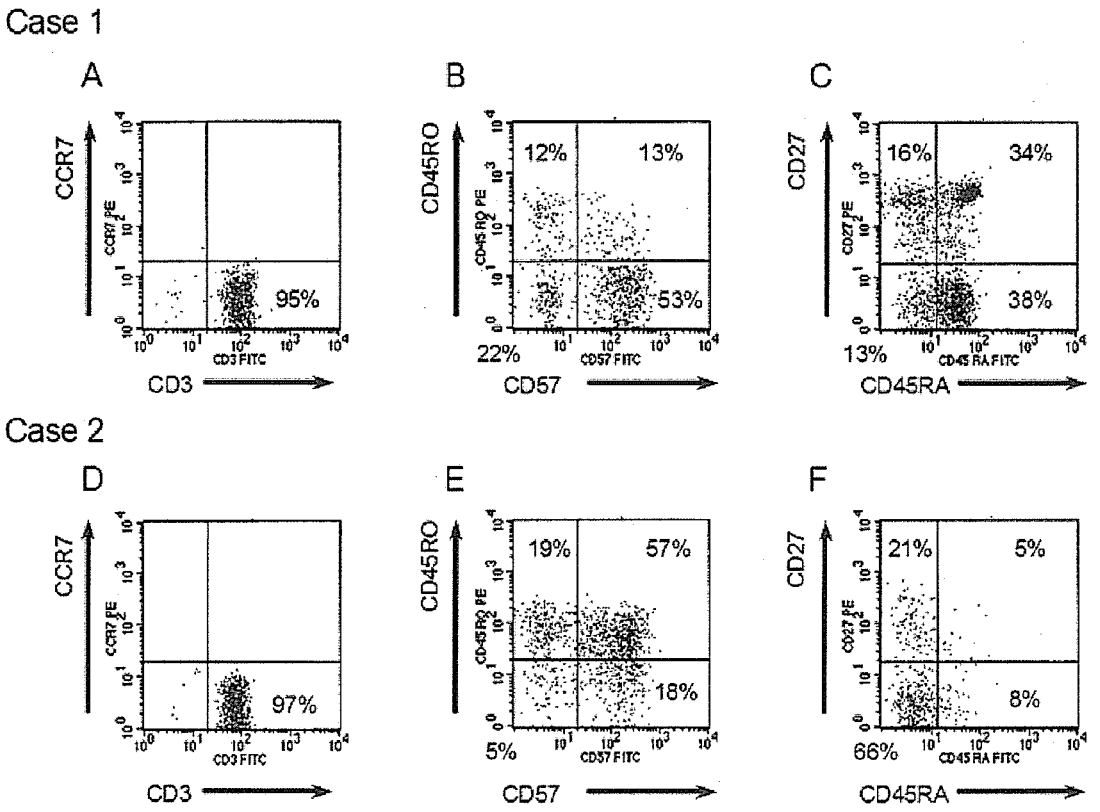

FIGURE 5 – Immunophenotype analysis of WT1-specific CTL. The immunophenotype of WT1-CTL in the first case of RCC was $CCR7^-$ and mainly $CD57^+/CD45RO^-/CDRA^+$ (*a–c*). The immunophenotype of WT1-CTL in the second case was $CCR7^-$ and $CD57^+/CD45RO^+$ and $CDRA^-/CD27^-$ (*d–f*).

notypes, including $CCR7^-$, $CD45RA^-$ and $CD45RO^+$, were shown to respond to antigen-specific peptide, while those with $CCR7^+$, $CD45RA^+$ and $CD45RO^-$ phenotypes were associated with a lack of response to antigen-specific peptide.[36] Hence, based on the phenotype analysis in correlation with the clinical course, WT1-CTL seemed to have played a major role in disease control in the second patient, while an antitumor cell other than WT1-CTL may have had an effect in the first patient, since the disease progression in this patient was slow even when the WT1-CTL disappeared. NK cells may be the predominant antitumor cells, since this patient had a high proportion of NK cells in the peripheral blood at day 296 (7.0%/lymphocyte), which had been only 0.72% on day 149. Further analysis of antigen-specific CTL is critically required to elucidate the precise relationship between the phenotype and cell function.

We have previously demonstrated that CMV epitope NLVPMVATV is presented in both HLA-A*0201 and HLA-A*0206.[37] It is quite likely that WT1 epitope RMFPNAPYL is also commonly presented in HLA-A02 phenotypes, since WT1-CTL was detected not only in HLA-A*0201 patients but also in those with HLA-A*0206. Monitoring of WT1-CTL by tetramer assay can be widely applied to the HLA-A02 phenotype, since over 95% of HLA-A02 are either A*0201 or A*0206.[38,39]

Although several studies on PR1-CTL detection in patients with leukemia have been reported,[19,25,27] PR1- and PRAME-specific CTL were not detected in our study. We considered that one time positivity of the tetramer assay is not sufficient, since there may be an interassay variability. Since neither PR1-CTL nor PRAME-CTL was detected even after cell culture, in which the expansion of WT1-CTL and CMV-CTL was successful, we speculate that PR1-CTL and PRAME-CTL were not induced in most of the patients after HSCT.

In conclusion, our results suggest that WT1-CTL is involved in a GVT effect and WT1 is currently the best antigen for immunomonitoring after HSCT, while PR1 and PRAME are less potent antigens to be used for wider application. Although WT1-CTL may occur after HSCT *per se* without additional immunotherapy, it would be ideal to induce GVT effect with minimal GVHD. Further development of a WT1-based immunotherapy is desired to induce optimal antitumor immune response.

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CASE REPORT

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Intensive multimodality therapy including paclitaxel and reduced-intensity allogeneic hematopoietic stem cell transplantation in the treatment of adrenal cancer with multiple metastases

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Abstract Adrenocortical carcinoma is a rare malignancy in adolescents and young adults. The prognosis of unresectable/metastatic adrenocortical carcinoma remains very poor because the rarity of the tumor has made it difficult to establish treatment guidelines, and diagnosis and the resultant treatment can be greatly delayed. We treated a 24-year-old woman who was diagnosed with adrenocortical carcinoma of the right adrenal gland which extended to the inferior vena cava. Although she underwent surgical resection of the extensive tumor as the primary treatment, the disease recurred in the lung and liver as multiple metastases shortly after surgery. She received intensive multimodality therapy, including chemotherapy with paclitaxel, ifosfamide, and cisplatin (TIP regimen), embolization of the feeding arteries, and proton irradiation for the liver mass. Finally, she underwent reduced-intensity allogeneic hematopoietic stem cell transplantation from an HLA 1-locus-mismatched sibling donor. A prolonged survival of 39 months after the onset of the disease was achieved. Although this experience is limited, we suggest that TIP chemotherapy was effective for adrenocortical carcinoma, and a graft-versus-tumor effect after reduced-intensity stem cell transplantation may have contributed to the prolonged survival.

Key words Allogeneic stem cell transplantation (allo-SCT) · Reduced-intensity stem cell transplantation (RIST) · Adrenocortical carcinoma (ACC) · Graft-versus-tumor (GVT) reaction · Graft-versus-host disease (GVHD)

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Introduction

Adrenocortical carcinoma (ACC) is a rare neoplasm with an incidence of approximately 0.5–2.0 per million per year.[1] The sex ratio is 2.5, with greater female involvement. Patients typically present with endocrine symptoms caused by the excessive production of hormones by the tumor, and abdominal symptoms including fullness, tenderness, nausea, and vomiting. It has been reported that tumor reduction contributes to long-term survival, and whenever possible radical surgery is recommended for all patients, including those with recurrent disease. More than three-forth of these patients have a functioning tumor independent of their clinical manifestations, and this suggests that early resection of the tumor could lead to a better chance of survival. However, in those without these early clinical manifestations, complete surgical resection becomes difficult or impossible since the diagnosis is delayed. The reported median survival time was 14.5 months, with a significantly lower survival in those aged 40 years or more, or those who had distant metastasis at the time of diagnosis.[2] Although it has been reported that chemotherapy with mitotane (o,p′-DDD, 1,1-dichlorodiphenyl-dichloroethane) could contribute to longer survival in an adjuvant setting,[2,3] the optimal therapeutic approach for those with systemic disease has not yet been established. Hence, an accumulation of experience is urgently required to identify an effective multidisciplinary strategy.

We describe here a patient with advanced ACC who was treated very intensively with radical resection of the primary tumor, followed by a combination of chemotherapy consisting of paclitaxel, ifosfamide, and cisplatin (TIP) for lung metastases, a combination of arterial embolization and proton irradiation therapy for liver metastases, and allogeneic stem cell transplantation (SCT) with a reduced-intensity regimen (RIST).

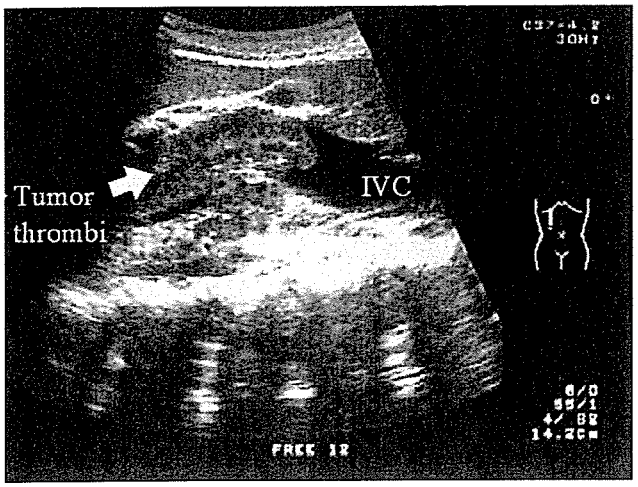

Fig 1. Abdominal echography at diagnosis showing a large right-side adrenal tumor with intravascular extension and massive liver metastasis. *IVC*, inferior vena cava

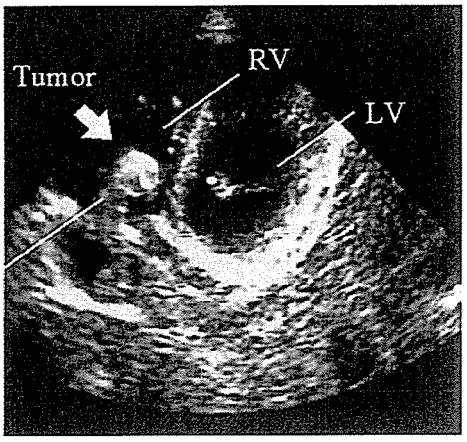

Fig 2. Cardiac echography showing a polypoid lesion in the right ventricle through the tricuspid valve during the diastolic phase. *RV*, right ventricular; *LV*, left ventricular

Case report

A 24-year-old woman developed general fatigue and amenorrhea in January 2000, and a giant tumor in the right adrenal gland was disclosed in October 2000 by evaluation, including ultrasound echography and computed tomography (CT). The liver mass extended into the inferior vena cava (IVC) and caused thrombi and mass lesion in the right atrium (Fig. 1). Cardiac echography revealed direct invasion of the cardiac tricuspid valve by the tumor that originated from the abdomen (Fig. 2). A systemic survey using CT scanning disclosed no other distant metastatic lesion. The patient underwent complete surgical resection of the tumor under support with an extracorporeal circulation device. A pathological diagnosis of adrenocortical carcinoma was established, and the production of androgen was confirmed by an elevated serum level of androgen.

