																			
TK9	Vβ12	GCC	AGC	AGT	TTA	GCT	AGC	GGG	AGG	GCC	TCC	CAT	GAG	CAG	TTC	TTC				
		A	S	S	L	A	S	G	R	A	S	H	E	Q	F	F				
TK10	Vβ2	GCC	AGC	AGT	GAC	TCT	ATC	GCG	GAT	GAG	CAG	TTC	TTC							
		A	S	S	D	S	I	A	D	E	Q	F	F							

ND, not detected.

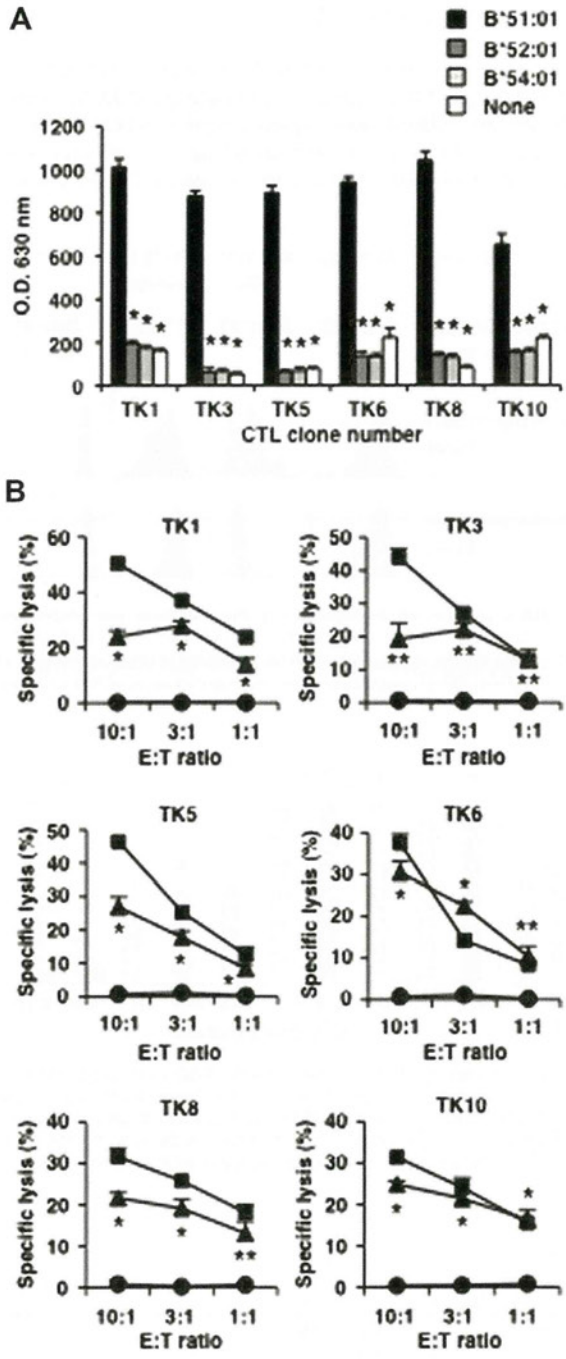

Fig. 3. Recognition of the HLA-B*51:01 molecule by CTLs. (A) COS cells were transfected with a plasmid encoding *B*51:01* cDNA, *B*52:01* cDNA, *B*54:01* cDNA, or no cDNA cocultured with CTL clones, and IFN-γ production was measured in the supernatant using ELISA. Data are the means and SD of triplicate determinations. *Significant difference ($p < 0.01$: Student's *t*-test) in the IFN-γ production stimulated by *B*52:01* cDNA, *B*54:01* cDNA or no cDNA compared with *B*51:01* cDNA. Data are representative of three experiments. (B) Recipient B-LCL (square), donor B-LCL (circle), and donor B-LCL transfected with *HLA-B*51:01* cDNA (triangle) were used as targets for CTL clones. Specific lysis is shown as the mean and SD of triplicate cultures at various E:T ratios. Significant difference (*$p < 0.01$; **$p < 0.05$) in the lysis of *B*51:01*-transfected donor B-LCL compared with donor B-LCL (negative control). Data are representative of three experiments.

were CD3+/CD4−/CD8+ (data not shown). The nucleotide sequences of the uniquely rearranged *TCR Vβ* gene of each clone were determined by direct DNA sequencing of the amplified PCR products of TCR (Table 2). The TK2 and TK3 clones had the same nucleotide sequences in the CDR3 regions of their TCR Vβ20, suggesting that these CTLs originated from a single clone. Similarly, TK4 and TK5, as well as TK6, TK7, and TK9, had the same nucleotide sequences in the CDR3 regions of their TCR Vβ19*1 and Vβ12, respectively, suggesting that each group also originated from a single clone. Thus, the 10 isolated alloreactive CTL clones appeared to have been derived from six independent clones.

