Interleukin (IL)-4 promotes T helper type 2-biased natural killer T (NKT) cell expansion, which is regulated by NKT cell-derived interferon-γ and IL-4

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Summary

CD1d-restricted natural killer T (NKT) cells can rapidly produce T helper type 1 (Th1) and Th2 cytokines and also play regulatory or pathological roles in immune responses. NKT cells are able to expand when cultured with α-galactosylceramide (α-GalCer) and interleukin (IL)-2 in a CD1d-restricted manner. However, the expansion ratio of human NKT cells is variable from sample to sample. In this study, we sought to determine what factor or factors are responsible for efficient *in vitro* expansion of NKT cells from various inbred mouse strains. Although the proportion of NKT cells in the spleen was nearly identical in each mouse strain, the growth rates of NKT cells cultured *in vitro* with α-GalCer and IL-2 were highly variable. NKT cells from the B6C3F1 and BDF1 mouse strains expanded more than 20-fold after 4 days in culture. In contrast, NKT cells from the strain C3H/HeN did not proliferate at all. We found that cell expansion efficiency correlated with the level of IL-4 detectable in the supernatant after culture. Furthermore, we found that exogenous IL-4 augmented NKT cell proliferation early in the culture period, whereas interferon (IFN)-γ tended to inhibit NKT cell proliferation. Thus, the ratio of production of IL-4 and IFN-γ was important for NKT cell expansion but the absolute levels of these cytokines did not affect expansion. This finding suggests that effective expansion of NKT cells requires Th2-biased culture conditions.

Keywords: natural killer T cell; interleukin-4; interferon-γ; glycolipid

Introduction

Mouse natural killer T (NKT) cells were initially identified as a T-cell subset that expresses NK cell receptors such as NK1·1, CD94 and Ly49.[1,2] The majority of NKT cells have the invariant T-cell receptor (TCR) α-chain rearrangement Vα14-Jα18 and recognize antigens presented by CD1d, a non-classical major histocompatibility complex (MHC) class I molecule.[3,4] NKT cells are continuously sensitized by endogenous antigens so that they display an effector-memory phenotype (such as $CD62L^{low}$ $CD44^{high}$)[5–7] and rapidly produce large amounts of T helper type 1 (Th1) and Th2 cytokines when stimulated with lipid antigens such as α-galactosylceramide (α-GalCer) in a CD1d-dependent manner.[2,8] NKT cells are regarded as immunoregulatory because of their cytokine profile. Moreover, NKT cells are thought to play an important role in response to infectious agents and in pathological responses such as allergies or autoimmune disease. NKT cells are cytotoxic to various tumour cell lines via Fas-ligand-, tumour necrosis factor-related apoptosis-inducing ligand (TRAIL)- and/or perforin-dependent pathways,[9–12] and play a role in tumour surveillance.[13] NKT cells activated by interleukin (IL)-12 or α-GalCer sequentially activate natural killer (NK) cells by producing interferon (IFN)-γ and induce antitumour immune responses. This in turn inhibits tumour metastasis and can suppress solid tumour growth. In some studies, it has been suggested that this ability helps to induce tumour antigen-specific CD8 T cells, thereby making an additional contribution to the immune response to cancer.[14]

In humans, counterparts of mouse NKT cells have also been found to be responsive to α-GalCer, which induces them to secrete IL-4 and IFN-γ. In addition, they have been shown to be cytotoxic to tumour cells via two different mechanisms, a CD1d-dependent and a CD1d-independent mechanism.[15] Human NKT cells have the

potential to induce antitumour responses *in vivo*. However, in patients with malignancies,[16,17] NKT cells are reduced in number and activity, and *in vivo* activation by α-GalCer leads to transient activation and long-term unresponsiveness of NKT cells.[18,19] For that reason, adaptive transfer of *in vitro* expanded and/or activated NKT cells is expected to induce effective antitumour responses.

To date, several combinations of cytokines with α-GalCer have been reported to expand NKT cells isolated from peripheral mononuclear cells. However, NKT cells present a diverse range of expansion ratios even among healthy individuals.[20,21] Although a previous study suggested that differences in NKT cell proliferation are associated with the age of the donor,[22] there is still much that remains to be determined concerning additional factors that influence NKT cell proliferation.

In this study, we used inbred mouse strains as an experimental system in which to reveal factors that affect variation in proliferation rates among individuals. Previously, we found that *in vitro* expanded NKT cells from C57BL/6 mice retained an effector-memory-like phenotype and retained the ability to produce cytokines.[23] In addition, we found that there was a marked difference in the NKT cell expansion ratio among various mouse strains and that the differences were closely related to the bias in production of Th1 or Th2 cytokines by NKT cells. Finally, we report that a relatively low rate of proliferation can be enhanced by the addition of IL-4, which creates Th2-biased culture conditions.

Materials and methods

Mice

Female C57BL/6N, BALB/cA, C3H/HeN, DBA/2N (C57BL/6 × DBA/2)F_1 (BDF1), (C57BL/6 × C3H/HeN)F_1 (B6C3F1), and SJL/J mice were purchased from Charles River Japan (Kanagawa, Japan). All mice, which were maintained in our animal facilities, were 8–11 weeks of age at the time of the experiment. All animal protocols for this study were reviewed and approved by the committee for ethics of animal experimentation at the National Cancer Center of Japan prior to the beginning of the study.

Monoclonal antibodies and reagents

Anti-IL-4 (clone 11B11) and anti-IFN-γ (clone R4-6A2) monoclonal antigen-neutralizing antibodies (mAbs) were obtained from the supernatant of a hybridoma culture maintained in serum-free medium in a CELLine CL-1000 flask (BD Biosciences, San Jose, CA) and purified by Protein G Sepharose (GE Healthcare Amersham Biosciences AB, Uppsala, Sweden) affinity column chromatography. Anti-CD16/32 (clone 2·4G2) was obtained from a hybridoma supernatant. Fluorescein isothiocyanate (FITC)-conjugated anti-CD3 (clone 145-2C11), allophycocyanin (APC)-conjugated anti-IL-4 (11B11), anti-IFN-γ (XMG1·2), and a rat immunoglobulin G1 (IgG1) isotype control (clone R3-34) and Golgi Stop™ were obtained from BD Biosciences. α-Galactosylceramide (α-GalCer) was kindly provided by the Pharmaceutical Research Laboratory, KIRIN Brewery Co., Ltd (Gunma, Japan). The phycoerythrin (PE)-conjugated CD1d/α-GalCer tetramer was prepared using a baculovirus expression system as previously described.[24] Human recombinant IL-2 (rIL-2) was kindly provided by Takeda Chemical Industries Ltd (Osaka, Japan). Mouse rIL-4 was obtained from PeproTech EC Ltd (London, UK).

