24. Ho AY, Pagliuca A, Kenyon M, et al. Reduced-intensity allogeneic hematopoietic stem cell transplantation for myelodysplastic syndrome and acute myeloid leukemia with multilineage dysplasia using fludarabine, busulphan, and alemtuzumab (FBC) conditioning. *Blood.* 2004;104:1616-1623.
25. van Besien K, Artz A, Smith S, et al. Fludarabine, melphalan, and alemtuzumab conditioning in adults with standard-risk advanced acute myeloid leukemia and myelodysplastic syndrome. *J Clin Oncol.* 2005;23:5728-5738.
26. Lim ZY, Ho AY, Ingram W, et al. Outcomes of alemtuzumab-based reduced intensity conditioning stem cell transplantation using unrelated donors for myelodysplastic syndromes. *Br J Haematol.* 2006;135:201-209.
27. de Lima M, Couriel D, Thall PF, et al. Once-daily intravenous busulfan and fludarabine: clinical and pharmacokinetic results of a myeloablative, reduced-toxicity conditioning regimen for allogeneic stem cell transplantation in AML and MDS. *Blood.* 2004;104:857-864.

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

Akira Iizuka,[1,4,5] Yoshinori Ikarashi,[1] Mitsuzi Yoshida,[1] Yuji Heike,[5] Kazuyoshi Takeda,[6] Gary Quinn,[3] Hiro Wakasugi,[2] Masanobu Kitagawa[4] and Yoichi Takaue[5]

[1]*Chemotherapy and* [2]*Pharmacology Divisions, and* [3]*Section for Studies on Metastasis, National Cancer Center Research Institute,* [4]*Department of Comprehensive Pathology, Aging and Developmental Sciences, Tokyo Medical and Dental University, Graduate School,* [5]*Hematopoietic Stem Cell Transplantation/Immunotherapy Unit, National Cancer Center Hospital, and* [6]*Department of Immunology, Juntendo University School of Medicine, Tokyo, Japan*

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Received 31 January 2007; revised 20 August 2007; accepted 4 September 2007.
Correspondence: Dr Y. Ikarashi, Chemotherapy Division, National Cancer Center Research Institute, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104-0045, Japan.
Email: yikarash@gan2.ncc.go.jp
Senior author: Dr Y. Ikarashi

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

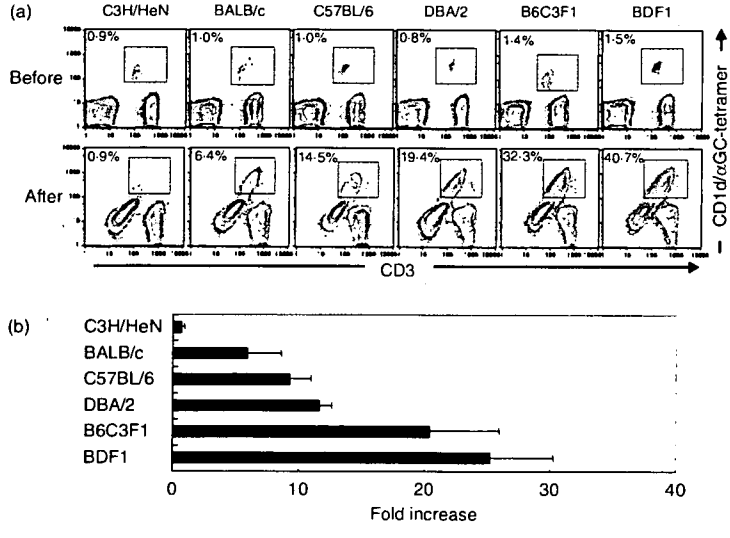

Figure 1. Expansion of natural killer T (NKT) cells *in vitro*. (a) Mouse spleen cells (1 × 10^6 cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml interleukin (IL)-2 for 4 days. Cells were stained with anti-CD3 monoclonal antibody (mAb) and CD1d/α-GalCer tetramer and 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).