3.3. CTL clones recognized the HLA-B*51:01 molecule

To evaluate the possibility that isolated CTL clones recognize the HLA-B*51:01 molecule, COS cells were first transfected with an *HLA-B*51:01*, *–B*52:01*, or *–B*54:01* cDNA construct, COS transfectants were cocultured with six independent CTL clones, and then the production of IFN-γ in the supernatant was measured. The COS cells transfected with *HLA-B*51:01* clearly stimulated IFN-γ production by six independent CTL clones, whereas neither *B*52:01* nor *B*54:01* stimulated them (Fig. 3A). Then, donor B-LCL were transfected with an *HLA-B*51:01* cDNA construct and used as target cells in a cytotoxicity assay. The donor B-LCL transfected with *HLA-B*51:01* were lysed by six CTL clones (Fig. 3B), indicating that all clones recognized the mismatched HLA-B*51:01 molecule as an alloantigen. On the other hand, these data suggest that the CTL response toward the HLA-B*51:01 molecule accounted for the majority of the recipient's CTL alloresponse during acute GVHD.

3.4. Recognition of HLA molecules by CTL clones was peptide-dependent

Various forms of T lymphocyte recognition of the allogeneic major histocompatibility antigen, ranging from peptide-dependent to peptide-independent, have been demonstrated [20]. To confirm peptide dependency in CTL recognition, examinations were focused on the difference in the amino acid sequences of the recipient B*51:01 and the donor B*52:01. They differed in two amino acids at positions 63 and 67 (Fig. 4A), which constitute peptide binding pockets A and/or B [21,22]. In particular, B-pocket has a critical role in peptide binding to HLA-B*51:01 molecules [23], and substitution of a single amino acid constituting peptide binding pocket can affect peptide binding [24]. Two mutated *B*51:01* cDNA constructs, *B*51:01-Asn63Glu* and *B*51:01-Phe67Ser*, in which individual amino acids were substituted with the corresponding amino acid in B*52:01 (Fig. 4A), were generated, as well as two more mutated *B*51:01* cDNA constructs, *B*51:01-Val194Ile* and *B*51:01-Ala199Val*, in which individual amino acids exist in *B*44:02* and other B alleles and localize outside the positions constituting peptide binding pockets. COS cells were then transfected with each wild or mutated cDNA construct and examined in the CTL stimulation assay. IFN-γ production of the TK3 clone was significantly decreased when stimulated by the *B*51:01-Phe67Ser* mutant in comparison with the wild-type *B*51:01* construct (Fig. 4B). IFN-γ production of all other CTL clones, TK1, TK5, TK6, TK8, and TK10, was significantly decreased when stimulated by *B*51:01-Asn63Glu* and *B*51:01-Phe67Ser* mutants in comparison with the wild-type *B*51:01* construct (Fig. 4B). However, both *B*51:01-Val194Ile* and *B*51:01-Ala199Val* mutants stimulated all CTL clones to the same degree as the wild-type *B*51:01* construct. Thus, these data suggest that recognition of the HLA-B*51:01 molecule by CTL clones was peptide-dependent.

Furthermore, CTL clones should recognize certain peptides other than leukemia antigens, presented by HLA-B*51:01 molecules, because *B*51:01*-transfected COS cells, which are derived from monkey kidney cells, stimulated IFN-γ production of CTLs