Flow cytometry

NKT cells were detected by multicolour flow cytometry as previously described.[23] Briefly, cells were preincubated with anti-CD16/32 mAb to block non-specific FcRγ binding and then stained with FITC-conjugated anti-CD3 and PE-conjugated CD1d/α-GalCer tetramer. Dead cells were excluded by propidium iodide staining and electronic gating. For detection of intracellular cytokines, cells were stimulated for 3 hr with phorbol 12-myristate 13-acetate (PMA) (25 ng/ml) and ionomycin (1 μg/ml), with the last 1 hr of stimulation in the presence of Golgi block, in a 37°, 5% CO_2 incubator, and then washed and incubated with anti-CD16/32 mAb, followed by incubation with FITC-conjugated anti-CD3 and PE-conjugated CD1d/α-GalCer tetramer. Cells were then permeabilized using Cytofix/Cytoperm (BD Biosciences) and IL-4 or IFN-γ was detected using APC-conjugated mAbs. Cells were analysed by flow cytometry (FACSCalibur; BD Biosciences).

NKT cell proliferation assay

Preparation of splenic mononuclear cells and *in vitro* expansion of NKT cells were performed as previously described.[23] Briefly, spleens of each mouse strain were macerated aseptically and pushed through a nylon mesh to obtain single-cell suspensions, and erythrocytes were lysed in ammonium chloride buffer. Mononuclear cells (1×10^6 cells/ml) were cultured with α-GalCer (50 ng/ml) and rIL-2 (100 IU/ml) in RPMI-1640 culture medium (Sigma-Aldrich, St. Louis, MO) supplemented with 8% fetal calf serum (JRH Biosciences, Lenexa, KS), 2-mercaptoethanol (5×10^{-5} M) 100 U/ml penicillin and 100 μg/ml streptomycin for 4 days in a 37°, 5% CO_2 incubator. After 4 days in culture, the absolute number of living cells was counted using a microscope after staining of cells with 0·2% trypan blue, and the relative percentages of NKT cells were determined by flow cytometry.

Cytokine production

The cell culture supernatant was collected after 24 hr or 4 days in culture and stored at −20°. The concentrations

of IL-4 and IFN-γ were determined by enzyme-linked immunosorbent assay (ELISA) (OptEIA ELISA set; BD Biosciences).

Results

α-GalCer-induced expansion of NKT cells from various mouse strains

Mouse NKT cells show a similar variation in expansion ratios to that observed for human NKT cells. We found that the expansion ratios were different for different mouse strains (Fig. 1). Before culture, spleen cell suspensions contained a small percentage (0·8–1·5%) and a small number (7–18 × 10^3 cells/ml) of NKT cells in each mouse strain. As shown in Fig. 1, culture of spleen cells with α-GalCer and IL-2 induced expansion of NKT cells, except for C3H/HeN mice. After 4 days of culture, NKT cells constituted 6·4–40·7% of cells in the culture and had expanded 7–25-fold in BALB/c, C57BL/6, DBA/2, B6C3F1 and BDF1 mice. The CD1d-restricted TCR α-chain Vα14 dominantly associates with the high-affinity TCR β-chain Vβ8·2, or the lower affinity chain Vβ8·3, Vβ7 or Vβ2, and a genetic defect in Vβ8 is reportedly the cause of the low responsiveness of NKT cells. We next asked if the TCR-β status of NKT cells had an effect on expansion. However, we found no significant differences among the six strains that were tested, and selective proliferation did not occur (data not shown).

NKT cell proliferation ratio correlates with amount of IL-4 in supernatant from a 4-day culture

Previously, a high concentration of IL-4 and IFN-γ in supernatant from a 4-day culture was observed.[23] Firstly, we measure amounts of IL-4 and IFN-γ in the culture supernatant.

An increase in the number of NKT cells was positively correlated with the production of IL-4 in the 4-day culture (Fig. 2a). However, high levels of IFN-γ were observed in all of the mouse strains, independent of an increase in either NKT cell number or IL-4 production. Almost all CD8 T cells acquired the ability to produce IFN-γ when activated indirectly via NKT cells by α-GalCer (data not shown), so it appears that, in C3H/HeN mice, NKT cells do not proliferate. Instead, it seems reasonable that a large amount of IFN-γ might be produced by the activated NK cells and CD8 T cells.[25,26]

A previous study reported cytokine secretion of NKT cells prior to their proliferation.[2,27] Thus, we harvested culture supernatants at 24 hr, before NKT cell expansion,[27] to determine the status of cytokine production at this early stage, which is the stage at which NKT cells initially respond to culture and initiate production of IL-4. This initial response positively correlated with NKT cell expansion to some degree, although the response was weaker than that observed for cells in culture for 4 days. It is notable that IL-4 production by C3H/HeN was more robust than that observed for C57BL/6, and IFN-γ

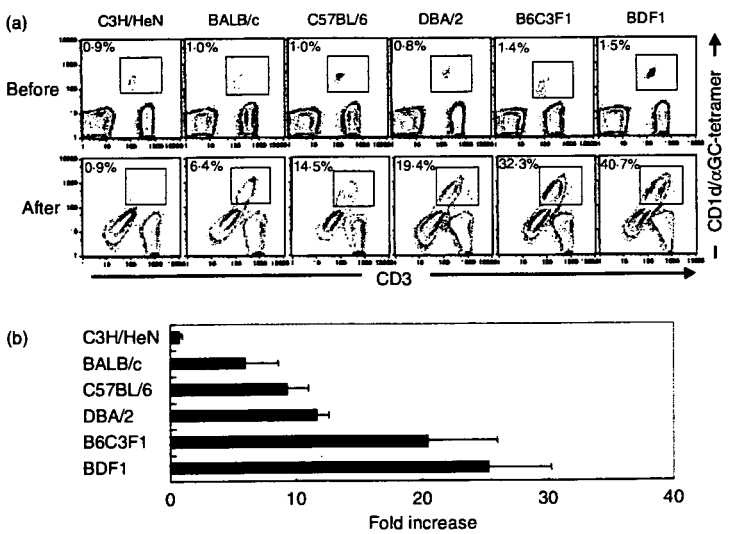

Figure 1. Expansion of natural killer T (NKT) cells *in vitro*. (a) Mouse spleen cells (1 × 10^6 cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml interleukin (IL)-2 for 4 days. Cells were stained with anti-CD3 monoclonal antibody (mAb) and CD1d/α-GalCer tetramer and analysed by flow cytometry. The percentage of NKT cells was determined for both fresh (upper row) and cultured (lower row) cells. Representative results from replicate experiments are shown. (b) The fold increase in NKT cells after culture was calculated based on living cell counts and the percentage of NKT cells in the total cell population. Data are shown as mean ± standard error of the mean (n = 9 for C3H/HeN, BALB/c and C57BL/6; n = 4 for DBA/2, B6C3F1 and BDF1).