Four months after surgery, the patient had tumor recurrence with multiple liver and lung metastases detected by a follow-up CT examination. Because the lesions grew rapidly, in April 2001 she began to receive chemotherapy which consisted of paclitaxel (TXL, 175 mg/m^2, day 1), ifosphamide (IFM, 1200 mg/m^2, days 2–6), and cisplatin (CDDP, 20 mg/m^2, days 2–6), i.e., the "TIP regimen," every 3–4 weeks. After 3 courses of TIP chemotherapy, lung lesions showed complete remission (CR), while liver metastases remained stable. For liver lesions, the patient underwent a transarterial embolism (TAE) procedure using a mixture of Lipiodol and falmorubicin following transarterial infusion (TAI) of cisplatin (CDDP) and mitomycin C (MMC). A total of 3 TAE/TAI procedures were performed up to August 2001, when all therapies were suspended owing to the development of severe myelosuppression. In November 2001, when the liver mass started to increase in size, 60 Gy proton-beam irradiation was administered concurrently with each course of TIP chemotherapy and TAE.

In April 2002, the patient decided to participate in a phase I trial of RIST for refractory solid tumors, which was approved by our institutional review board (IRB), in the expectation of a powerful graft-versus-tumor (GVT) effect. Prior to the patient's registration on the trial, the medical team held a thorough discussion with the patient and her family regarding possible treatment options such as mitotane. Because of the lack of evidence of curability with the mitotane therapy, the patient did not decide to receive mitotane. She therefore chose the option to receive RIST from an HLA 1-locus-mismatched brother under sufficient informed consent (recipient's HLA typing: A33, A24, B60, B52, DR12, and DR2; donor's HLA typing: A33, A11, B60, B52, DR12, and DR2). The RIST regimen consisted of fludarabine (30 mg/m^2 for 6 days), busulfan (4 mg/kg/day for 2 days) and antithymocyte globulin (25 mg/m^2 for 2 days). At the time of RIST, her disease was CR in the lung, while the liver lesion was not evaluable because of the earlier intensive local treatments. A combination of cyclosporine (3 mg/kg) and methotrexate (10 mg/m^2 on day 1, and 7 mg/m^2 on days 3 and 6) was administered for graft-versus-host disease (GVHD) prophylaxis. The early course after RIST was uneventful except for transient neutropenic fever for 4 days, which was successfully treated with antimicrobial therapy. Hematopoietic engraftment was observed on day 13. On day 33 after RIST, the patient developed grade 1, stage II, skin GVHD, which resolved spontaneously in 3 weeks. The dose of cyclosporine was gradually tapered until it was discontinued on day 80, while the serum androgen level decreased remarkably between day 60 and day 150. The relationship between the occurrence of GVHD and tumor response is illustrated in Fig. 3. Although a CT scan examination on day 90 confirmed PD in the lung lesions, the tumors appeared to be slowly progressive. Thereafter, the patient maintained a relatively high performance status with indolent tumors until April 2003, when she suddenly experienced serious hematemesis. A upper gastrointestinal endoscopy revealed extensive mucosal damage disproportionally located in the lesser curvature, which ap-

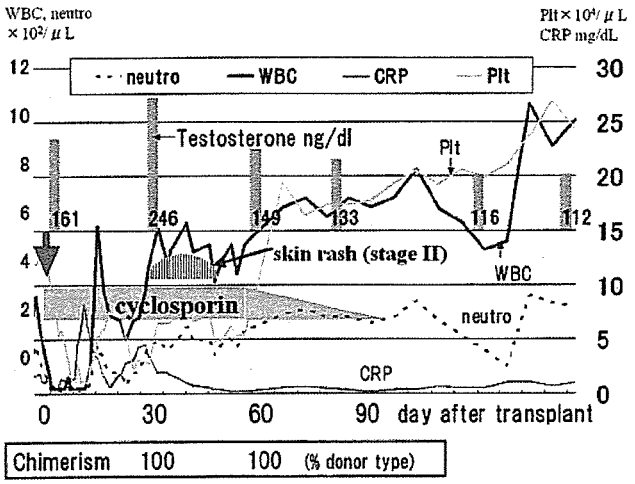

Fig 3. Clinical course of transplantation. The serum level of testosterone (normal range 10–60 ng/dl) as a tumor marker continued to decrease after the ocurrence of acute graft-versus-host disease. White blood cell count (*WBC*) and platelets (*plt*) slowly increased after hematopoietic engraftment, which was observed at day 13. Chimerism achieved 100% donor type at 30 days after SCT and continued to keep 100% donor type. *CRP*, C-reactive protein

pears secondary to proton irradiation. The patient experienced a silent perforation of the gastric wall resulting panperitonitis the next day, which led to her death 12 months after transplantation. Necropsy was not approved.

Discussion

The prognosis of localized ACC primarily depends on the histology[4] and tumor size,[5] and there have been reports on the value of radical tumor resection and the efficacy of mitotane therapy.[2] In contrast, no effective therapy has been reported for patients with distant metastases or unresectable tumor, and the reported median survival period is 8 months.[6] Here, we report the case of a patient who had an extended tumor and survived 39 months after the onset of the disease, which was much longer than the reported median value. In addition, this patient maintained a high performance status and qualified daily life for 9 months after progression until the day before her death, although the tumor recurred shortly after RIST.

We believe that three factors may have contributed to this patient's ability to achieve a durable asymptomatic period. First, the TIP regimen, which has been shown to be effective for germ cell tumors and other types of endocrine tumor as a salvage chemotherapy,[7] might also be effective for ACC. Considering the lack of adequate therapeutic options, we feel that even this limited single-patient experience could still be of value for future evaluations. Second, this patient underwent an experimental RIST procedure from a partially HLA-mismatched sibling as a final consolidation therapy, as part of clinical trials in patients with various types of solid tumor. Clinical studies that have been reported to date provide proof-of-principle that allogeneic T cells can induce clinically relevant GVT effects in solid tumors, including renal cell carcinoma (RCC)[8] and others.[9,10] It is widely accepted that the GVT effect is closely associated with GVHD. Hence, it is possible that the GVT effect may be enhanced by selecting a mismatched graft. Although this patient had no measurable lesions at the time of RIST, the decrease in the serum level of androgen between day 60 and day 150 when GVHD emerged and the indolent course of tumor progression after RIST might be considered evidence of a GVT effect. Third, this patient received intensive procedures for local tumor control, including extensive primary tumor resection, TAE/TAI, and proton-beam irradiation, which may have made the tumor more sensitive to a GVT effect, as previously suggested.[11]

In summary, successful allogeneic SCT requires a highly integrated program of donor selection, preparative regimen, and management of GVHD. Nevertheless, it is possible that the combination of TIP chemotherapy, intensive local tumor controls, and a GVT effect may have contributed to the prolonged survival of this patient.

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Coincidental Outbreak of Methicillin-Resistant *Staphylococcus aureus* in a Hematopoietic Stem Cell Transplantation Unit

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Background: Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most common nosocomial pathogens among hospital-acquired infections, and immunocompromised patients are highly susceptive to infection. The molecular typing of isolated strains is a common method for tracing an outbreak of MRSA, but experience with this approach is still limited in the hematopoietic stem cell transplantation (HSCT) ward.

Methods: We experienced 6 cases of MRSA infection/colonization in our 26-bed HSCT ward during a 4-week period. This unusual outbreak strongly suggested that the same MRSA strain was involved despite strict isolation and aseptic patient care. Clarification of the transmission pattern was critical, and we applied pulsed-field gel electrophoresis (PFGE) and amplified fragment length polymorphism (AFLP) assays for evaluation.

Results and conclusion: In four of the six cases, the pattern of bands examined by PFGE and AFLP analyses supported the idea that direct person-to-person transmission was very unlikely and the outbreak was coincidental. This experience highlights the clinical value of molecular typing methods for the clinical epidemiological assessment of MRSA outbreak.
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Key words: outbreak; MRSA; stem cell transplantation

INTRODUCTION

The rapid increase in the incidence of hospital-acquired infection by methicillin-resistant *Staphylococcus aureus* (MRSA) is making infection control procedures very critical, particularly for immunocompromised patients [1]. Hospital-acquired infections also serve as a hallmark of the effectiveness and quality of infection control maneuvers [2]. Outbreaks of infection caused by MRSA have time-consuming and expensive consequences, and genetic analysis is useful, since it can be used to determine the route and origin of MRSA infection [3]. Currently available laboratory methods for determining DNA fragment sizes or sequences in MRSA isolates include Southern blotting [4], ribotyping [5], polymerase chain reaction (PCR) [6], and pulsed-field gel electrophoresis (PFGE) [4,7]. PFGE has become the most common tool for the rapid discrimination of MRSA strains due to its convenience, reliability, and cost-effectiveness [8,9]. However, the interpretation of PFGE bands still needs to be standardized [10]. Alternatively, the amplified fragment length

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TABLE I. Clinical Characteristics of 6 Cases*

UNP	Age	Sex	Disease	Day after transplantation	Cause of admission	Admission to hospital	First admission ward	Admission to SCT unit
1	53	M	MDS	109	GVHD	2002/11/13	Ward A	11/15/2002
2	35	M	NHL	219	GVHD	2002/11/20	Ward B	11/27/2002
3	35	M	GCT	43	GVHD	2002/11/29	Ward C	12/5/2002
4	58	M	MDS	−13	SCT	2002/10/31	SCT unit	10/31/2002
5	54	F	NHL	−22	SCT	2002/11/5	SCT unit	11/5/2002
6	63	M	CML	210	GVHD	2002/11/5	SCT unit	11/5/2002

*Cases 4 to 6 were admitted before case 1 showed severe intestinal symptoms induced by gut GVHD, complicated with continuous gastrointestinal bleeding. In 4 of these 6 cases, hospitalization was due to GVHD after transplantation (3 acute and 1 chronic), and 3 of these patients, including case 1, received corticosteroid therapy for the treatment of GVHD.