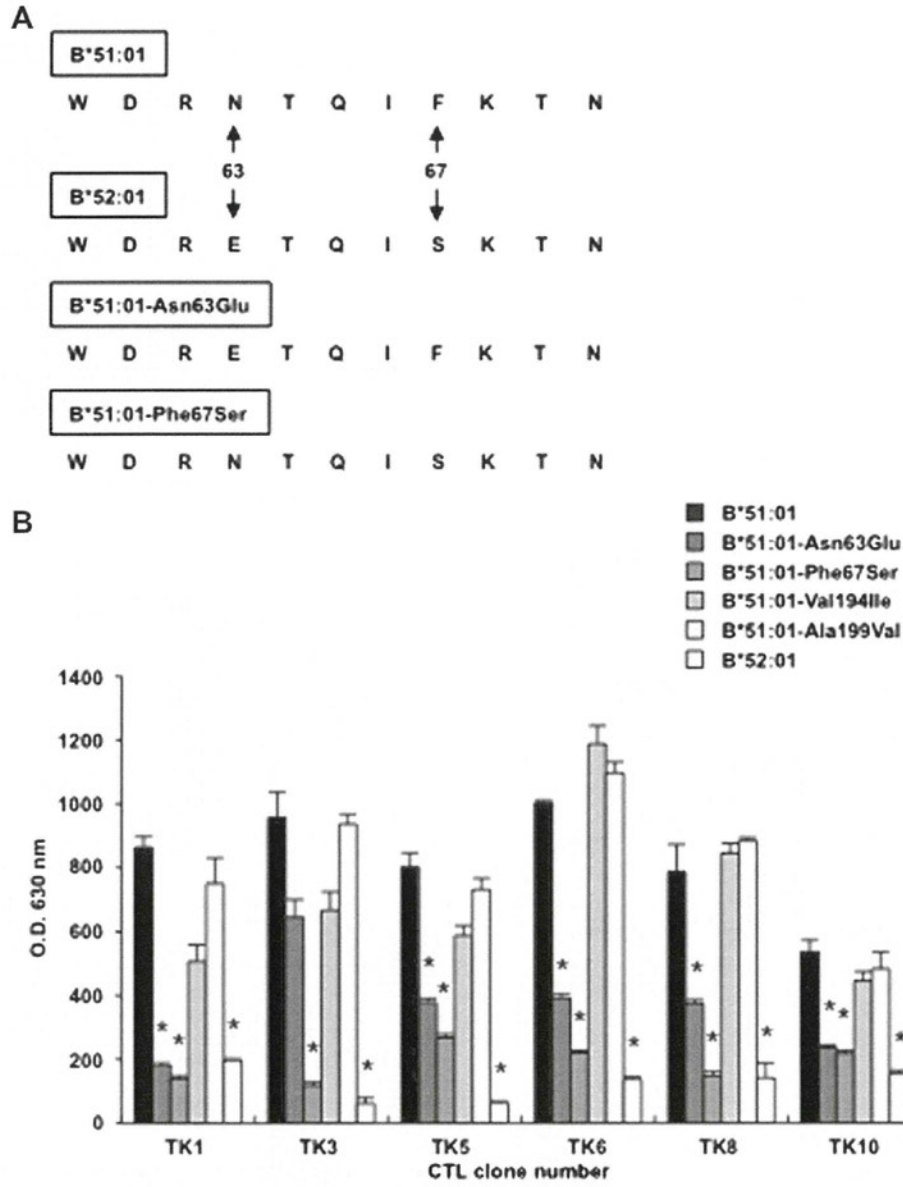

Fig. 4. Recognition of the HLA-B*51:01 molecule by CTLs is peptide-dependent. (A) The amino acid sequences at position 60 to 70 of the *B*51:01*, *B*52:01*, *B*51:01-Asn63Glu*, and *B*51:01-Phe67Ser* cDNAs are shown. Asn at position 63 was substituted with the corresponding amino acid in B*52:01, Glu, in the *B*51:01-Asn63Glu* mutant. Phe at position 67 was substituted with the corresponding amino acid in B*52:01, Ser, in the *B*51:01-Phe67Ser* mutant. (B) COS cells were transfected with a plasmid encoding *B*51:01*, *B*51:01-Asn63Glu*, *B*51:01-Phe67Ser*, *B*51:01-Val194Ile*, *B*51:01-Ala199Val* or *B*52:01* cDNA construct, cocultured with CTL clones, and IFN-γ production was measured in the supernatant using ELISA. Data are the means and SD of triplicate determinations. *Significant difference ($p < 0.05$: Student's *t*-test) in the IFN-γ production stimulated by each mutant or *B*52:01* cDNA construct compared with the wild-type *B*51:01* cDNA construct. Data are representative of three experiments.

(Fig. 3A), and *B*51:01*-transfected donor B-LCL, which are derived from B lymphocytes, were lysed by CTLs (Fig. 3B).