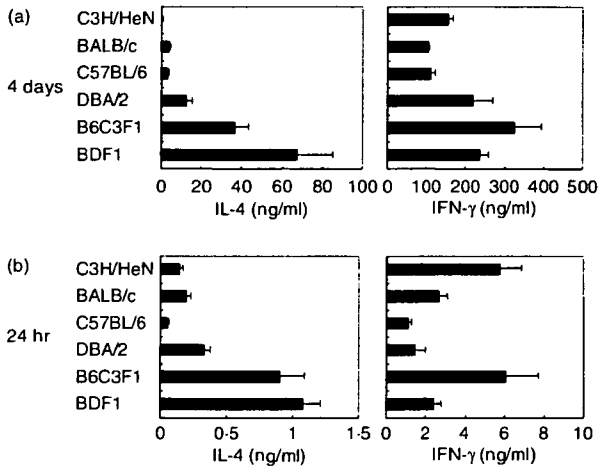

Figure 2. Production of interleukin (IL)-4 and interferon (IFN)-γ in expansion cell culture supernatants. Mouse spleen cells (1×10^6 cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml IL-2 for 4 days. Supernatants were collected after 24 hr (b) or 4 days (a). The levels 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

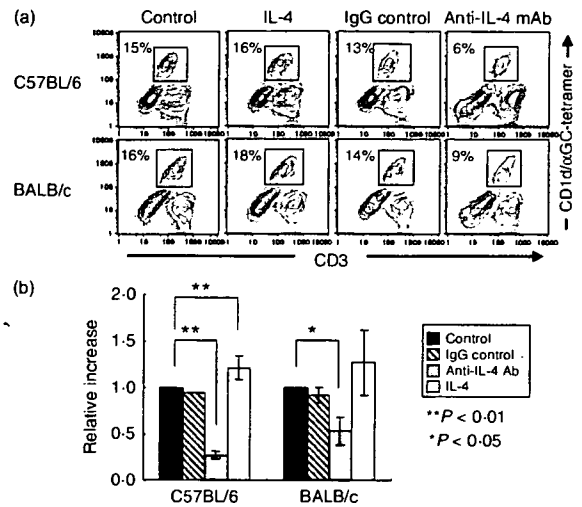

Figure 3. Expansion of natural killer T (NKT) cells in the presence or absence of interleukin (IL)-4. (a) Spleen cells (1×10^6 cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml IL-2 for 4 days with IL-4 (10 ng/ml) or anti-IL-4 monoclonal antibody (mAb) (1 mg/ml). The 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 (*$P < 0·05$; **$P < 0·01$).

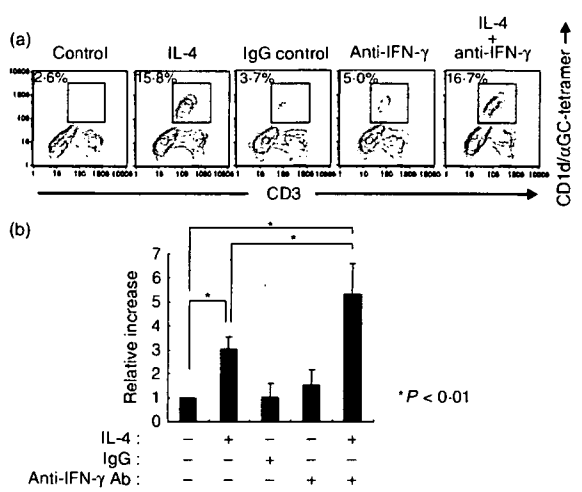

Figure 4. Expansion of natural killer T (NKT) cells from C3H/HeN strain mice in conditions that favour production of T helper type 2 (Th2)-biased cytokines. (a) Spleen cells (1×10^6 cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml interleukin (IL)-2 and with IL-4 (10 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 (*$P < 0·01$).

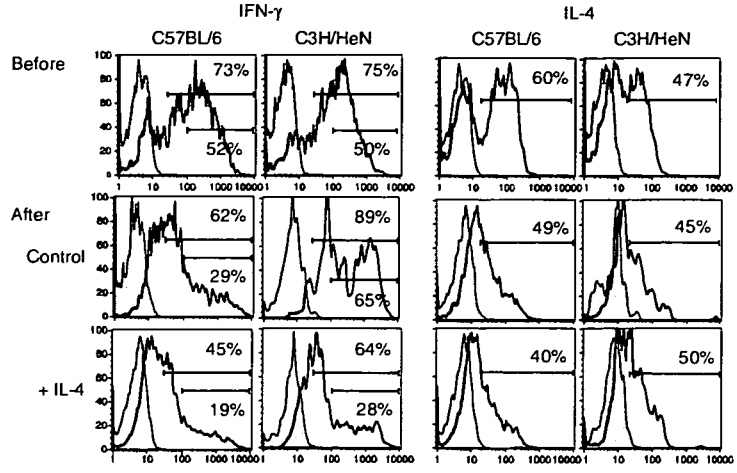

Figure 5. Cytokine production profile of natural killer T (NKT) cells treated with interleukin (IL)-4. Intracellular cytokine staining for interferon (IFN)-γ and IL-4 in NKT cells that were fresh (upper), cultured (middle), or cultured with additional IL-4 (lower) is shown. The cells were stimulated 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).