Note: MDS, myelodysplastic syndrome; NHL, non-Hodgkin lymphoma; GCT, germ cell tumor; CML, chronic myelogeneous lymphoma; GVHD, graft versus host disease; PSL, prednisolone; SCT, stem cell transplantation.

polymorphism (AFLP) method is based on the selective amplification by PCR of a subset of restriction fragments from a digest of the whole bacterial genome [11,12]. AFLP has advantages over PFGE since it has more power for discriminating between different strains more quickly with higher specificity in the recognition of digestive fragments of whole bacterial genome [13].

The goal of these laboratory tests is to provide firm evidence that isolates, which are epidemiologically related during an outbreak of the infection, are also genetically related and thus represent the same strain. To enhance the reliability of such molecular laboratory results, the combined use of various genotyping methods appears to be effective [14,15]. An outbreak has been defined as infectious disease derived from the same pathogen, while an outbreak that originates from strains that are indistinguishable from each other by typing methods but for which no direct linkage can be demonstrated is called an "endemic outbreak" [16]. It has been suggested that in disease outbreak due to endemic strains, the common origin may be temporally distant from those in outbreak strains. From the perspective of infection control, this difference is critical, since different procedures are needed to prevent the spread of disease.

We experienced an outbreak of MRSA in our hematopoietic stem cell transplantation (SCT) ward that was initially suspected to be derived from a single origin. To address this serious problem, we tracked down the route of infection and obtained results that highlighted the clinical value of molecular typing using these methods.

PATIENTS AND METHODS

Patients

The routine infection-monitoring procedure in the SCT ward includes surveillance cultures and identification for specific pathogens in the nasal swab, pharyngeal swab or sputum, urine, or stool, which are collected from patients who are suspected to have infection or colonization of the target pathogen including MRSA at the time of admission. In a 4-week period, we experienced six cases (UPN 1 to 6) of MRSA infection or colonization in the SCT ward, while the preceding incidence of MRSA detection in the SCT ward had been only one or two cases per month (mean 0.8/month, range 0–2/month, SD 0.61). Therefore, this was epidemiologically defined as an MRSA outbreak. The patient characteristics are summarized in Table I. We reviewed the medical records of the patients to collect the clinical information required to track down the transmission route. We documented the time course of MRSA identification in relation to patient characteristics, risks of nosocomial infection, and room assignment.

Samples

Isolates were grown from culturing sputum, urine, stool, pus, and blood, and a few were grown from culturing miscellaneous sites such as pharynx and nasal cavity. We examined the first sample isolated in each patient by molecular typing, PFGE, and AFLP analysis.

DNA Isolation and PFGE

Targeted bacterial strains were cultured at 37°C in Luria-Bertani broth. The cell component was lysed by proteinase K to extract DNA. Genomic DNA was digested with *Sma*I and resolved with the CHEF-DRII system (Bio-Rad Laboratories) as described by the manufacturer (traditional typing strategies) [17]. As a control strain, we used MRSA isolated from two groups: (1) two strains isolated from past patients in the same ward, which has no temporal relationship to our present cases (cases 7 and 8) and (2) five strains isolated from a different hospital

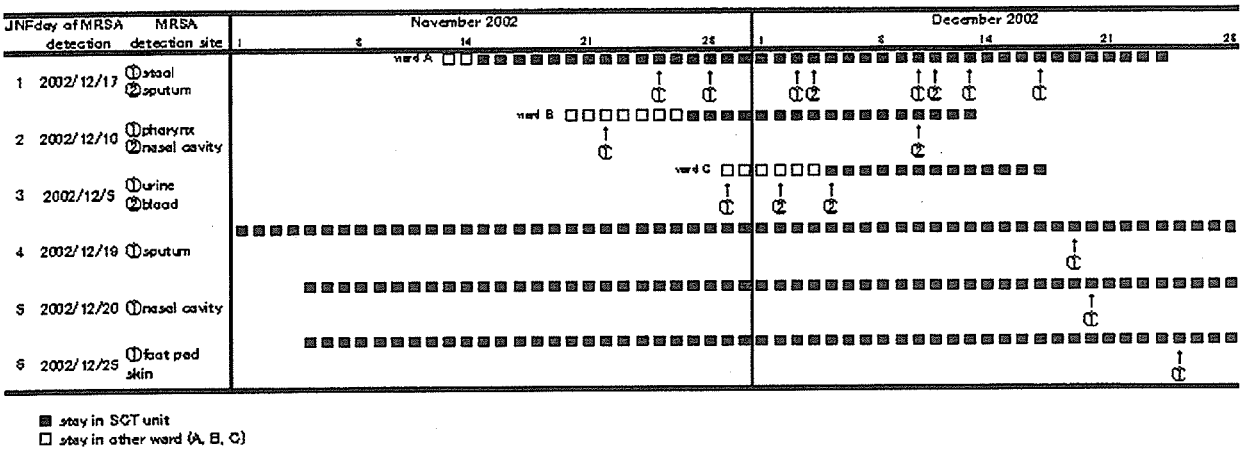

Fig. 1. Time course of MRSA detection in the SCT unit. MRSA was first isolated in the stool of case 1 on 25 November 2002. MRSA had been identified prior to admission to the SCT ward in cases 2 and 3. In contrast, in the other three cases (cases 4, 5, and 6), MRSA was detected after admission to the SCT ward.

(University of Tokyo Hospital, 1150 beds), which was not epidemiologically associated with our hospital (cases 9 to 13). PFGE banding was compared with that in case 1, who was thought to be the origin of this outbreak episode. The criteria described by Tenover et al. [16] were used for the molecular epidemiological interpretation of PFGE banding as follows:

(i) indistinguishable: outbreak was derived from the same isolate;
(ii) closely related: different isolates, closely related to the outbreak pattern;
(iii) possibly related: different isolates, possibly related to the outbreak pattern;
(iv) unrelated: different isolates, unrelated to the outbreak pattern.

One genetic event detected by PFGE was considered meaningful enough as different isolates.

AFLP

Bacterial DNA was prepared with a QIAamp DNA Mini kit (Qiagen) according to the manufacturer's recommendations. DNA was then manipulated with an AFLP Microbial Fingerprinting kit (Applied Biosystems) according to the manufacturer's instructions based on a previous study [11]. Briefly, DNA was digested with *Eco*RI and *Mse*I and then ligated to the corresponding adapters. This was followed by preselective amplification and selective amplification, where *Eco*RI-A (FAM), *Eco*RI-C (NED), *Eco*RI-G (JOE), and *Mse*I-C primers were used. The AFLP reactions were evaluated by analyzing data from samples loaded and run on an ABI 310 Genetic Analyzer with GeneScan software. A dendrogram was constructed from a pairwise distance matrix with the Clustal W version 1.8 software package.

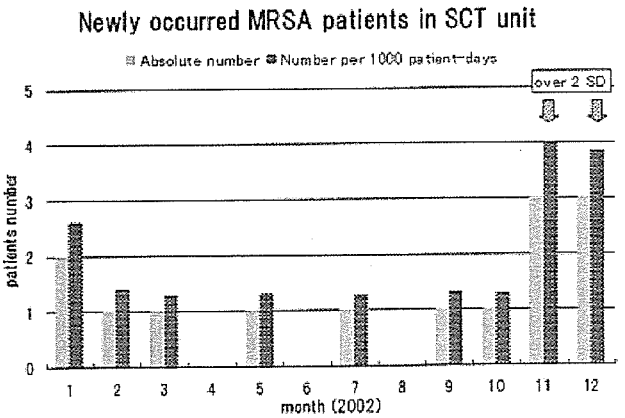

Fig. 2. The incidences of newly detected MRSA cases in SCT unit in 2002. Each bar indicates a number of patients clinically identified as MRSA in 2002.

Definitions [18]

"Methicillin-resistant" is defined according to NCCLS MIC criteria by dilution susceptibility tests. An "outbreak" of MRSA is defined as an increase in the rate of MRSA cases or a clustering of new cases in a specific place during a given period. In this report, we defined an unusual increase in MRSA cases as a multiply repeated isolation of MRSA from a physically independent ward (transplantation unit) with an incidence $\geq$ 2 SD over the baseline. The SCT unit is geographically separate from other wards and has an independent space that is managed to maintain sterilization. Patients from whom MRSA was isolated and who had any concomitant symptoms in the MRSA-detected part were referred to as "MRSA