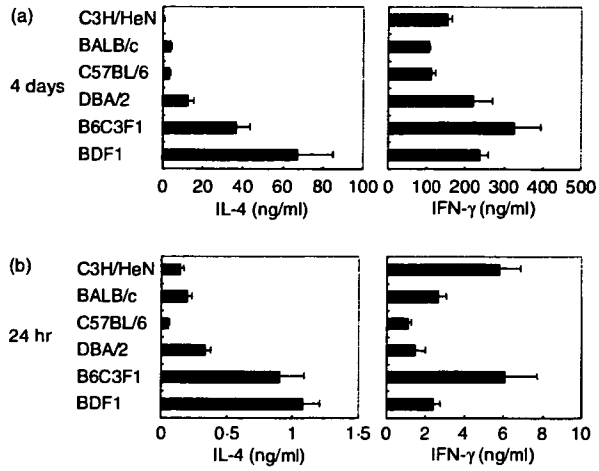

Figure 2. Production of interleukin (IL)-4 and interferon (IFN)-γ in expansion cell culture supernatants. Mouse spleen cells (1×10^6 cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml IL-2 for 4 days. Supernatants were collected after 24 hr (b) or 4 days (a). The levels of IFN-γ and IL-4 in the supernatants were determined by enzyme-linked immunosorbent assay (ELISA). Data are shown as mean ± standard error of the mean ($n = 9$ for C3H/HeN, BALB/c and C57BL/6; $n = 4$ for DBA/2, B6C3F1 and BDF1).

production of C3H/HeN mice was much higher than that of other strains (Fig. 2b). These observations lead us to speculate that IL-4 and IFN-γ produced by NKT cells work as promoting and suppressing factors, respectively, during NKT cell proliferation.

NKT cell proliferation partially depends on IL-4 and is enhanced by Th2 cytokines

We next examined the influence of IL-4 on NKT cell proliferation *in vitro*. Proliferation of these cells was accelerated by addition of IL-4 at the start of the culture period, an effect that could be partially suppressed by neutralization of IL-4 (Fig. 3). In the C3H/HeN strain, where proliferation of NKT cells was not robust, a more significant induction of proliferation by IL-4 was observed (Fig. 4). In addition, neutralization of IFN-γ using antibodies did not significantly change the proportion of NKT cells in the total cell population. However, this did appear to up-regulate the total number of living cells and lead to a concomitant increase in the total number of NKT cells (Fig. 4b). Only NKT cells can produce IL-4 when cultured with α-GalCer and IL-2,[23] so IL-4 must act as an autocrine growth factor in the expansion of NKT cells in this context.

The proportion of intracellular IFN-γ high positive NKT cells is reduced by addition of IL-4

Exogenous IL-4 promoted NKT cell expansion in C3H/HeN mice, as shown in Figs 3 and 4. We next examined

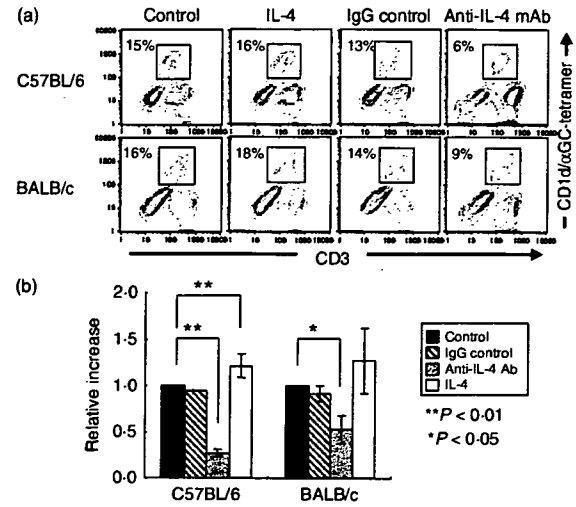

Figure 3. Expansion of natural killer T (NKT) cells in the presence or absence of interleukin (IL)-4. (a) Spleen cells (1×10^6 cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml IL-2 for 4 days with IL-4 (10 ng/ml) or anti-IL-4 monoclonal antibody (mAb) (1 mg/ml). The percentages of NKT cells are shown. Data are representative of replicate experiments. (b) The relative increase was based on absolute numbers of NKT cells and was compared with control expansion culture. Data are shown as mean ± standard deviation for five independent experiments. A paired two-tailed Student's *t*-test was used for statistical analysis (*$P < 0·05$; **$P < 0·01$).

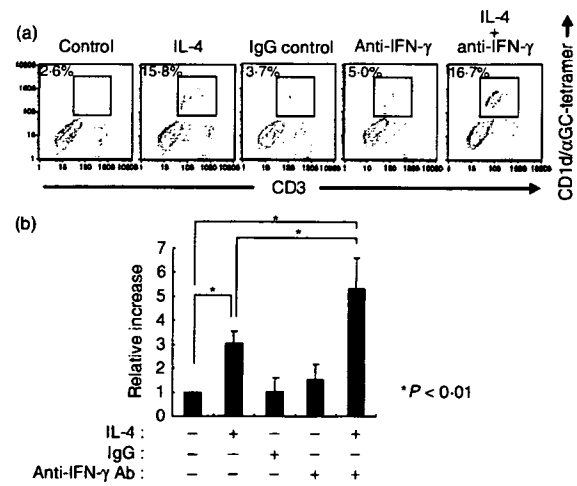

Figure 4. Expansion of natural killer T (NKT) cells from C3H/HeN strain mice in conditions that favour production of T helper type 2 (Th2)-biased cytokines. (a) Spleen cells (1×10^6 cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml interleukin (IL)-2 and with IL-4 (10 ng/ml) and/or anti-interferon (IFN)-γ monoclonal antibody (mAb) (1 mg/ml) for 4 days. The percentages of NKT cells are shown. Data are representative of replicate experiments. (b) The relative increase was based on absolute numbers of NKT cells and was compared with the control expansion culture. Data are shown as mean ± standard deviation for seven independent experiments. A paired two-tailed Student's *t*-test was used for statistical analysis (*$P < 0·01$).

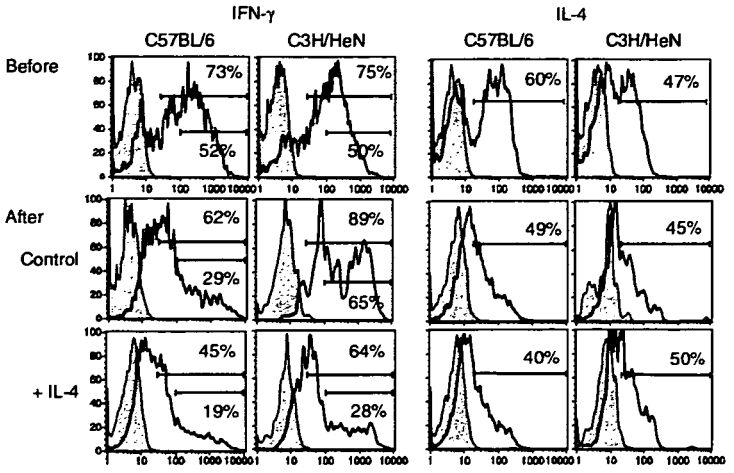

Figure 5. Cytokine production profile of natural killer T (NKT) cells treated with interleukin (IL)-4. Intracellular cytokine staining for interferon (IFN)-γ and IL-4 in NKT cells that were fresh (upper), cultured (middle), or cultured with additional IL-4 (lower) is shown. The cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 3 hr, stained with anti-CD3 monoclonal antibody (mAb), CD1d/α-galactosylceramide (α-GalCer) tetramer and anti-IFN-γ, anti-IL-4, or an isotype control mAb, and then detected and sorted via flow cytometry. Histogram panels for CD1d/α-GalCer-tetramer$^+$ CD3$^+$ cells are shown. Closed histograms indicate isotype controls. The percentage of total positive and high positive cells are indicated in the histograms. Data are representative of replicate experiments.

whether NKT cells cultured in Th2 conditions produced IFN-γ and IL-4. After 4 days of culture with α-GalCer and IL-2, intracellular IFN-γ- and IL-4-positive NKT cells were observed in both strains of mice. However, the proportion of intracellular IFN-γ high positive NKT cells was reduced when the cells were cultured with additional IL-4 (Fig. 5). In contrast to IFN-γ, the proportion of IL-4-positive NKT cells did not differ between cultures with and without IL-4. Therefore, NKT cells expanding as a result of induction with additional IL-4 displayed a polarized Th2 phenotype.