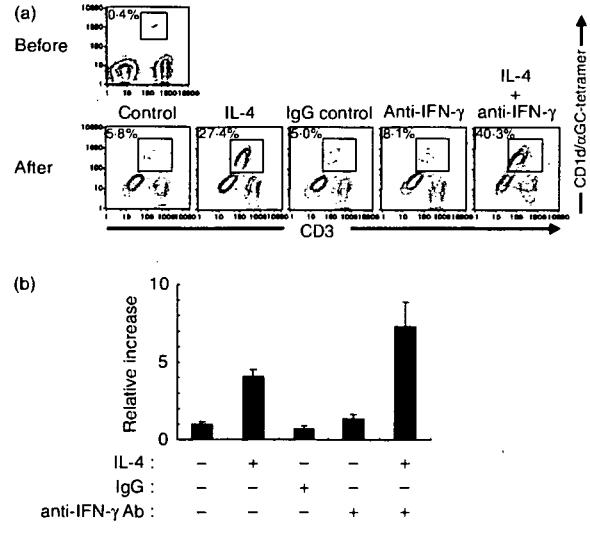

Figure 6. Expansion of natural killer T (NKT) cells from SJL/J mice *in vitro*. (a) Spleen cells (1×10^6 cells/ml) were cultured with 50 ng/ml α-galactosylceramide (α-GalCer) and 100 U/ml interleukin (IL)-2 for 4 days with IL-4 (10 ng/ml) and/or anti-interferon (IFN)-γ monoclonal antibody (mAb) (1 mg/ml). 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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References

1 Ballas ZK, Rasmussen W. NK1.1$^+$ thymocytes. Adult murine CD4$^-$, CD8$^-$ thymocytes contain an NK1.1$^+$, CD3$^+$, CD5^{hi}, CD44^{hi}, TCR-Vβ 8$^+$ subset. *J Immunol* 1990; **145**:1039–45.

2 Godfrey DI, Hammond KJ, Poulton LD, Smyth MJ, Baxter AG. NKT cells: facts, functions and fallacies. *Immunol Today* 2000; 21:573–83.

3 Makino Y, Koseki H, Adachi Y, Akasaka T, Tsuchida K, Taniguchi M. Extrathymic differentiation of a T cell bearing invariant Vα14 Jα281 TCR. *Int Rev Immunol* 1994; 11:31–46.

4 Gapin L, Matsuda JL, Surh CD, Kronenberg M. NKT cells derive from double-positive thymocytes that are positively selected by CD1d. *Nat Immunol* 2001; **2**:971–8.

5 Mattner J, Debord KL, Ismail N *et al.* Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature* 2005; **434**:525–9.

6 Nishimura T, Santa K, Yahata T *et al.* Involvement of IL-4-producing Vβ 8.2$^+$ CD4$^+$ CD62L$^-$ CD45RB$^-$ T cells in non-MHC gene-controlled predisposition toward skewing into T helper type-2 immunity in BALB/c mice. *J Immunol* 1997; **158**:5698–706.

7 van Der Vliet HJ, Nishi N, de Gruijl TD, von Blomberg BM, van den Eertwegh AJ, Pinedo HM, Giaccone G, Scheper RJ. Human natural killer T cells acquire a memory-activated phenotype before birth. *Blood* 2000; **95**:2440–2.

8 Godfrey DI, Kronenberg M. Going both ways: immune regulation via CD1d-dependent NKT cells. *J Clin Invest* 2004; **114**:1379–88.

9 Arase H, Arase N, Kobayashi Y, Nishimura Y, Yonehara S, Onoe K. Cytotoxicity of fresh NK1.1$^+$ T cell receptor α/β$^+$ thymocytes against a CD4$^+$8$^+$ thymocyte population associated with intact Fas antigen expression on the target. *J Exp Med* 1994; **180**:423–32.

10 Nieda M, Nicol A, Koezuka Y *et al.* TRAIL expression by activated human CD4$^+$Vα24NKT cells induces in vitro and in vivo apoptosis of human acute myeloid leukemia cells. *Blood* 2001; **97**:2067–74.