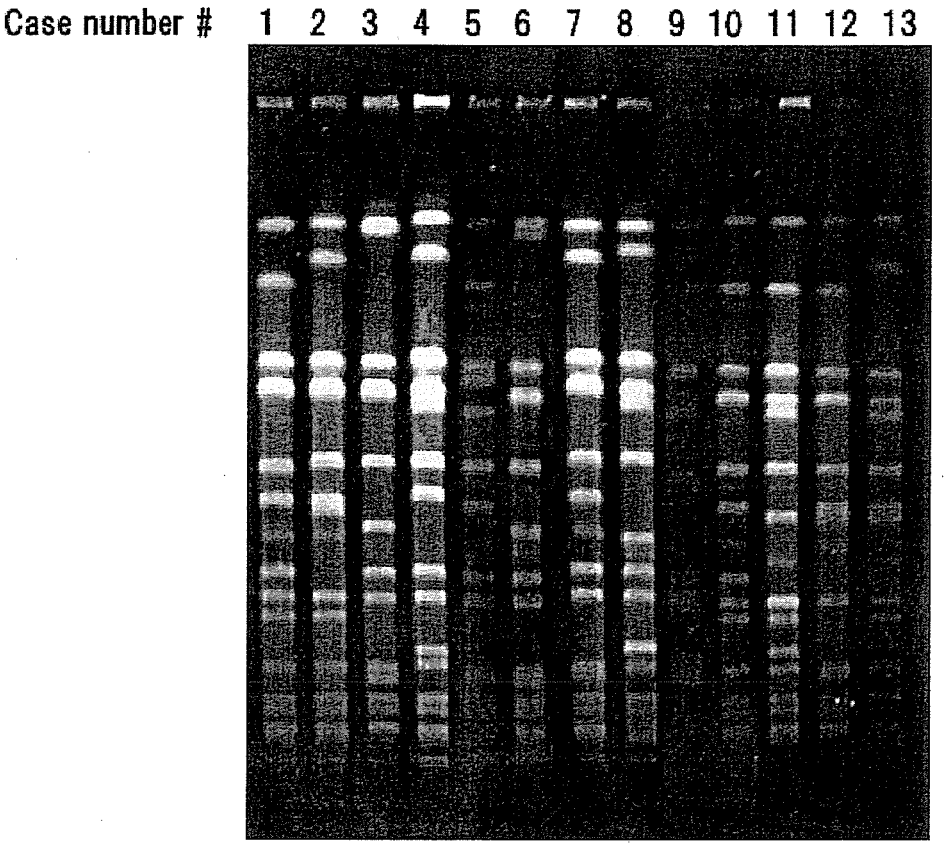

Fig. 3. PFGE analysis of isolated MRSA in the SCT unit. The PFGE pattern showed that there were no detectable differences in bands between cases 3 and 6, but more than two bands were identified in the other four cases (cases 1, 2, 4 and 5). Additionally, two strains that had been previously isolated in the SCT ward (cases 7 and 8) were distinguishable, and the five epidemiologically different isolates (cases 9 to 13) from the University of Tokyo Hospital (1,150 beds) were also distinguishable, with differences in more than two bands.

infection," while those without symptoms were considered "MRSA colonization."

RESULTS

Clinical Course of MRSA Outbreak

The clinical characteristics of six patients in whom MRSA was isolated are presented in Table I. The first patient (case 1) was admitted to the SCT ward because of severe intestinal symptoms induced by gut GVHD, chronic diarrhea, and continuous gastrointestinal bleeding, which occurred at 107 days after SCT. The patient received corticosteroid and intravenous antimicrobial therapy. For 4 weeks prior to his admission, there had been no case of MRSA infection or colonization in the ward. At 13 days after admission, the first isolation of MRSA in his stool was recorded (Table I). Subsequently, five other patients newly developed MRSA events over the next 4 weeks (Figure 1), while the incidence of MRSA detection of SCT ward had remained at one or two cases per month (mean 0.8 /month, range 0–2 /month, SD 0.61, Figure 2). Among these five cases, three (cases 4, 5, and 6) had been admitted to the SCT ward directly from the outpatient clinic without a past history of MRSA infection. The other two cases (cases 2 and 3) were transferred from other wards after the admission of case 1, and MRSA was isolated prior to transfer to the SCT ward (Figure 1). Since, in these two cases, MRSA was identified again in different site in SCT with different drug-sensitivity profile (data not shown) from a previous strain, we included these two cases in the analysis. There were no other patients who were previously identified with MRSA infection or colonization.

Tracing Procedure

The transmission, if any, appeared to take a random pattern, as illustrated in Figure 1. To better evaluate whether the transmission pattern was direct or indirect, we identified the layout of the patients' bed assignments. This revealed that there were nei-

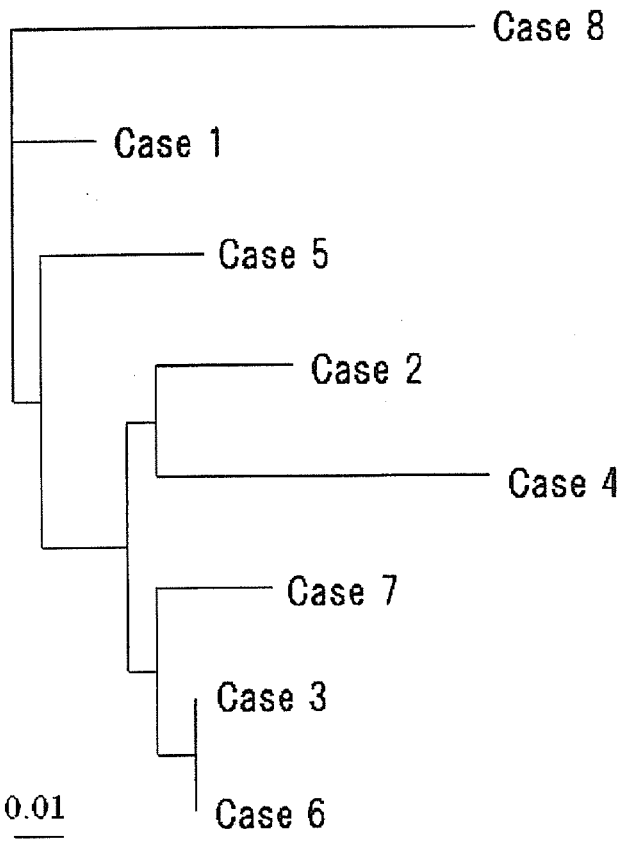

Fig. 4. AFLP pattern of isolated MRSA. AFLP analysis was performed for the same six isolates (case 1 to 6) described in Figure 4. Gene polymorphism showed same result of similarity as PFGE pattern had already indicated, i.e., cases 3 and 6 had the same polymorphism pattern and the others were different strains. The mutual relation of gene polymorphism is presented in the dendrogram and relatedness is indicated by the length of line. The scale bar drawn in the lower part indicated 1.0 % relatedness.

ther overlaps nor coexistence with preceding patients, except that cases 1 and 3 used the same room on different days without an overlap.

PFGE and AFLP Assays of MRSA Isolates

PFGE analysis of the six MRSA strains isolated (Figure 3) showed that two strains (cases 3 and 6) were indistinguishable and therefore considered to be derived from the same isolate, while the remaining four cases (cases 1, 2, 4, and 5) were considered to have different strains. Seven epidemiologically different isolates, i.e., two strains isolated from another ward at different times (cases 7 and 8) and five strains isolated from another hospital (cases 9 to 13) were used as in-hospital and extra-hospital controls, respectively. The results were confirmed by AFLP analyses as a dendrogram shown in Figure 4.

DISCUSSION

The spread of MRSA in highly protected care units, including ICU [19] and neonatal ICU [20], is a well-known major complication in compromised patients. Although few reports have been published on the outbreak of MRSA in a SCT unit, a continuous rise in the incidence of hospital-acquired MRSA infection [21] should influence the incidence of MRSA infection in SCT recipients [22]. Collin et al. reported that the incidence of multidrug resistant *S. aureus* was 15% in isolates from BMT patients with blood stream infection in 1991–1997 [23]. Prolonged neutropenia has been found to be a risk factor for the development of infectious complications in SCT recipients [24]. Since the outbreak of MRSA among immunocompromised patients can greatly affect their mortality, appropriate methods for infection control are strongly warranted. The Consensus Panel's guidelines for preventing the spread of MRSA recommend contact precautions and the isolation of infected or colonized patients in a single room or cohort, i.e., grouping them geographically with designated staff [18]. Also, since MRSA colonization precedes infection because of inpatient circumstances and rather strong treatments [25,26], a local control is very important for controlling MRSA outbreak in selected circumstances such as SCT ward in which many immunocompromised patients are taken care of.

In this report, we described an MRSA outbreak in the SCT ward during a limited period of 4 weeks. Initially, we suspected that all MRSA infections were caused by a single source, such as highly contaminated stool. However, unexpectedly, no direct contact was identified among patients and staff who were involved in their care. The transmission of MRSA mostly occurs through direct person-to-person contact, and transmission from the environment is extremely rare in places where strict precautions are taken and careful decontamination procedures are used. Hence, we undertook a molecular epidemiological analysis to critically examine the suspected break in our procedure. We found that four of the six isolates were genetically different, and our Infection Control Team concluded that horizontal transmission was unlikely. Nevertheless, the interest raised with this event resulted in further enforcement of essential precautions against droplets and contact,and the elimination of new MRSA cases for subsequent months.

Although our observation was well anticipated, in that molecular typing techniques are effective in the diagnosis and tracking of MRSA, the results are still unique, since they highlight the value of these methods over clinical judgment in a critical care situation

with highly immunocompromised patients. Since the molecular typing properties of MRSA are very similar in Japan, especially in the local areas [27], we focused on the genetic event detected by molecular typing and diagnosed those differences as different strain from outbreak. Thus, this report should be helpful for evaluating whether the routine application of these measures should be critically considered in the assessment of outbreak.

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Modulation of acute graft-versus-host disease and chimerism after adoptive transfer of *in vitro*-expanded invariant Vα14 natural killer T cells

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Abstract

Mouse natural killer T cells with an invariant Vα14-Jα18 TCR rearrangement (Vα14i NKT cells) are able to regulate immune responses through rapid and large amounts of Th1 and Th2 cytokine production. It has been reported that *in vivo* administration of the Vα14i NKT cell ligand, α-galactosylceramide (α-GalCer) significantly reduced morbidity and mortality of acute graft-versus-host disease (GVHD) in mice. In this study, we examined whether adoptive transfer of *in vitro*-expanded Vα14i NKT cells using α-GalCer and IL-2 could modulate acute GVHD in the transplantation of spleen cells of C57BL/6 mice into (B6 × DBA/2) F_1 mice.

We found that the adoptive transfer of cultured spleen cells with a combination of α-GalCer and IL-2, which contained many Vα14i NKT cells, modulated acute GVHD by exhibiting long-term mixed chimerism and reducing liver damage. Subsequently, the transfer of Vα14i NKT cells purified from spleen cells cultured with α-GalCer and IL-2 also inhibited acute GVHD. This inhibition of acute GVHD by Vα14i NKT cells was blocked by anti-IL-4 but not by anti-IFN-γ monoclonal antibody. Therefore, the inhibition was dependent on IL-4 production by Vα14i NKT cells. Our findings highlight the therapeutic potential of *in vitro*-expanded Vα14i NKT cells for the prevention of acute GVHD after allogeneic hematopoietic stem cell transplantation.