NKT cell expansion is accelerated by Th2-biased cytokine conditions

The SJL/J mouse strain is defective in cytokine production by NKT cells, as a consequence of a loss of high-affinity TCR to CD1d, which results from a deletion of the TCR Vβ8 subfamily genomic loci.[28,29] The proportion of NKT cells in the spleens of these mice was lower than that observed for other strains (Fig. 6a), and IFN-γ and IL-4 production after α-GalCer stimulation was also lower than that observed for other strains tested in this study (data not shown). NKT cells from SJL/J mice proliferated even in the absence of additional IL-4, as was observed for NKT cells from C57BL/6 mice. Moreover, similar to findings for NKT cells from C3H/HeN mice, the NKT cell proliferation effect could be enhanced by addition of IL-4 and further enhanced by addition of IL-4 combined with neutralization of IFN-γ (Fig. 6b).

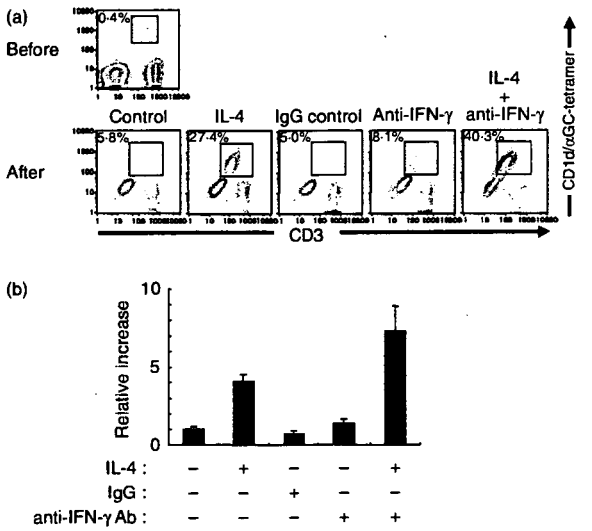

Figure 6. Expansion of natural killer T (NKT) cells from SJL/J mice *in vitro*. (a) Spleen cells (1×10^6 cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml interleukin (IL)-2 for 4 days with IL-4 (10 ng/ml) and/or anti-interferon (IFN)-γ monoclonal antibody (mAb) (1 mg/ml). The percentages of NKT cells are shown. Data are representative of replicate experiments. (b) The relative increase was based on absolute numbers of NKT cells and was compared with the control expansion culture. Data are shown as the mean of three wells ± standard deviation. Similar results were obtained in two independent experiments.

Discussion

In a previous study in which we induced expansion of NKT cells collected from human peripheral blood, we

observed wide variation in the efficiency of NKT cell expansion.[21] Similarly, when mouse NKT cells were induced to proliferate using similar methods in the present study, the ratios of expanding cell types were distinctly different in cells obtained from different mouse strains (Fig. 1). This suggests that genetic background influences or controls the difference in proliferation efficiency observed in humans and mice. However, we could not rule out the alternative possibility that the effect was a result of bipolar expansion of the cells, rather than originating from genetic variation in one or a few loci.

In this study, we have shown that the amount of IL-4 in the culture supernatant was related to the efficiency of NKT cell expansion induced by α-GalCer and IL-2. Previous studies revealed that addition of exogenous IL-2, IL-7 and IL-15 was able to augment NKT cell expansion by α-GalCer.[30–34] Similarly, in the present study we found that exogenous IL-2 augmented α-GalCer-induced NKT cell expansion in various mouse strains, with the exception of C3H/HeN mice. Moreover, addition of exogenous IL-4 promoted α-GalCer-induced NKT cell expansion in spleen cells from C3H/HeN mice. It has been shown that only NKT cells have the ability to produce IL-4 in this culture.[23] IL-4 might therefore be an autocrine or paracrine growth factor in α-GalCer-induced NKT cell expansion.

NKT cells, NK cells and some T cells when cultured with α-GalCer and IL-2 produce IFN-γ.[23] In contrast to IL-4, the amount of IFN-γ did not correlate with the efficiency of NKT cell expansion. Furthermore, we found that NKT cell proliferation in C3H/HeN mice was slightly increased by neutralization of IFN-γ in the culture. These results suggest that IFN-γ partially inhibits NKT cell expansion by α-GalCer. Interestingly, we found an inverse correlation between the IFN-γ:IL-4 ratio in the culture supernatant after 24 hr of culture and the efficiency of NKT cell proliferation (data not shown). Although higher amounts of IL-4 were detected in the culture of cells from C3H/HeN mice than in the culture of cells from C57BL/6 mice after 24 hr of culture, α-GalCer stimulated spleen cells from C3H/Ne mice produced higher amounts of IFN-γ and exhibited the highest IFN-γ:IL-4 ratio of all mouse strains tested. These results may explain the failure of NKT cell expansion in spleen cells from C3H/HeN mice.

The balance between the production of IFN-γ and the production of IL-4 by NKT cells is influenced by microenvironmental factors such as cytokines and antigen-presenting cells.[20,35–38] IL-7 and IL-12 selectively enhance IL-4 production by NKT cells.[35,36] Antigen-presenting cells such as α-GalCer-pulsed B cells selectively elicit weak IL-4 but not IFN-γ production from NKT cells.[37] There is a high IFN-γ:IL-4 ratio in cultures of spleen cells from C3H/HeN mice, which is caused by splenic NKT cells (A. Iizuka *et al.*, unpublished data) Moreover, it has been reported that the balance of IFN-γ:IL-4 production by NKT cells is developmentally controlled.[39,40] At immature stages, NKT cells predominantly produce IL-4, whereas IFN-γ secretion increases during the course of development.[39] Moreover, immature NKT cells have the ability to proliferate as compared with mature NKT cells.[39] Therefore, NKT cells in the spleen of C3H/HeN mice may be more mature than those of C57BL/6 mice, or contain only a few immature NKT cells. We assume that the failure of proliferation and the high IFN-γ:IL-4 cytokine production ratio of NKT cells in the spleen of C3H/HeN mice were attributable to their maturation stage.

Although IL-4 has opposite effects to IFN-γ and suppresses the Th1 immune response, IL-4 induces proliferation of human IL-13$^+$ NK cells[41] and CD8$^+$ T cells.[42] We found that Th2 culture conditions (in the presence of IL-4 and anti-IFN-γ mAb) facilitated NKT cell expansion induced by α-GalCer and IL-2 even in C3H/HeN and SJL/J mice. IL-4 also induces IFN-γ production by NK and NKT cells *in vivo*.[43] However, the proportion of IFN-γ-positive, but not IL-4-positive, NKT cells decreased when cells were cultured in the presence of IL-4. As in human immature IL-13$^+$ NK cells,[41] IL-4 may induce expansion of developmentally immature NKT cells which have a Th2-biased phenotype.