11 Nicol A, Nieda M, Koezuka Y, Porcelli S, Suzuki K, Tadokoro K, Durrant S, Juji T. Human invariant Vα24$^+$ natural killer T cells activated by α-galactosylceramide (KRN7000) have cytotoxic anti-tumour activity through mechanisms distinct from T cells and natural killer cells. *Immunology* 2000; **99**:229–34.

12 Mattarollo SR, Kenna T, Nieda M, Nicol AJ. Chemotherapy pretreatment sensitizes solid tumor-derived cell lines to Vα24$^+$ NKT cell-mediated cytotoxicity. *Int J Cancer* 2006; **119**:630–7.

13 Smyth MJ, Thia KY, Street SE *et al.* Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med* 2000; **191**:661–8.

14 Seino K, Motohashi S, Fujisawa T, Nakayama T, Taniguchi M. Natural killer T cell-mediated antitumor immune responses and their clinical applications. *Cancer Sci* 2006; **97**:807–12.

15 Metelitsa LS, Naidenko OV, Kant A, Wu HW, Loza MJ, Perussia B, Kronenberg M, Seeger RC. Human NKT cells mediate antitumor cytotoxicity directly by recognizing target cell CD1d with bound ligand or indirectly by producing IL-2 to activate NK cells. *J Immunol* 2001; **167**:3114–22.

16 Giaccone G, Punt CJ, Ando Y *et al.* A phase I study of the natural killer T-cell ligand α-galactosylceramide (KRN7000) in patients with solid tumors. *Clin Cancer Res* 2002; **8**:3702–9.

17 Shimizu K, Hidaka M, Kadowaki N *et al.* Evaluation of the function of human invariant NKT cells from cancer patients using alpha-galactosylceramide-loaded murine dendritic cells. *J Immunol* 2006; **177**:3484–92.

18 Parekh VV, Wilson MT, Olivares-Villagomez D, Singh AK, Wu L, Wang CR, Joyce S, Van Kaer L. Glycolipid antigen induces long-term natural killer T cell anergy in mice. *J Clin Invest* 2005; **115**:2572–83.

19 Ishikawa A, Motohashi S, Ishikawa E *et al.* A phase I study of α-galactosylceramide (KRN7000)-pulsed dendritic cells in patients with advanced and recurrent non-small cell lung cancer. *Clin Cancer Res* 2005; **11**:1910–7.

20 van der Vliet HJ, Molling JW, Nishi N *et al.* Polarization of Vα24$^+$ Vβ11$^+$ natural killer T cells of healthy volunteers and cancer patients using α-galactosylceramide-loaded and environmentally instructed dendritic cells. *Cancer Res* 2003; **63**:4101–6.

21 Harada Y, Imataki O, Heike Y *et al.* Expansion of α-galactosylceramide-stimulated Vα24$^+$ NKT cells cultured in the absence of animal materials. *J Immunother* 2005; **28**:314–21.

22 Kadowaki N, Antonenko S, Ho S, Rissoan MC, Soumelis V, Porcelli SA, Lanier LL, Liu YJ. Distinct cytokine profiles of neonatal natural killer T cells after expansion with subsets of dendritic cells. *J Exp Med* 2001; **193**:1221–6.

23 Ikarashi Y, Iizuka A, Heike Y, Yoshida M, Takaue Y, Wakasugi H. Cytokine production and migration of in vitro-expanded NK1.1$^-$ invariant Vα14 natural killer T (Vα14i NKT) cells using α-galactosylceramide and IL-2. *Immunol Lett* 2005; **101**:160–7.

24 Matsuda JL, Naidenko OV, Gapin L, Nakayama T, Taniguchi M, Wang CR, Koezuka Y, Kronenberg M. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J Exp Med* 2000; **192**:741–54.

25 Smyth MJ, Crowe NY, Pellicci DG, Kyparissoudis K, Kelly JM, Takeda K, Yagita H, Godfrey DI. Sequential production of interferon-gamma by NK1.1$^+$ T cells and natural killer cells is essential for the antimetastatic effect of α-galactosylceramide. *Blood* 2002; **99**:1259–66.

26 Kambayashi T, Assarsson E, Lukacher AE, Ljunggren HG, Jensen PE. Memory CD8$^+$ T cells provide an early source of IFN-γ. *J Immunol* 2003; **170**:2399–408.

27 Eberl G, MacDonald HR. Rapid death and regeneration of NKT cells in anti-CD3ε- or IL-12-treated mice: a major role for bone marrow in NKT cell homeostasis. *Immunity* 1998; **9**:345–53.