Keywords: Graft-versus-host disease; NKT cell; α-Galactosylceramide; Chimerism

1. Introduction

Mouse natural killer T cells with an invariant Vα14-Jα18 TCR rearrangement (Vα14i NKT cells) are a unique T cell population that is specifically activated by a synthetic glycolipid, α-galactosylceramide (α-GalCer) in a non-classical MHC class I molecule CD1d-restricted manner [1]. Vα14i NKT cells are known as immunomodulating cells influencing the Th1/Th2 balance, mainly via rapid secretion of robust amounts of Th1 (such as IFN-γ) and Th2 (IL-4, IL-10 and IL-13) cytokines. Thus, Vα14i NKT cells have a critical role for various immune responses including autoimmune disease [2], tumor-immunity [3,4], infection and allogeneic transplantation [5].

Graft-versus-host disease (GVHD) is an intractable and severe obstacle in allogeneic hematopoietic stem cell transplantation (HSCT). To resolve this, various treatments such as donor T cell depletion [6] and immunosuppressive drugs [7] have been attempted. In mouse acute GVHD models, a Th1 dominant cytokine secretion profile and expansion of donor $CD8^+$ T cells have been reported [8–10]. Hence it is suggested that acute GVHD is reduced by the skewed Th2 polarization of host immunity and the suppression of donor $CD8^+$ T cell

Abbreviations: GVHD, graft-versus-host disease; HSCT, hematopoietic stem cell transplantation; α-GalCer, α-galactosylceramide; Vα14i NKT cells, natural killer T cells with an invariant Vα14-Jα18 TCR rearrangement; B6, C57BL/6; BDF_1, (B6 × DBA/2) F_1; SC, spleen cells; α-GCSC, SC were cultured with IL-2 and α-GalCer; mAb, monoclonal antibody; Vα24i NKT cells, NKT cells with an invariant Vα24-JαQ TCR rearrangement; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase

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expansion. It has been reported that immune-regulatory cells such as $CD4^+$ $CD25^+$ T cell [11], $NK1.1^+$ or $DX5^+$ T cells [5,12] reduced acute GVHD. Recently, it has been demonstrated that the administration of α-GalCer to induce IL-4 production by host Vα14i NKT cells suppressed acute GVHD in a mouse model [13,14], which suggests the potential of α-GalCer/NKT cell-based immunotherapy for the prevention of acute GVHD. Nevertheless, the frequency of human NKT cells with an invariant Vα24-JαQ TCR rearrangement paired with Vβ11 TCR (Vα24i NKT cells) is very low (less than 0.5%) in peripheral blood mononuclear cells [15]. Furthermore, it has been reported that the number of NKT cells in recipients of HSCT with acute GVHD is lower compared to those without acute GVHD [16]. Given that *in vivo* administration of α-GalCer could not expand host NKT cells in some cases [17,18], we hypothesized that an adoptive transfer of *in vitro*-expanded NKT cells would be more effective than *in vivo* administration of α-GalCer alone in patients with acute GVHD.

Several investigators have reported that human Vα24i NKT cells were effectively expanded using α-GalCer plus a combination of cytokines, such as IL-2, IL-7 and IL-15 *in vitro* [19–22], while mouse Vα14i NKT cells could also be expanded with α-GalCer *in vitro* [1,23]. We found that the culture of spleen cells with α-GalCer and IL-2 for 4 days efficiently induced the expansion of Vα14i NKT cells [24]. Moreover, we revealed that *in vitro*-expanded Vα14i NKT cells retained the ability to produce IL-4 and IFN-γ and migrated into peripheral organs after adoptive transfer [24]. Therefore, adoptive transfer of *in vitro*-expanded Vα14i NKT cells may reduce acute GVHD.

In this study, we demonstrated that adoptive transfer of *in vitro*-expanded Vα14i NKT cells reduced acute GVHD such as liver injury and maintained long-term mixed chimerism. This effect is dependent on IL-4 using neutralizing anti-IL-4 monoclonal antibody. Our findings indicate the therapeutic potential of *in vitro*-expanded Vα14i NKT cells for the prevention of acute GVHD.

2. Materials and methods

2.1. Mice

Female C57BL/6N (B6, H-2^b), DBA/2N (DBA/2, H-2^d) and (C57BL/6 × DBA/2) F_1 (BDF_1, H-$2^{b/d}$) mice were purchased from Charles River Japan (Kanagawa, Japan). All mice maintained in our animal facilities were 8–12 weeks of age at the time of transplantation. All animal protocols for this study were reviewed and approved by the committee for ethics of animal experimentation in the National Cancer Center.

2.2. Monoclonal antibodies and reagents

Fluorescein isothiocyanate (FITC)-conjugated mAb against H-$2K^d$ and phycoerythrin (PE)-conjugated mAb against CD3, CD4, CD8, B220, DX-5, NK1.1 were all purchased from BD Pharmingen (San Diego, CA). For blocking IL-4 and IFN-γ *in vivo*, anti-IL-4 (clone: 11B11) and anti-IFN-γ (clone: R4-6A2) mAb were obtained from the ascites of nude mice inoculated with the hybridomas. α-GalCer was kindly provided by Pharmaceutical Research Laboratory, KIRIN Brewery Co. Ltd. (Gunma, Japan). Recombinant human IL-2 was kindly donated by Takeda Chemical Ind. Ltd. (Osaka, Japan). PE or APC-conjugated CD1d/α-GalCer tetramer was prepared in a baculovirus expression system as previously described [25]. Mouse CD1d/β2-microglobulin expression vector was provided by Dr. M. Kronenberg (La Jolla Institute for Allergy and Immunology, San Diego, CA).

2.3. Cell culture and purification of Vα14i NKT cells

In vitro expansion of Vα14i NKT cells was performed as previously described [24]. Briefly, spleen cell (SC) suspensions (5×10^5 cells/ml) were cultured with α-GalCer (50 ng/ml) and recombinant human IL-2 (100 IU/ml) in RPMI 1640 culture medium (Sigma–Aldrich, Saint Louis, MO) supplemented with 8% fetal calf serum (JRH Biosciences, Lenexa, KS), penicillin (50 U/ml), streptomycin (50 μg/ml) and 2-mercaptoethanol (5×10^{-5} M) for 4 days in a 37 °C, 5% CO_2 incubator. In some experiments, *in vitro*-expanded Vα14i NKT cells were positively selected with PE-conjugated CD1d/α-GalCer tetramer, anti-PE microbeads and SuperMACS system (Miltenyi Biotec, Bergisch Gladbach, Germany), as preciously described [24]. In brief, dead cells were removed from cultured SC as described above using a dead cell removal kit (Miltenyi Biotec), LS column (Miltenyi Biotec) and SuperMACS system (Miltenyi Biotec). Then, the SC were preincubated with anti-CD16/32 (2.4G2, BD PharMingen), stained with appropriate diluted PE-conjugated CD1d/α-GalCer tetramer on ice in the dark for 30 min, and washed three times by buffer (phosphate buffered saline supplemented with 0.5% bovine serum albumin and 2 mM EDTA). The stained cells were then incubated with anti-PE microbeads (Miltenyi Biotec) (1×10^7 cells/microbeads in 40 μl) on ice in the dark for 30 min, suspended in 2 ml buffer, and finally passed through a LS column using the SuperMACS system with additive 3× 3 ml of buffer for washing column. Consequently, we acquired the purified Vα14i NKT cells as residual cells in the column. The purity of CD1d/α-GalCer $tetramer^+$ $CD3^+$ cells was more than 96%.

2.4. Cell transfer and treatment with antibodies

For the induction of GVHD, 7×10^7 spleen cells from B6 mice were transferred into BDF_1 mice intravenously through the tail vein (GVHD mice) as previously described [8]. One day later, 2×10^7 spleen cells cultured with α-GalCer and IL-2 for 4 days (α-GCSC) were injected intravenously into BDF_1 mice with GVHD. In other experiments, purified Vα14i NKT cells were transferred into GVHD mice. In some experiments, GVHD mice were administered with anti-IL-4 mAb (3 mg/mouse) or anti-IFN-γ mAb (1 mg/mouse) intraperitoneally on the day of α-GCSC transfer, referring previous reports for effective doses of mAbs [26–28].

2.5. Flow cytometry

The phenotype of cells was determined by multicolor flow cytometory as previously described [24]. To prevent non-specific binding of mAb, cells were pre-incubated with anti-CD16/32 (2.4G2, BD PharMingen). The relative percentages of host- and donor-origin cells in the recipient spleens were determined by anti-H2-K^d (recipient type) as an indicator of GVHD in which donor chimerism was elevated [29]. The relative percentage of donor-origin cells (% donor chimerism) in chimeric recipients was calculated by the following formula: 100 − %H-$2K^d$ positive cells.

In addition, for the determination of a lineage-specific chimerism, recipient spleens were stained with FITC-conjugated antibody against H-$2K^d$ and PE-conjugated antibodies against CD3, CD4, CD8, B220, DX-5. Vα14i NKT cell frequency was determined by FITC-conjugated CD3 and APC-conjugated CD1d/α-GalCer tetramer. Propidium iodide was used to exclude dead cells. The stained cells were analyzed using FACSCalibur (BD Biosciences, San Jose, CA) and Flow Jo software (Tree Star Inc., San Carlos, CA).

2.6. Assessment of GVHD

Recipient mice were sacrificed on day 14. The serum glutamic oxaloacetic transaminase (GOT) and glutamic pyruvic transaminase (GPT) levels were detected (SRL Inc., Tokyo, Japan) by serological examination using standard methodologies. Additionally, liver and small bowel were embedded in paraffin, cut into 5 μm-thick sections, and stained with H&E for histological examination.

3. Results

3.1. Adoptive transfer of spleen cells cultured with α-GalCer and IL-2 (α-GCSC) inhibit donor T cell engraftment in mice with acute GVHD.