NKT cell maturation is controlled by the transcription factor T-bet.[44,45] Terminally differentiated NKT cells acquire a strong ability to produce IFN-γ and elicit cytotoxicity.[44] Assuming that expanded Th2-biased NKT cells after culture with α-GalCer, IL-2 and IL-4 are immature cells, it will be possible to induce terminally differentiated Th1-biased NKT cells for Th1 cell immunotherapy, such as cancer cell therapy.

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Bone marrow transplantation in children with severe aplastic anemia using a conditioning regimen containing 3 Gy of total body irradiation, cyclophosphamide with or without antithymocyte globulin

Inagaki J, Nagatoshi Y, Kawano Y, Saito Y, Takahashi D, Nagayama J, Shinkoda Y, Hirata H, Okamura J. Bone marrow transplantation in children with severe aplastic anemia using a conditioning regimen containing 3 Gy of total body irradiation, cyclophosphamide with or without antithymocyte globulin.
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Abstract: We have employed the 3 Gy toal body irradiation (TBI) containing conditioning regimen to bone marrow transplantation (BMT) for severe aplastic anemia (SAA) in pediatric patients irrespective of donor type since March 1986. The outcome of BMT for 17 SAA patients is favorable. Eight patients received BMT from human leukocyte antigen matched-related donors (MRD) and nine received BMT from alternative donors. The conditioning regimen consisted of 3-Gy TBI and cyclophosphamide of 200 mg/kg in the BMT from MRD. In the case of BMT from alternative donor, antithymocyte globulin 10 mg/kg was added to the regimen. Fifteen of 17 patients (88%) engrafted on median of day 18 (range, 11–26) and all 13 evaluable patients showed complete donor chimerism by median 30 (range, 13–47) days after BMT. Fourteen patients have survived with a median follow-up of 67 (range, 2–228) months and the probability of survival was 81.9% (95% CI, 63.3–100%). No late complications including second malignancies caused by TBI have been observed and all three female patients have regular menstruation. In conclusion, TBI of 3 Gy appears to be an appropriate dose regarding to ensure engraftment and avoid the risk of late adverse event for SAA patients.

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Key words: 3 Gy total body irradiation – severe aplastic anemia – children – engraftment – late complication

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HSCT from an HLA-MRD is an effective curative treatment for patients with SAA (1, 2). The standard conditioning regimen consists of CY and ATG has been widely used. However, in 2001, Kojima et al. (3) reported an unexpectedly high incidence of the graft rejection and mixed chimerism in patients with SAA who were transplanted with the CY/ATG regimen from MRD in a Japanese population. Since no similar high rejection rate after the same regimen has ever been previously reported, the reason for this observation was not clear. That regimen might be insufficient, from an immunosuppressive point of view, to sustain the engraftment at least in Japanese SAA patients. On the other hand, there is no standard conditioning regimen for HSCT from PMRD or MUD, and HSCT from alternative donors has a high rate of graft rejection and severe GVHD, thus resulting in a poor

Abbreviations: ATG, antithymocyte globulin; BMT, bone marrow transplantation; CMV, cytomegalovirus; CsA, cyclosporine A; CY, cyclophosphamide; DLI, donor lymphocyte infusion; FLU, fludarabine; G-CSF, granulocyte colony-stimulating factor; GVHD, graft-vs.-host disease; HLA, human leukocyte antigen; HSCT, hematopoietic stem cell transplantation; MRD, matched-related donor; MUD, matched-unrelated donor; PMRD, partially mismatched-related donor; SAA, severe aplastic anemia; sMTX, short-term methotrexate; TBI, total body irradiation.

outcome (4–6). In transplantation for SAA, the important risk factors for rejection responsible for the lower survival rate were the repeated episodes of prior transfusion, the prolonged duration of disease (7) and the disparity of HLA especially in class I alleles (8–10). To overcome graft rejection, many investigators have employed more intensive conditioning regimens including TBI and the outcomes of alternative donor transplantation have thus steadily improved (11, 12).

TBI is a powerful and reliable procedure to induce an intensive suppressive effect for the immune system as well as an anti-leukemic effect. The TBI dose of equal or over 12 Gy has been used for patients with malignant diseases such as leukemia and lymphoma. However, it is well known that TBI causes many late adverse events including hormonal disturbance, pulmonary complications, cataract, and neurological sequelae especially in children (13, 14). The risk of a second malignant tumor also increases in patients receiving the TBI-containing regimen (16–19). To obtain durable engraftment with minimal adverse effects of TBI, the appropriate dose of TBI should thus be determined in a conditioning regimen of HSCT for SAA patients.

Since March 1986, to avoid graft rejection, we have employed the 3-Gy TBI-containing regimen for BMT from MRD as well as for alternative donors in patients with SAA. We herein report the results of BMT for 17 SAA patients and the efficacy and safety for engraftment and late adverse effects with this regimen.

Patients and methods

Patients

A total of 17 patients with acquired SAA were transplanted at the Section of Pediatrics, National Kyushu Cancer Center, between March 1986 and June 2005. The characteristics of patients are shown in Table 1. The patients consisted of 14 males and three females with a median age of 10 (range, 2–19) yr at the time of BMT. The median interval from diagnosis to BMT was three (range, 1–126) months and 11 of 17 patients had received various systemic immunosuppressive therapies including ATG, CsA, methylprednisolone with or without G-CSF before BMT. One of the 17 patients (UPN 13) suffered from hepatitis-associated SAA.

Donor selection was based on the serological typing for HLA-A, HLA-B, and DR until 1997. Since 1998, high-resolution typing of HLA-A, HLA-B, and DRB1 loci was examined by DNA-based methods. Eight patients received BMT from HLA-MRD. Two patients received BMT from HLA one-locus mismatched-related donor. One (UPN 22) of these patients received BMT from her mother and the other (UPN 94) from his uncle. All related BMT were performed within four months from diagnosis. Seven patients underwent unrelated BMT. All of them failed to respond to conventional immunosuppressive therapies and had been dependent on transfusions of red blood cells or platelets. Two (UPN 202 and UPN 290) of seven patients were found to have mismatches in HLA by high-resolution typing. Eight recipient–donor pairs were ABO-incompatible and nine pairs were sex mismatched.