28 Serizawa I, Koezuka Y, Amao H, Saito TR, Takahashi KW. Functional natural killer T cells in experimental mouse strains, including NK1.1$^-$ strains. *Exp Anim* 2000; **49**:171–80.

29 Beutner U, Launois P, Ohteki T, Louis JA, MacDonald HR. Natural killer-like T cells develop in SJL mice despite genetically distinct defects in NK1.1 expression and in inducible interleukin-4 production. *Eur J Immunol* 1997; **27**:928–34.

30 Asada-Mikami R, Heike Y, Harada Y *et al.* Increased expansion of Vα24$^+$ T cells derived from G-CSF-mobilized peripheral blood stem cells as compared to peripheral blood mononuclear cells following α-galactosylceramide stimulation. *Cancer Sci* 2003; **94**:383–8.

31 Imataki O, Heike Y, Ishida T, Takaue Y, Ikarashi Y, Yoshida M, Wakasugi H, Kakizoe T. Efficient ex vivo expansion of Vα24$^+$ NKT cells derived from G-CSF-mobilized blood cells. *J Immunother* 2006; **29**:320–7.

32 Brossay L, Chioda M, Burdin N, Koezuka Y, Casorati G, Dellabona P, Kronenberg M. CD1d-mediated recognition of an α-galactosylceramide by natural killer T cells is highly conserved through mammalian evolution. *J Exp Med* 1998; **188**:1521–8.

33 Nishi N, van der Vliet HJ, Koezuka Y, von Blomberg BM, Scheper RJ, Pinedo HM, Giaccone G. Synergistic effect of KRN7000 with interleukin-15, -7, and -2 on the expansion of human Vα 24$^+$V β11$^+$ T cells *in vitro*. *Hum Immunol* 2000; **60**:357–65.

34 van der Vliet HJ, Nishi N, Koezuka Y *et al.* Potent expansion of human natural killer T cells using α-galactosylceramide

(KRN7000)-loaded monocyte-derived dendritic cells, cultured in the presence of IL-7 and IL-15. *J Immunol Meth* 2001; **247**:61–72.

35 Hameg A, Gouarin C, Gombert JM, Hong S, Van Kaer L, Bach JF, Herbelin A. IL-7 up-regulates IL-4 production by splenic NK1.1$^+$ and NK1.1$^-$ MHC class I-like/CD1-dependent CD4$^+$ T cells. *J Immunol* 1999; **162**:7067–74.

36 Zhu R, Diem S, Araujo LM *et al.* The Pro-Th1 cytokine IL-12 enhances IL-4 production by invariant NKT cells: relevance for T cell-mediated hepatitis. *J Immunol* 2007; **178**:5435–42.

37 Bezbradica JS, Stanic AK, Matsuki N *et al.* Distinct roles of dendritic cells and B cells in Va14Ja18 natural T cell activation in vivo. *J Immunol* 2005; **174**:4696–705.

38 Minami K, Yanagawa Y, Iwabuchi K, Shinohara N, Harabayashi T, Nonomura K, Onoe K. Negative feedback regulation of T helper type 1 (Th1)/Th2 cytokine balance via dendritic cell and natural killer T cell interactions. *Blood* 2005; **106**:1685–93.

39 Benlagha K, Kyin T, Beavis A, Teyton L, Bendelac A. A thymic precursor to the NK T cell lineage. *Science* 2002; **296**:553–5.

40 Pellicci DG, Hammond KJ, Uldrich AP, Baxter AG, Smyth MJ, Godfrey DI. A natural killer T (NKT) cell developmental pathway involving a thymus-dependent NK1.1$^-$CD4$^+$ CD1d-dependent precursor stage. *J Exp Med* 2002; **195**:835–44.

41 Loza MJ, Perussia B. Final steps of natural killer cell maturation: a model for type 1-type 2 differentiation? *Nat Immunol* 2001; **2**:917–24.

42 Ueda N, Kuki H, Kamimura D *et al.* CD1d-restricted NKT cell activation enhanced homeostatic proliferation of CD8$^+$ T cells in a manner dependent on IL-4. *Int Immunol* 2006; **18**:1397–404.

43 Morris SC, Orekhova T, Meadows MJ, Heidorn SM, Yang J, Finkelman FD. IL-4 induces in vivo production of IFN-γ by NK and NKT cells. *J Immunol* 2006; **176**:5299–305.