In order to obtain a large number of Vα14i NKT cells, spleen cells from BDF_1 mice were cultured with α-GalCer and IL-2 for 4 days as previously reported [24]. As shown in Fig. 1A, the percentage of $CD3^+$ CD1d/α-GalCer $tetramer^+$ cells increased approximately 20-fold after expansion in culture. In

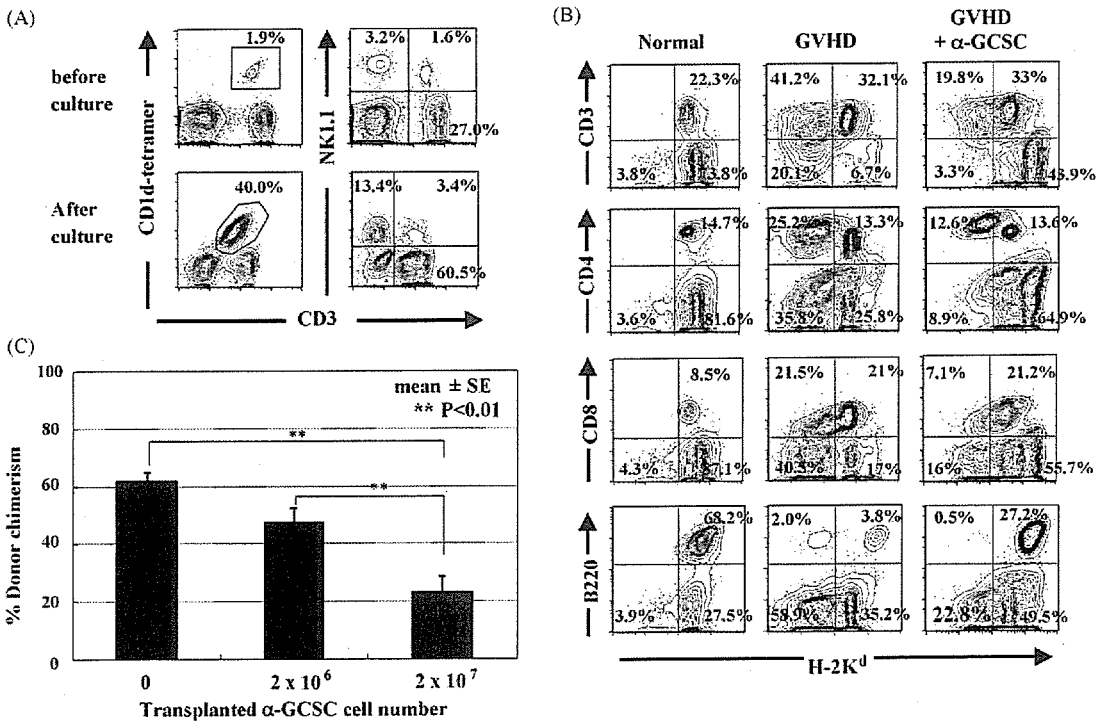

Fig. 1. Adoptive transfer of spleen cells cultured with α-GalCer and IL-2 reduced percentages of donor chimerism depending on cell number. (A) Spleen cells (SC) of BDF_1 mice were cultured with 50 ng/ml α-GalCer and 100 IU/ml IL-2 for 4 days. The percentage of Vα14i NKT cells and $NK1.1^+$ $CD3^-$ NK cells were determined. Before and after culture cells were stained with anti-CD3-FITC, anti-NK1.1-PE mAb and CD1d/α-GalCer tetramer-APC. The numbers in each of the quadrants represent the percentage of total analyzed cells. The fluorescence profiles are representative of at least three independent experiments. (B, C) BDF_1 mice were transferred with 7×10^7 B6 SC on day 0 for GVHD induction and with or without α-GCSC as indicated at 2×10^7 or 2×10^6 on day 1 and then donor chimerism and surface phenotype of SC were analyzed by flow cytometory on day 14. (B) SC of untreated (normal: left column), transplanted with B6 SC alone (GVHD: center column) and transplanted with both B6 SC and 2×10^7 α-GCSC (GVHD + α-GCSC: right column) mice were stained with anti-H-$2K^d$-FITC and each of anti-CD3, CD4, CD8, B220, DX-5-PE mAb at day 14. The numbers in each of the quadrants represent the percentage of total analyzed cells. The fluorescence profiles are representative of at least three independent experiments. (C) The bars indicate averages of percentage of donor chimerism with standard error of the mean. The number of each group is α-GCSC transfer of 0: $n=6$; 2×10^6: $n=6$; 2×10^7: $n=7$. $^{**}p<0.01$ versus group of α-GCSC transfer of 0. The differences between groups were analyzed using non-repeated measures ANOVA with Bonferroni correction. Data are representative of three independent experiments.

addition, $CD3^-$ $NK1.1^+$ cells (NK cells) in α-GCSC were also expanded 2.5-fold. To investigate whether α-GCSC containing large amounts of Vα14i NKT cells could inhibit acute GVHD, we transplanted BDF_1 mice with 7×10^7 spleen cells from B6 on day 0 for GVHD induction and α-GCSC (2×10^7 or 2×10^6 cells) on day 1. Normal mice received saline only on day 1. At day 14, mice transplanted with spleen cells from B6 mice alone (GVHD mice) exhibited donor-dominant chimerism (% donor chimerism (mean ± S.E.M.): 61.7 ± 3.0%) and expansion of donor $CD3^+$ cells (41%) including both $CD4^+$cells (25.2%) and $CD8^+$ cells (21.5%), while mice transplanted with B6 SC plus 2×10^7 α-GCSC had reduced donor chimerism (% donor chimerism (mean ± S.E.M.): 22.9 ± 5.6), lower engraftment of donor $CD4^+$ cells (12.6%) and $CD8^+$ cells (7.1%) as compared with GVHD mice (Fig. 1B and 1C). These data indicate that the transfer of α-GCSC suppressed early donor T cell engraftment. However, lower number of α-GCSC (2×10^6 cells) did not significantly inhibit donor cell engraftment (Fig. 1C).

3.2. Adoptive transfer of α-GCSC reduces symptoms of acute GVHD

Next, we examined whether the transfer of α-GCSC ameliorate serological and histological findings of acute GVHD, by analyzing the serum levels of GOT and GPT, and histology of liver tissue specimens. The serum GOT levels of GVHD mice

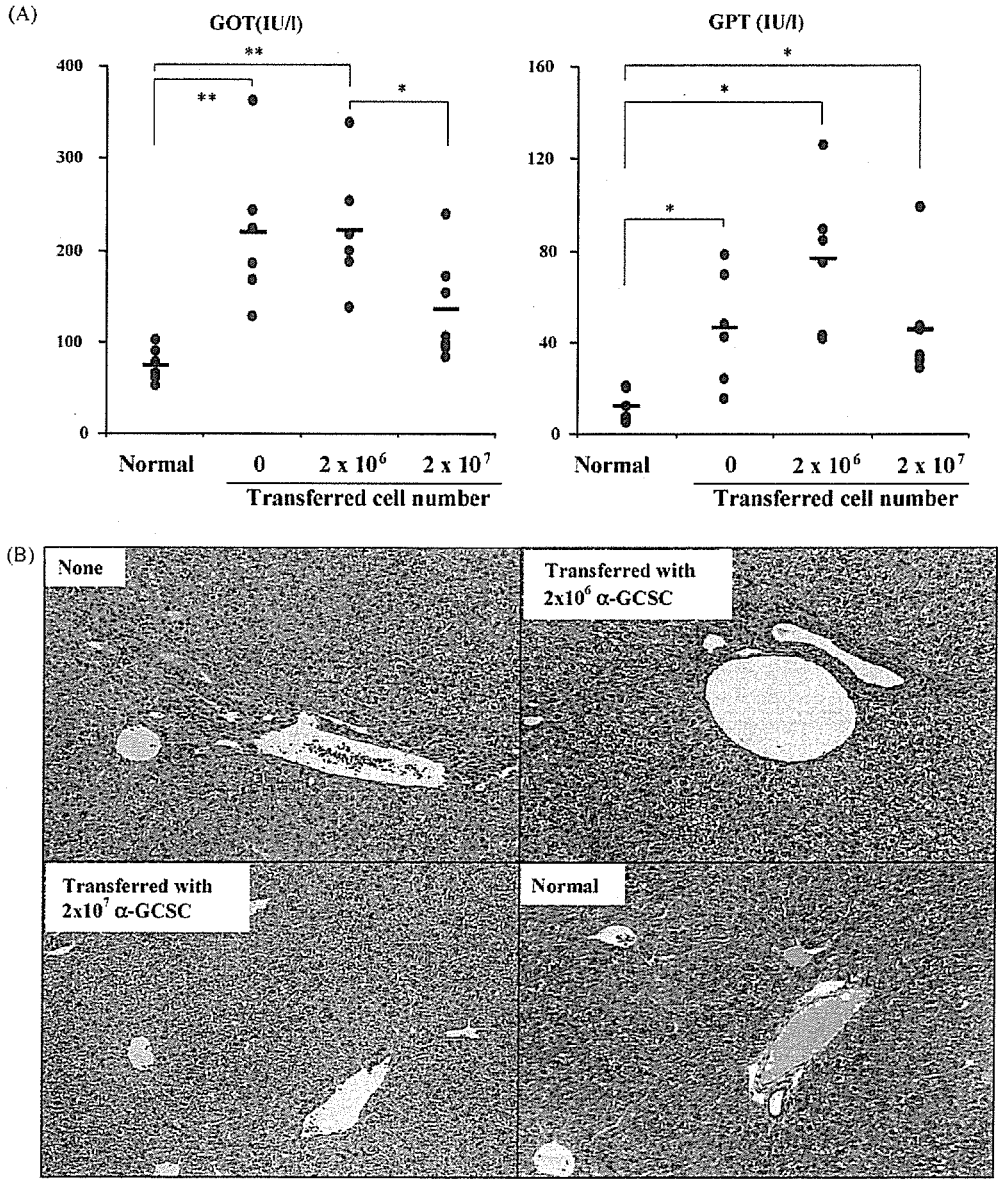

Fig. 2. Transplanted BDF_1 α-GCSC alleviated GVHD signs serologically and histologically. (A) Induction of GVHD and infusion of α-GCSC were performed as described in Fig. 1. The serum GOT levels of mice group with 2×10^7 α-GCSC were low compared with other GVHD mice groups ($^*p < 0.05$ and $^{**}p < 0.01$ by non-repeated measures ANOVA and Bonferroni correction), although the serum GPT levels of all mice groups were significantly high compared with normal ($n = 6$). (B) Histology of liver tissue of GVHD mice with or without α-GCSC as described above and normal. Representative of three independent experiments of each group is shown.

were significantly higher as compared with untreated control mice, whereas the serum GOT levels in GVHD mice transferred with 2×10^7 α-GCSC were reduced by 50% as compared with GVHD mice (Fig. 2A). The reduction of serum GOT was not observed when transferred with 2×10^6 α-GCSC. Serum GPT levels were not significantly different among all groups (Fig. 2A). Histological analysis showed remarkable hepatic lymphocyte infiltration in the portal area in GVHD mice, while very little or no infiltration was detected in GVHD mice transferred with 2×10^7 α-GCSC. Mice treated with 2×10^6 α-GCSC showed no reduction in lymphocyte infiltration (Fig. 2B). These results indicate that α-GCSC alleviated acute GVHD and retarded donor T cell engraftment. However, spleen cells containing about 3% Vα14i NKT cells cultured with IL-2 alone could not inhibit acute GVHD and rapid donor T cell engraftment (data not shown), suggesting that the inhibitory effect of α-GCSC on GVHD is mainly attributable to the potential of Vα14i NKT cells.