Conditioning regimen

All patients received TBI of the 3 Gy-based conditioning regimen irrespective of the donor type and HLA disparity. The regimen consists of TBI 3 Gy in a single fraction at a dose rate of 0.07 Gy/min using 10 MeV X-rays with R-L

Table 1. Patient characteristics

UPN	Sex	Age at BMT (yr)	Interval from diagnosis to BMT (months)	Prior treatment	Pretransplant transfusions (times)		Donor		Donor type	HLA disparity		Conditioning regimen	GVHD prophylaxis
					Red cells	Platelets	Sex	Age		Antigen	DNA		
13	M	3	2	+	1	6	F	6	Sibling	0	NE	TBI/CY	CsA
55	F	6	2	–	2	1	F	5	Sibling	0	NE	TBI/CY	CsA
76	M	14	1	–	0	?	F	21	Sibling	0	NE	TBI/CY	CsA
110	M	2	1	–	4	8	M	4	Sibling	0	NE	TBI/CY	CsA
125	M	10	3	+	1	2	F	15	Sibling	0	NE	TBI/CY	CsA + sMTX
152	F	6	1	–	3	5	F	9	Sibling	0	0	TBI/CY	CsA
216	F	9	4	+	4	5	F	47	Parent	0	0	TBI/CY/ATG	CsA + sMTX
300	M	16	2	–	2	2	F	12	Sibling	0	0	TBI/CY	CsA + sMTX
22	M	10	1	+	11	12	F	36	Parent	1	NE	TBI/CY	CsA + sMTX
94	M	16	2	–	6	12	M	38	Uncle	1	NE	TBI/CY/ATG	CsA + sMTX
158	M	6	21	+	?	?	F	43	Unrelated	0	0	TBI/CY	CsA + sMTX
193	M	17	99	+	>10	>50	M	28	Unrelated	0	0	TBI/CY/ATG	FK506 + sMTX
202	M	9	22	+	48	100	M	28	Unrelated	0	1	TBI/CY/ATG	FK506 + sMTX
206	M	15	55	+	32	148	F	28	Unrelated	0	0	TBI/CY/ATG	FK506 + sMTX
232	M	7	38	+	>50	>50	F	24	Unrelated	0	0	TBI/CY/ATG	FK506 + sMTX
286	M	19	72	+	15	1	M	47	Unrelated	0	0	TBI/CY/ATG	FK506 + sMTX
290	M	17	126	+	>10	28	F	40	Unrelated	0	2	TBI/CY/ATG	FK506 + sMTX

UPN, unique patient number; GVHD, graft-versus-host disease; NE, not examined; TBI, total body irradiation; CY, cyclophosphamide; ATG, antithymocyte globulin; CsA, cyclosporine A; sMTX, short term methotrexate; FK506, tacrolimus.

portals on day −6 and CY 50 mg/kg/day on day −5 to −2 in the BMT from MRD. When the donor is PMRD or unrelated, ATG (Zetbulin®; Fresenius Biotech GmbH, Hamburg, Germany) 2.5 mg/kg/day on day −5 to −2 was added to the regimen. UPN 22, undergoing BMT from one-locus mismatched mother on December 1987, did not receive ATG because ATG was difficult to obtain in those days. In the case of UPN 158, he was conditioned TBI/CY without ATG notwithstanding unrelated BMT.

Prophylaxis for GVHD

In the case of BMT from MRD, CsA with sMTX 15 mg/m^2 on day 1 and 10 mg/m^2 on days 3, 6 and 11 were used when the patient was equal to or over 10 yr of age and CsA alone for others. In BMT from PMRD, CsA with sMTX was used. Tacrolimus (FK506) with sMTX was given for BMT from MUD except for one patient (UPN 158) because FK506 could not be used at that time in Japan.

Transplantation

All patients received bone marrow without *ex vivo* T-cell depletion. In seven cases of major ABO incompatibilities with their donors, red cells were removed from the harvested marrow by centrifugation in the cell separator. The median transplanted nuclear cell count was 3.1 (range, 0.3–8.5) × 10^8 per recipient body weight. For recent patients, recombinant human G-CSF was given intravenously to facilitate the engraftment of neutrophils from day 7 and immunoglobulin was given weekly for prophylaxis against CMV infection.

Evaluation of engraftment and chimerism

Engraftment was defined as the first day the absolute neutrophil count reached 0.5 × 10^9/L for three consecutive days within 28 days post-transplantation. Both the platelets and reticulocytes were considered to have recovered when they achieved counts of over 20 × 10^9/L and over 1% without transfusion support.

A chimerism analysis was performed using bone marrow samples when engraftment was observed. Three early patients who received sex-mismatched BMT were analyzed by G-banding in sex chromosome of bone marrow samples. For recent patients, fluorescence *in situ* hybridization with sex chromosomes for five sex-mismatched BMT and short-tandem repeat polymerase chain reaction for five sex-matched BMT were carried out.

Results

Engraftment and chimerism

The clinical data of patients after BMT are shown in Table 2. Fifteen of 17 patients (88%) engrafted on median of day 18 (range, 11–26). The platelet and reticulocyte recovery was achieved on median of day 39 (range, 15–160) and day 35 (range, 13–88), respectively. Two patients donated from MUD rejected their grafts. In one of the two patients (UPN 290), the graft was genotypically two-loci mismatched for HLA-A and HLA-B loci. He received an unrelated cord blood transplantation 40 days after first BMT. In another case (UPN 202), the graft was one-locus mismatched in allelic level at DRB1 and only 0.3 × 10^8/kg mononuclear cells were transplanted. He received CD34-positive peripheral blood stem cell transplantation from his mother 45 days after first BMT. They died from encephalitis and sepsis without engraftment.

The data on 13 patients were available for a chimerism analysis, and all 13 patients showed

Table 2. Outcome of BMT

UPN	Engraftment (days)			Complete donor chimerism (days)	GVHD		Major complications	Outcome (follow-up months)
	ANC	Plt	Reti		Acute	Chronic		
13	20	15	13	17	Grade I	Limited	–	Alive (228)
55	11	17	22	NE	0	–	–	Alive (125)
76	12	25	32	13	Grade II	–	Hemorrhagic cystitis	Alive (84)
110	26	40	55	NE	Grade II	–	–	Alive (106)
125	15	160	67	13	Grade III	Limited	Avascular necrosis	Alive (67)
152	12	18	21	21	0	–	–	Alive (76)
216	23	84	33	43	Grade II	Limited	EBV-LPD	Alive (43)
300	15	20	33	47	Grade III	–	Pulmonary aspergillosis	Alive (4)
22	23	23	46	25	Grade I	Limited	Hemorrhagic cystitis	Alive (188)
94	19	30	88	28	Grade II	–	Pulmonary aspergillosis	Alive (121)
158	20	22	19	30	Grade II	Extensive	–	Alive (74)
193	18	25	22	32	0	–	–	Alive (38)
202	NA	NA	NA				Graft rejection	Died (2)
206	20	36	38	31	Grade IV	Limited	TMA, Adenoviremia	Died (5)
232	13	18	18	30	0	–	–	Alive (36)
286	18	25	22	30	0	–	–	Alive (7)
290	NA	NA	NA				Graft rejection	Died (2)

ANC, absolute neutrophil counts; Plt, platelet counts; Reti, reticulocyte counts; NA, not achieved; NE, not evaluable; TMA, thrombotic microangiopathy; EB-LPD, Epstein–Barr virus-associated lymphoproliferative disorder.

complete donor chimerism by a median of 30 (range, 13–47) days after BMT.

GVHD

Ten patients (59%) developed acute GVHD. Acute GVHD of grades II–IV occurred in 63% of matched-related BMT and 33% of alternative donor BMT. Acute GVHD of grade III or IV occurred in two patients. Six patients (35%) developed chronic GVHD and one of these six patients had extensive type (skin and oral mucosa). All survivors were free from immunosuppressive therapy at the time of the last follow-up.