3.5. Leukemia blasts escaped from immunological pressure by HLA-B-specific CTLs

Whether the leukemia blasts escaped from the cytotoxicity of HLA-B*51:01-specific CTL clones was then examined. Pre-transplant and post-transplant leukemia blasts were purified by fluorescence-activated cell sorter (purity, ~62% and ~99%, respectively), and a cytotoxicity assay was performed only for the TK1 CTL clone because of the limited number of cryopreserved blasts. Weak but clear lysis of pre-transplant leukemia blasts by the TK1 CTL clone was observed, whereas post-transplant leukemia blasts were not lysed (Fig. 5A). All other CTL clones (TK3, TK5, TK6, TK8, and TK10) also did not lyse post-transplant leukemia blasts (Fig. 5B).

In addition, whether HLA-B*51:01-specific CTL pressure persisted until leukemia relapse was examined. The IFN-γ ELISPOT assay was performed to detect HLA-B*51:01-reactive CTLs in patient blood on day 232, 1 month before clinical leukemia relapse (Fig. 6). IFN-γ-producing B*51:01-reactive T lymphocytes were detected at a level nearly equal to the level of recipient B-LCL-reactive CTLs, that is, the total CTL alloresponse.

4. Discussion

The mechanism of leukemia relapse in this recipient can be explained as follows. CTLs specific for HLA-B*51:01 molecule/

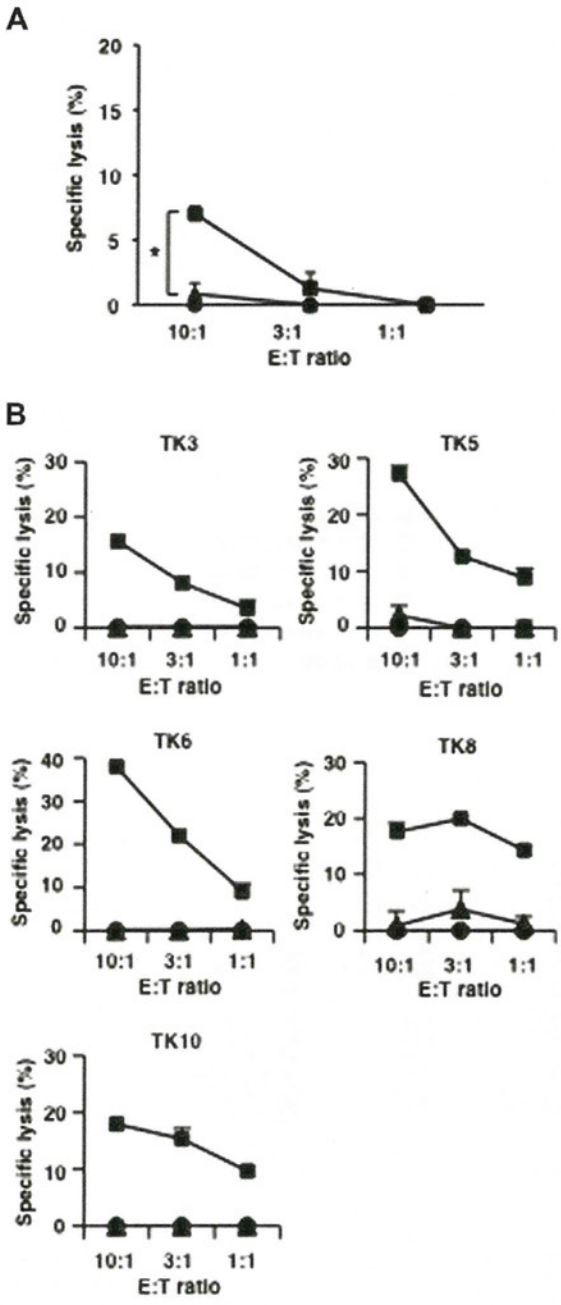

Fig. 5. Cytotoxicities of CTLs against leukemia blasts. (A) Purified pre-transplant leukemia blasts (purity, ~62%) (square), purified post-transplant leukemia blasts (purity, ~99%) (triangle) and donor B-LCL (circle) were used as targets for TK1 CTL clones. Specific lysis is shown as the mean and SD of triplicate cultures at various E:T ratios. *Significant difference ($p = 0.024$: Student's t-test) in the lysis of the pre-transplant leukemia blasts compared with the post-transplant leukemia blasts. Data are representative of three experiments. (B) Purified post-transplant leukemia blasts (purity, ~99%) (triangle), B-LCLs from the patient (square) and donor (circle) were used as targets for CTL clones. Specific lysis is shown as the mean and SD of triplicate cultures at various E:T ratios. Data are representative of three experiments. There was no significant difference in the lysis of the post-transplant leukemia blasts compared with B51-negative donor B-LCL (negative control).