3.3. Maintenance of donor cell engraftment and mixed chimerism in GVHD mice requires IL-4 but not IFN-γ following adoptive transfer of α-GCSC

Next, we examined whether the inhibition of acute GVHD was due to rapid rejection and/or graft failure by α-GCSC. Long-term donor chimerism was observed in GVHD mice with or without α-GCSC (2×10^7 cells) at 14, 42 and 100 days after the induction of GVHD (Fig. 3A). GVHD mice exhibited complete donor chimerism at day 100. Approximately 20% donor chimerism was observed in GVHD mice when treated with α-GCSC at day 14, and this gradually increased to 35% by day 100. Therefore, GVHD mice with α-GCSC sustained mixed chimerism for a significant period of time. Donor-derived lymphocytes in these mice contained T cells ($CD4^+$ and $CD8^+$) and B cells (Fig. 3B). Although very few donor-derived B cells (0.5%) were detected at 14 days after induction of GVHD with α-GCSC administration, 6% donor-derived B cells were appeared in GVHD mice with α-GCSC at 100 days. These results suggest that the transfer of α-GCSC did not impair donor cell engraftment or maintenance of long-term mixed chimerism.

It has been known that activated Vα14i NKT cells rapidly produced IL-4 and IFN-γ [23,24]. We therefore examined whether IL-4 and/or IFN-γ produced by Vα14i NKT cells is the cytokine(s) responsible for mediating inhibition of GVHD. Neutralizing mAbs against IL-4 and IFN-γ were administered intraperitoneally into GVHD mice with or without α-GCSC. As shown in Fig. 4, administration of anti-IL-4 or anti-IFN-γ mAb had no effect on donor chimerism of GVHD mice. However, the inhibitory effect on GVHD by a transfer of α-GCSC

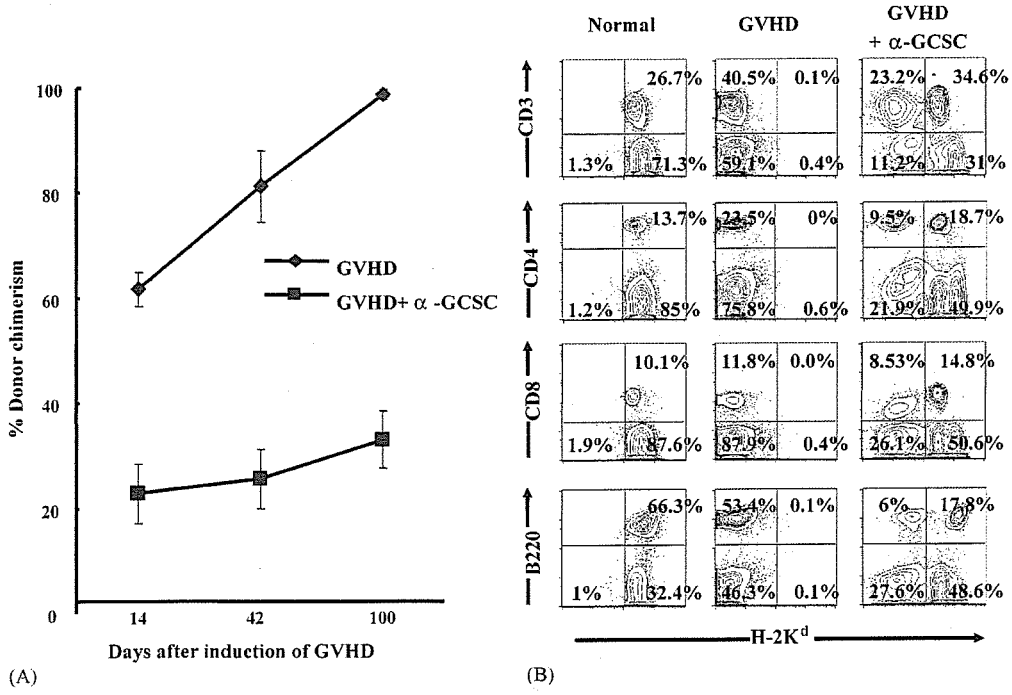

Fig. 3. Donor cells were not rejected and mixed chimerism was maintained in GVHD mice with α-GCSC for a long term. (A) Induction of GVHD and a transfer of α-GCSC were performed as described in Fig. 1. SC of GVHD mice transplanted with or without α-GCSC were stained with anti-H-$2K^b$ mAb and then donor chimerism was determined on days 14, 42 and 100 after GVHD induction. Percentages of donor chimerism in SC of GVHD mice transplanted with α-GCSC gradually increased as days passed. GVHD indicates GVHD mice without transfer of α-GCSC ($n=6$ on day 14, $n=6$ on day 42, $n=4$ on day 100); GVHD + cultured SC, GVHD mice with transfer of α-GCSC ($n=7$ on day 14, $n=5$ on day 42, $n=4$ on day 100). Values are mean ± S.E.M. on days 14, 42 and 100. $^{**}p<0.01$ versus group of GVHD. (B) SC of untreated (normal: the left column), transplanted with B6 SC alone (GVHD: the center) and transplanted with both B6 SC and 2×10^7 α-GCSC (GVHD + cultured SC: the right) mice were stained with anti-H-$2K^d$-FITC and each of anti-CD3, CD4, CD8, B220, DX-5-PE mAb on day 100. The numbers in each of the quadrants represent the percentage of total analyzed cells. The fluorescence profiles are representative of at least three independent experiments.

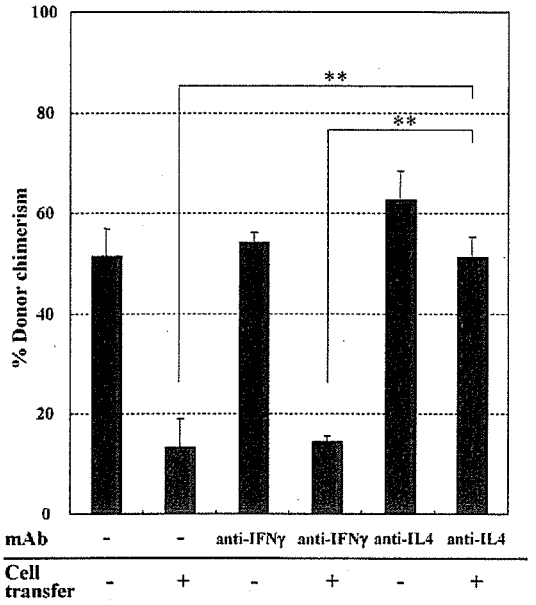

Fig. 4. GVHD was inhibited by the function of BDF_1 α-GCSC depending on IL-4, but not IFN-γ. Induction of GVHD and a transfer of α-GCSC were performed as described in Fig. 1. GVHD mice with or without α-GCSC were injected with or without anti-IL-4 (3 mg/mouse) or IFN-γ (1 mg/mouse) neutralizing mAbs on day 1. Donor chimerism was determined by anti-H-$2K^b$ mAb on day 14. Values are mean ± SEM on day 14. The number of each group is from the left $n = 5, 6, 6, 4, 4$ and 5, respectively.

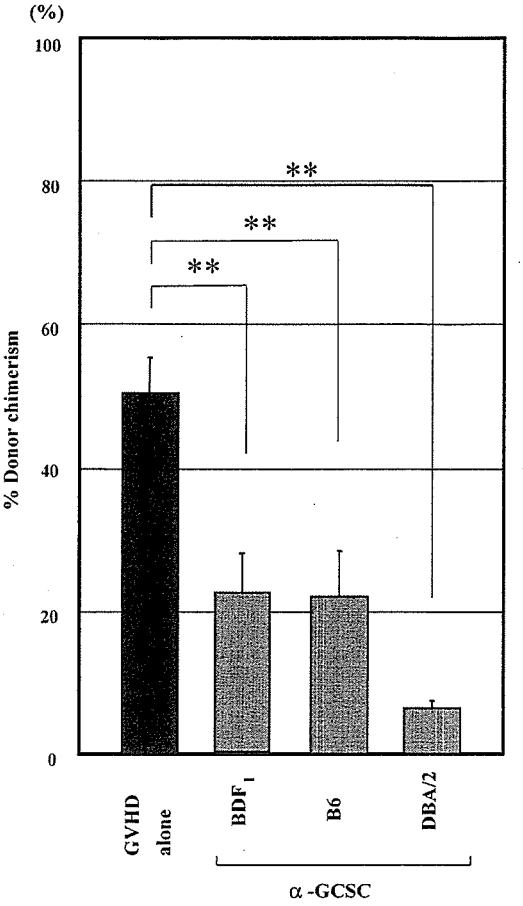

Fig. 5. α-GCSC from B6 or DBA/2 mice could also inhibit GVHD. Induction of GVHD and infusion of α-GCSC were performed as described in Fig. 1. The percentages of donor cell chimerism at day 14 in GVHD mice with any α-GCSC was significantly lower compared with GVHD mice with none (GVHD). The number of each group is GVHD (GVHD mice with none), $n = 15$; BDF_1, $n = 7$; B6, $n = 6$; DBA/2, $n = 6$. $^{**}p < 0.01$ versus group of GVHD by non-repeated measures ANOVA and Bonferroni correction.

was blocked by anti-IL-4, but not by anti-IFN-γ mAb (Fig. 4). Therefore, the retardation of donor cell engraftment and alleviation of acute GVHD by α-GCSC appears to be mediated by an IL-4-dependent mechanism.