Complications

Viral infections were the most frequent complication. CMV reactivation occurred in four patients and they were treated successfully with ganciclovir. No patient developed CMV disease. One patient (UPN 216) developed an Epstein–Barr virus-associated lymphoproliferative disorder and thus received a DLI twice. She recovered from the disease after DLI although acute GVHD of grade II occurred post-DLI. Invasive pulmonary aspergillosis occurred in two patients. One patient required a right upper lobectomy six months after BMT while the other was treated successfully with intravenous amphotericin B followed by voriconazole. Two patients developed hemorrhagic cystitis a few days after the administration of CY. They were treated with hydration and diuretics and soon thereafter improved. The conditioning regimen was well tolerated and neither grade IV mucositis/stomatitis nor febrile neutropenia was seen in our patients.

Survival

Fourteen patients survived with a median follow-up of 67 (range, 2–228) months and the probability of event-free survival was 82% (95% CI, 63–100%). All eight patients who received matched-related BMT and six of nine patients who were transplanted from alternative donors were alive with a median follow-up of 80 (range, 4–228) and 36 (range, 2–188) months, respectively [overall survival rate, 100% and 67% (95% CI 36–97%), respectively]. Two patients died of infectious complications after the rejection in unrelated BMT. One patient died of a disseminated adenoviral infection following acute GVHD of grade IV.

Late adverse effect

At the time of the last follow-up, no short stature of more than 2 SD below the mean was observed in eight patients younger than 18 yr of age and the remaining six older patients achieved their expected final heights. There were no obvious adverse events of TBI such as cataracts, hormonal disturbances including the gonadal and thyroid function or pulmonary complications. Regular menstruation was observed in all three female patients. To date, no patient has developed any secondary malignant disease.

Discussion

In the 1970s, most SAA patients who had MRD received high-dose CY alone as a conditioning for BMT and graft rejection was observed in 30–60% (20, 21). Rejection was thought to result from the sensitization of patients to the minor histocompatibility antigens of donors induced by the previous blood product transfusion (22, 23). To reduce the risk of graft rejection, more intensive conditioning regimens which combine CY with other agents such as ATG, procarbazine or with radiation (24–28) and newly devised methods such as the addition of the donor's peripheral blood buffy-coat cells to the marrow (29, 30) were developed. In 1983, Feig et al. (27) showed encouraging results in this setting with a new conditioning regimen consisting of CY and TBI of 3 Gy. In this report, only one of 46 consecutive patients rejected the grafts and the two-yr survival rate was 82% for patients < 25 yr of age. This result was instructive for us and since then we have employed the CY and 3 Gy TBI-based regimen in BMT for SAA patients. On the other hand, Storb et al. (29) reported favorable results for matched-related BMT using a regimen consisting of CY and ATG with CsA plus sMTX for the prophylaxis of GVHD. To date, they have published a series of outcomes and proved the feasibility of this strategy (1, 31).

Because it is well known that TBI causes many late adverse events, a non-TBI regimen is indeed preferable to avoid graft rejection. However, Kojima et al. (3) reported the results of 40 Japanese SAA patients transplanted from MRD using the CY/ATG regimen and the incidence of early or late graft rejection and mixed chimerism was 20% and 25%. As a result, only 71% of the patients were able to achieve a normal marrow function. The presentation suggested that immunosuppressive effect of the regimen might be insufficient to sustain engraftment at least in Japanese SAA patients. Although our study consisted of a small number of patients, we experienced no cases who showed graft rejection in matched-related BMT and all evaluated patients achieved complete chimerism. In

addition, no late adverse complications of TBI were observed in all survivors including nine patients followed more than five yr. However, it would be controversial to adopt TBI-containing regimen for matched sibling BMT in SAA nowadays. The substitution for TBI such as FLU might be considered in this setting.

In the case of HSCT from an alternative donor, the prevention of graft rejection becomes a more crucial problem because of the high incidence of rejection (4, 6, 32, 38). TBI has commonly been included in conditioning regimens to prevent graft rejection because of its powerful and reliable immunesuppressive effect (11, 12, 33, 34). However, the optimal dose of TBI remains to be elucidated. In this regard, the National Marrow Donor Program conducted a prospective study to determine the minimum dose of TBI to sustain the engraftment (35, 36). In this study, in case of HLA-identical transplant, only one of the 35 patients who were conditioned with 2 Gy TBI regimen experienced graft failure. No other patients who received TBI with doses higher than 4 Gy showed graft rejection regardless of HLA disparity. This study demonstrated that, in the HLA-identical unrelated BMT, TBI of 2 Gy in combination with CY 200 mg/kg and ATG are effective enough to allow for a durable engraftment with a low toxicity and high survival rate. In addition, the importance of low-dose TBI was thus proven to sustain engraftment even in the HLA-mismatched unrelated BMT. The retrospective analysis of the outcome for 154 SAA patients identified through the Japan Marrow Donor Program demonstrated the possibility that the regimen consist of low-dose TBI (2–5 Gy), CY 200 mg/kg and ATG can be sufficient to sustain engraftment in patients who receive a transplantation even from HLA-mismatched unrelated donor (8). In our two rejected patients, UPN 290 had received the multiple transfusions for 125 months before BMT and he received two-loci mismatched graft in HLA-A and HLA-B allele. The other rejected patient (UPN 202) had been heavily transfused prior to BMT and transplanted only 0.3×10^8/kg mononuclear cells from an HLA one-locus mismatched donor in HLA-DRB1 allele. Given these disadvantages of the transplant procedures in two unrelated BMT cases, our study showed that the conditioning regimen with TBI of 3 Gy is feasible to allow for the rapid and sustained engraftment of an alternative marrow in pediatric SAA patients. However, the feasibility of this regimen for HLA-mismatched unrelated BMT remains unclear. Recently, an interesting study in 38 SAA patients transplanted from alternative donor was reported from Severe Aplastic Anemia Working Party of the European Group for Blood and Marrow Transplantation (37). To avoid the adverse effect of TBI, they employed non-TBI conditioning regimen consisted of CY, ATG and FLU resulted in a preferable survival (73%), however, the incidence of graft failure was reported to be relatively high (18%). Given the fact that graft rejection has been a major cause of death according to previous studies, we consider that the low-dose TBI should thus be included in the conditioning regimen for the successful engraftment and a better prognosis, especially in alternative donor HSCT, even though there have also been several small studies employing FLU or FLU plus alemtuzumab instead of TBI which have shown favorable results (39–41). Certainly, FLU is promising agent for inducing profound immunosuppression and it has been frequently adopted as a part of a conditioning regimen for reduced-intensity stem cell transplantation. It is warranted to determine whether FLU could be substituted for TBI.

In our series, in the BMT from alternative donors, the incidence of grades II–IV acute GVHD was 33%, which was comparable or lower than those reported in other studies of unrelated HSCT (11, 12, 42). The use of ATG in the conditioning regimen and FK506 plus sMTX for the prophylaxis of GVHD is considered to contribute to the low incidence of acute GVHD. On the other hand, in matched-related BMT, 63% patients developed grades II and III acute GVHD, and this percentage was higher than that of the other studies in matched-related HSCT (1, 3, 24, 31) although only acute GVHD of grade II, a non-severe form, occurred in most of our cases. Chronic GVHD occurred in six patients of whom only one patient experienced extensive type chronic GVHD, while all of them were finally able to discontinue the immunosuppressive agent. The major factor of morbidity and mortality for transplanted SAA patients was chronic GVHD (15) and because the nature of the underlying disease are required no graft-vs.-tumor effect, thus reducing the incidence of chronic GVHD of the extensive type is one of the most important factors to improve survival. In addition, it is known that the major risk factor of chronic GVHD is preceding severe acute GVHD. Although the number of cases investigated in this study was too small to draw any definitive conclusions, the cause of the high incidence of acute GVHD in matched-related BMT in this study is thus open to further discussion.