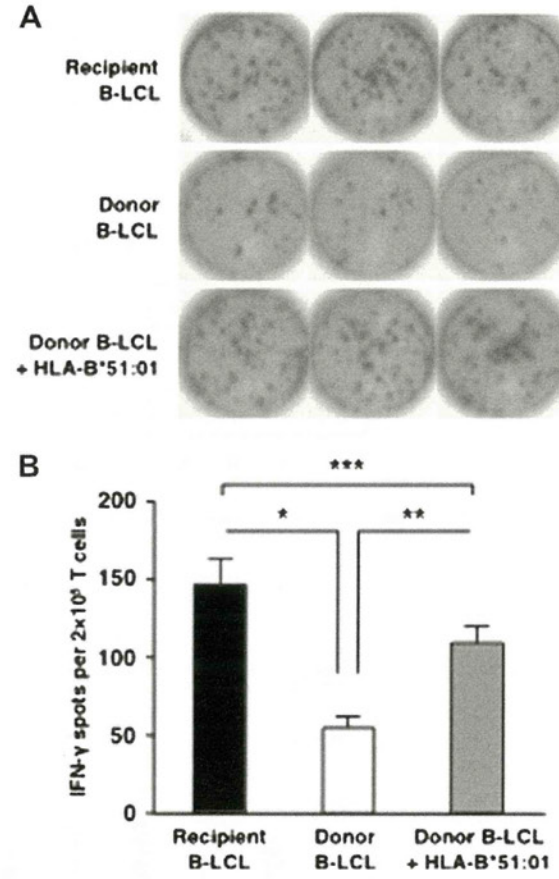

Fig. 6. Detection of HLA-B*51:01-specific CTLs in T lymphocytes obtained from the recipient on day 232 after transplantation. (A) Representative ELISPOT wells show triplicate results of T lymphocytes stimulated by recipient B-LCL, donor B-LCL, and *HLA-B*51:01*-transfected donor B-LCL. Data are representative of three experiments. (B) The frequency of CTLs in T lymphocytes recognizing the HLA-B*51:01 molecule was measured by IFN-γ ELISPOT analysis. The frequency of IFN-γ-producing cells is shown against recipient B-LCL (black), donor B-LCL (white), and *HLA-B*51:01*-transfected donor B-LCL (gray). Data are the means and SD of triplicate determinations. *$p = 0.0057$; **$p = 0.0077$; ***$p = 0.090$ (Student's t-test). Data are representative of three experiments.

non-leukemia peptide complex were generated in the recipient blood during acute GVHD, and these CTLs continued to produce immunological pressure on leukemia blasts for at least 8 months after transplantation, but B*51:01-down-regulated leukemia blasts escaped from the pressure of B*51:01-specific CTLs, and then the leukemia clinically relapsed.

CTLs recognizing mismatched HLA molecules play an important role in the immune reaction after HLA-mismatched HSCT, including graft rejection [25–27], GVHD [28], and the GVL effect [11]. In this study, the mismatched HLA-B*51:01-specific CTLs could participate both in GVHD and the GVL effect in the recipient. Ten CTL clones were isolated from the recipient's blood just after the onset of grade III acute GVHD involving skin, gut, and liver, and all clones demonstrated HLA-B*51:01-specific cytotoxicity in a non-leukemia peptide-dependent manner (Fig. 3 and 4). The patient was suffering from GVHD until his death on day 279, and in the ELISPOT assay for T lymphocytes obtained from recipient blood on day 232, HLA-B*51:01-reactive T lymphocytes accounted for the majority of alloreactive T lymphocytes (Fig. 6). Meanwhile, weak but clear lysis of pre-transplant leukemia blasts by an HLA-B*51:01-specific CTL clone was confirmed (Fig. 5A), and the primary refractory T lymphoblastic leukemia/lymphoma was in remission until day 261. These data are consistent with participation of the recipient HLA-B locus-specific CTLs both in GVHD and the GVL effect.

Selective HLA down-regulation was seen in this patient's post-transplant leukemia blasts. Mechanisms that alter HLA class I expression have been investigated and summarized as follows [29]: (1) loss of heterozygosity in chromosome 6 and/or 15, in