3.4. Purified in vitro-expanded Vα14i NKT cells ameliorated acute GVHD

We next determined whether α-GCSC derived from the parental strain (B6 or DBA/2) could also inhibit rapid donor cell engraftment. Firstly, α-GCSC containing 30 or 15% of Vα14i NKT cells in B6 mice or DBA/2 mice, respectively, were transferred (2×10^7) into GVHD mice. Expectedly, transfer of α-GCSC, originating from both B6 and DBA/2 mice reduced the percentage of donor chimerism in GVHD mice (Fig. 5). The results suggest that the inhibitory effect of α-GCSC on GVHD was not related to their strain of origin.

To examine which cell compartment in the α-GCSC inhibits acute GVHD, Vα14i NKT cells were purified from α-GCSC by using CD1d/α-GalCer tetramer and MACS system. The purity of Vα14i NKT cells was more than 96% (Fig. 6A). Donor chimerism of GVHD mice injected with 4×10^6 purified Vα14i NKT cells (nearly equivalent to 2×10^7 α-GCSC) was lower than that of mice with GVHD alone, although it was higher than that of GVHD mice transplanted with 2×10^7 of α-GCSC (Fig. 6B). This data indicates that transfer of purified Vα14i NKT cells alone can ameliorate GVHD.

4. Discussion

Vα14i NKT cells play an important role in immune regulation including autoimmunity, tumor immunity and infection. Furthermore, it has been demonstrated that $NK1.1^+$ NKT cells from donor bone marrow [5] or residual host [30] can inhibit acute GVHD. Recently, several groups reported that *in vivo* administration of the Vα14i NKT cell specific ligand, α-GalCer, modulated acute GVHD and prolonged survival [13,14,31]. These studies suggest the therapeutic potential of α-GalCer or Vα14i NKT cells for the prevention of acute GVHD after allogeneic HSCT.

Although we also obtained similar results regarding the inhibition of GVHD by α-GalCer-activated Vα14i NKT cells, we used *in vitro*-expanded Vα14i NKT cells and non-myeloablative F_1 mice as recipients. It is likely that this difference led to the distinct results in regard to the difference in donor chimerism. We found that GVHD mice with a transfer of Vα14i NKT cells could maintain mixed chimerism (donor chimerism frequency of 20–30%) for a long period. By contrast, Morecki et al. trans-

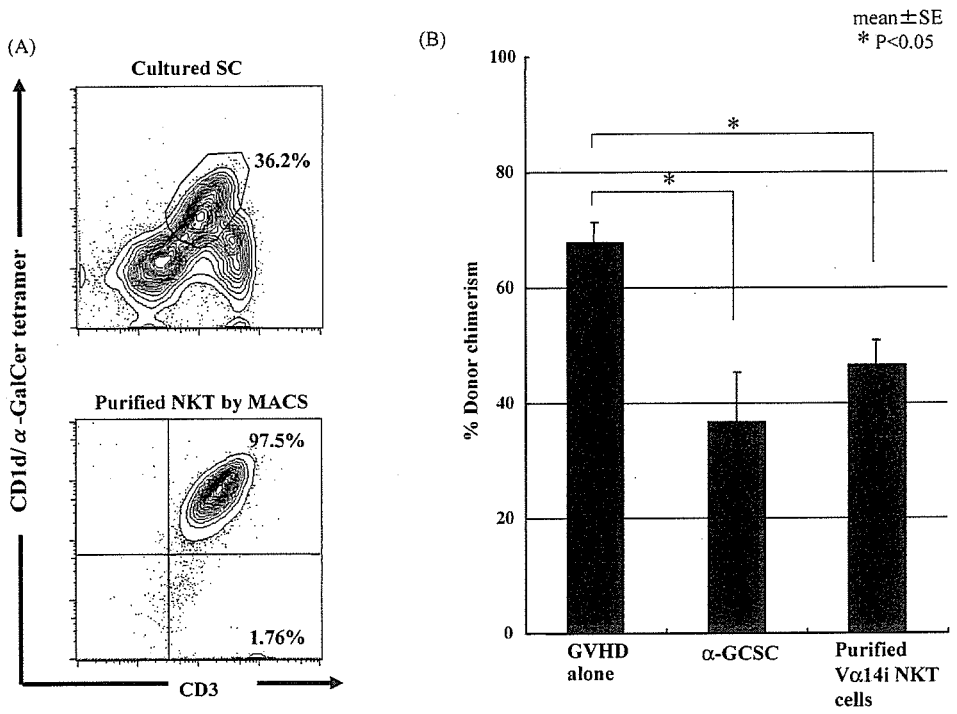

Fig. 6. Purified Vα14i NKT cells from BDF_1 α-GCSC also alleviated GVHD. (A, B) Induction of GVHD and cell culture were performed as described in Fig. 1. The purification of NKT cells from BDF_1 α-GCSC was done as described in materials and methods. (A) Before and after the purification of NKT cells, BDF_1 α-GCSC were stained with anti-CD3-FITC and CD1d/α-GalCer tetramer-APC or CD1d/α-GalCer tetramer-PE mAb. The numbers in each of the quadrants represent the percentage of total analyzed cells. The fluorescence profiles are representative of at least three independent experiments. (B) Mice were additionally infused with or without 2×10^7 BDF_1 α-GCSC or 4×10^6 purified NKT cells from α-GCSC by MACS system. The percentage of donor cell chimerism in SC in each mouse was determined on day 14. The number of each group is GVHD (GVHD mice with none), $n=6$; α-GCSC 2×10^7, $n=5$; isolated Vα14i NKT cells 4×10^6, $n=6$. $^{**}p < 0.01$ versus group of GVHD by non-repeated measures ANOVA and Bonferroni correction.

planted spleen cells of parental B6 mice into low dose total body irradiated (BALB/c × B6) F_1 mice similar to our GVHD model and showed that the donor chimerism of α-GalCer- administered mice was very low (2% of donor chimerism). This difference in donor chimerism between a transfer of Vα14i NKT cells and an injection with α-GalCer seems to be attributable to IL-4, although previous studies [14,31] and our current results demonstrated that IL-4 from Vα14i NKT cells mainly contributed to the inhibition of acute GVHD. It has been reported that the amount and time course of serum IL-4 levels were distinct between direct administration of α-GalCer and the transfer of α-GalCer pulsed dendritic cells [32]. Direct administration of α-GalCer induced more rapid and higher levels of serum IL-4 levels as compared with α-GalCer-pulsed dendritic cells. We propose that the distinct donor chimerism in GVHD mice between a transfer of Vα14i NKT cells and an injection with α-GalCer may be attributable to the different amount and time course of serum IL-4 levels. Furthermore, we measured Th polarization (IL-4 and IFN-γ production) in total (donor plus recipient) cells 7 days after transplantation by ELISA assay and found a Th2 dominant response (data not shown). However, we do not know if a Th2 dominant response differed between donor and recipient derived cells.

Previous reports [29,33,34] indicated that rapid engraftment of donor cells was always accompanied by severe GVHD, and that, conversely, slow engraftment led to a reduction of GVHD. Pan et al. [35] also showed that a stable and lower level of donor chimerism should be enough to induce donor–recipient reciprocal tolerance. Thus, their and our data show that IL-4-dependent retention of mixed chimerism or a gradual transition from a mixed to a complete chimera by transfer of α-GCSC leads to alleviation of GVHD. It should be noted, however, that systemic administration of IL-4 is ineffective or toxic [36]. Moreover, a stable mixed chimerism of GVHD mice transplanted with Vα14i NKT cells was sustained for an expanded period. These results suggest that the stable chimerism induced by Vα14i NKT cells is not due to graft rejection.

α-GCSC including $CD1d^+$ cells loaded with α-GalCer activated recipient Vα14i NKT cells (data not shown). Therefore, both recipient and transferred Vα14i NKT cells might contribute to alleviation of GVHD as previously reported [31]. However, we showed that inhibition of GVHD by Vα14i NKT cells was due to IL-4 produced exclusively by Vα14i NKT cells among α-GCSC [24], and that a transfer of purified Vα14i NKT cells alone prevented acute GVHD. These data suggest that transferred Vα14i NKT cells were sufficient to modulate acute GVHD.

Recently, Haraguchi et al. [31] reported the effect of *in vivo* administration of α-GalCer and the adoptive transfer of NKT cells on the prevention of GVHD. This seems to be logical considering the relationship between host-residual and transferred NKT cells as they mentioned that host-residual, but not

transferred, NKT cells are essential for amelioration of GVHD. Although the authors indicated that maximal GVHD reduction and survival were mainly accompanied by graft rejection, our data demonstrated that effective GVHD reduction was accompanied by maintenance of mixed chimerism. This discrepancy may be explained by the different GVHD settings, non-myeloablative and myeloablative recipients, and by the balance between the dose of alloreactive donor cells and the activity of host-residual NKT cells.

Although several studies have reported that the number of circulating NKT cells was reduced in cancer patients [17,37,38], direct injection of α-GalCer is not expected to induce anti-tumor effects. On the other hand, adoptive *in vitro*-expanded NKT cell immunotherapy may be useful for cancer therapy. In support of this, we found that adoptive transfer of *in vitro*-expanded Vα14i NKT cells could prevent lung tumor metastasis in a mouse model (unpublished data Ikarashi et al.). We believe that adoptive transfer of NKT cell therapy combined with allogenic HSCT may be beneficial for cancer patients, because of NKT cell function for prevention of GVHD and anti-tumor effects.

Acknowledgments

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