Recovery of the gonadal function and pregnancy has only been reported in a small number of patients conditioned with high-dose TBI-containing regimen (43–46). Sanders et al. (43) described the outcome of pregnancies after HSCT. In 995 patients conditioned with TBI of equal or over 10 Gy, 10% of adult female and 18% of adult male recovered their ovarian and testicular function. Thirteen female patients and partners of five male patients had 24 pregnancies after transplantation. The risk of secondary malignant neoplasm, especially of solid tumors after BMT increased in patients conditioned with high-dose TBI or limited-field irradiation (16, 18, 19). Curtis et al. (16) reported that the risk of solid cancer increased significantly with the increasing doses of radiation such as a single dose of more than 10 Gy or fractionated doses of more than 12 Gy. As radiation-induced tissue damage depends on the total dose, the number of fractionations, the dose rate and distribution (34), lower-dose TBI regimen might thus make it possible to reduce the risk of infertility and secondary malignancy. All of our three female patients have normal gonadal functions and regular menstruations although a careful follow-up is needed regarding their fertility. In addition, none of our patients, including the four cases observed for over 10 yr after BMT, has so far developed any secondary malignant disease. Further, large-sized studies with a long-term follow-up are required to estimate the influence of 3-Gy TBI.

We consider that the most important precedence for HSCT is to achieve durable engraftment and this priority became more crucial for SAA patients. In our study, as well as in recent publications, low-dose TBI has played an essential role in successful engraftment. In the present study, 15 of 17 patients achieved engraftment and complete donor chimerism was confirmed in all 13 evaluable patients soon after engraftment. In addition, the survival rate was 82% and all eight patients transplanted from MRD sustained durable engraftment. In alternative donor BMT, except for two patients with high-risk conditions for engraftment, seven patients achieved durable engraftment and six of these seven patients were alive and well. At present, no late complications caused by TBI have been observed. Our regimen has thus shown satisfactory results and the TBI of 3 Gy therefore appears to be an appropriate dose for combination with CY with or without ATG in order to achieve successful engraftment and reduce the risk of late complications for SAA patients.

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Successful Engraftment in Reduced-Intensity Cord Blood Transplantation (CBT) as a Salvage Therapy for Graft Failure After Primary CBT in Adults

Accumulating evidence strongly supports the efficacy of umbilical cord blood transplantation (CBT) in adults (*1*, *2*). This now becomes a standard alternative to bone marrow or peripheral blood stem cell transplantation for patients who lack a human leukocyte antigen (HLA)-matched donor. However, surprisingly high incidence of graft failure (GF) after CBT (7–40%) has been reported (*2–4*). The second CBT could be a therapeutic strategy to rescue patients with GF, but very few cases of successful engraftment by the second CBT for patients with GF after primary CBT have been reported (*4–6*).

In the past few years, we performed the second CBT in four cases with primary GF after CBT and all cases successfully achieved engraftment as summarized in Table 1. In these salvage CBTs, we paid attention to following three points. First, we tried to make a confirmation of GF and decision to perform the salvage CBT as quickly as possible. The confirmation of GF was made by no donor chimerism in bone marrow cells on day 28 or by no sign of hematopoietic recovery until day 35 (week 5) after primary CBT. Finally, the salvage CBT was performed before day 42 (week 6). The earlier application of salvage CBT while patients still have better performance status without infection or organ toxicities may improve the engraftment and survival.

Second, considering toxicities of conditioning regimen used for primary CBT, reduced-intensity CBT was chosen for the second transplant to avoid regimen-related toxicity and mortality. Because strong immunosuppression has a clear advantage over engraftment, we used fludarabine-based preparative regimen. Subsequent conditioning therapy including fludarabine within a short duration after primary transplant and strong graft-versus-host disease (GVHD) prophylaxis could cause a high risk of infection, particularly cytomegalovirus (CMV) in CBT. However, only subclinical CMV infection occurred, which was well controllable with preemptive administration of ganciclovir. Acute GVHD was also mild.

Third, to intensify the immunosuppression in combination with a key drug tacrolimus, we utilized mycophenolate mofetil (MMF) instead of methotrexate (MTX) which was used in the first CBT in cases 1, 2 and 3 for the following two reasons.

1. MMF has been reported to cause lower incidence of mucositis compared with MTX (*7*).
2. Although mechanism has not been elucidated, several reports have suggested that a GVHD prophylaxis regimen containing MMF after allogeneic transplantation is associated with faster engraftment (*7–10*).

Our retrospective observation also shows the promotional effect of MMF in hematopoietic engraftment (data not shown), but further studies are necessary to decide the optimal dose of MMF for stem cell transplantation.

TABLE 1. Patient characteristics

	Case 1		Case 2		Case 3		Case 4	
Age/sex	55/female		53/male		45/female		23/female	
Disease status	ALL, 2nd CR		APL, 2nd CR		DLBL, 2nd CR		SAA-post CBT secondary GF	
Transplantation	1st	2nd	1st	2nd	1st	2nd	2nd	3rd
Conditioning regimen	TBI-CY	Flu-BU	TBI/CY	Flu-BU	TBI-CY	Flu-BU	TBI-Flu-Mel	TBI-Flu-Mel
HLA matching	5/6	5/6	4/6	4/6	4/6	5/6	4/6	4/6
Total cell dose ($\times 10^7$/kg)	2.81	2.44	2.07	2.01	4.01	2.28	2.41	4.1
CD34$^+$ cell dose ($\times 10^5$/kg)	3.7	0.43	0.77	0.52	0.63	1.16	0.63	1.64
GVHD prophylaxis	CyA+sMTX	FK506+MMF	CyA+sMTX	FK506+MMF	FK506+sMTX	FK506+MMF	FK506	FK506+MMF
Day of second transplant	Day 37		Day 39		Day 39		Day 42	
Days to neutrophils $>0.5\times 10^9$/L	Day 42		Day 32		Day 31		Day 19	
Days to platelets $>20\times 10^9$/L	Day 129		Not reached		Not reached		Day 166	

ALL, acute lymphoblastic leukemia; APL, acute promyelocytic leukemia; DLBL, diffuse large B-cell lymphoma; SAA, severe aplastic anemia; CR, complete remission; CBT, cord blood transplantation; GF, graft failure; TBI, total body irradiation; Flu, fludarabine; BU, busulfan; Mel, melphalan; GVHD, graft-versus-host disease; CyA, cyclosporine A; sMTX, short-term methotrexate; MMF, mycophenolate mofetil; FK506, tacrolimus.

We all have to recognize the fact that GF can possibly occur in approximately one third of adult CBT, particularly in the case of low transplant cell numbers. It would be important to make sure of a cord blood unit for salvage transplant as early as possible, and not to lose a chance to make a decision of the salvage CBT to avoid life-threatening complications. Further clinical studies are necessary to establish reduced-intensity CBT as a salvage therapy for primary GF.

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