44 Townsend MJ, Weinmann AS, Matsuda JL, Salomon R, Farnham PJ, Biron CA, Gapin L, Glimcher LH. T-bet regulates the terminal maturation and homeostasis of NK and Vα14i NKT cells. *Immunity* 2004; **20**:477–94.

45 Matsuda JL, Zhang Q, Ndonye R, Richardson SK, Howell AR, Gapin L. T-bet concomitantly controls migration, survival, and effector functions during the development of Vα14i NKT cells. *Blood* 2006; **107**:2797–805.

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ORIGINAL ARTICLE

Functional analysis of cytomegalovirus-specific T lymphocytes compared to tetramer assay in patients undergoing hematopoietic stem cell transplantation

Y Morita-Hoshi[1,2], Y Heike[1], M Kawakami[1], T Sugita[3], O Miura[2], S-W Kim[1], S-I Mori[1], T Fukuda[1], R Tanosaki[1], K Tobinai[1] and Y Takaue[1]

[1]*Department of Medical Oncology, National Cancer Center Hospital, Tokyo, Japan;* [2]*Department of Hematology and Oncology, Graduate School of Medicine, Tokyo Medical and Dental University, Tokyo, Japan and* [3]*Cellular Immunology Section, SRL Inc., Hachioji-city, Tokyo, Japan*

In order to evaluate whether we could predict reactivation of CMV by monitoring the number of CMV-specific cytotoxic T-lymphocytes (CTL), tetramer analysis was performed in 37 patients who underwent hematopoietic stem cell transplantation (HSCT). The results disclosed that the mean number of CMV-specific CTL at day 30 did not differ among patients who developed CMV antigenemia (22/μl) and those who did not (12/μl). Serial tetramer analysis showed that 21% of the patients had >10/μl CMV-specific CTL at the first detection of CMV antigenemia and 67% of the patients had more than 10/μl CMV-specific CTL at the onset of CMV disease. Intracellular staining upon stimulation by CMV lysates and peptide in patients with CMV colitis revealed that both IFN-γ producing CD4+ and CD8+ lymphocytes were suppressed at the onset of CMV colitis (1.6 and 8/μl), which increased with recovery of the disease (19 and 47/μl). These data suggest that it is difficult to predict CMV reactivation solely by the number of CMV-specific CTL. We suggest that additional functional analysis by intracellular cytokine assay may be useful for immunomonitoring against CMV.

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Keywords: CMV; intracellular IFN-γ; CTL; HSCT; HLA-A02

Introduction

Reactivation of CMV is one of the major complications in patients undergoing hematopoietic stem cell transplantation (HSCT) and is significantly related to morbidity and mortality despite the recent development of potent antiviral medications.[1,2] The decision to administer antiviral therapy is currently based on the clinical risk and the detection of viremia by various methods including PCR for CMV-derived DNA or CMV antigenemia assay. However, treatment with antiviral drugs such as ganciclovir and foscarnet increases the risk for secondary graft failure and other infectious complications due to myelotoxicity. To optimize the therapy with minimum drug exposure, it is important to monitor the recovery of CMV-specific immunity accurately. For this purpose, tetramer-based monitoring of CMV-specific cytotoxic T-cells (CTL) has been widely performed in patients with an HLA-A02 or HLA-B07 serotype.[3–11] Some of the results have demonstrated that the reconstitution of CMV-specific CTL as evaluated by quantitative tetramer to levels >10–20/μl is adequate for protection against CMV infection.[5–7] However, some patients with CMV-specific CTL above this level still experience CMV reactivation.[9] It has also been reported that the cellular response to CMV in immunosuppressed patients reflects functional impairment,[10] and CMV reactivation following HSCT has been shown to be associated with the presence of dysfunctional CMV-specific T-cells.[11] Therefore, by itself, the quantification of CMV-specific CTL seems to be insufficient and a simultaneous qualitative analysis of CMV-specific lymphocytes is needed. Furthermore, it is essential that we should develop a universal monitoring method, which is not limited to HLA to cover larger populations, since an epitope that is potent enough for immunomonitoring is not obtained in some HLA types such as HLA-A24.[12] In this study, simultaneous functional analysis of CMV-specific lymphocytes by intracellular cytokine assay upon stimulation with CMV lysate and antigen peptide were performed with tetramer-based CTL quantification in patients who underwent HSCT to identify an optimal monitoring system.

Materials and methods

Study patients

CMV seropositive patients with an HLA-A*0201 or HLA-A*0206 genotype who had undergone allogeneic non-T-cell

Correspondence: Dr Y Takaue, Director, Department of Medical Oncology, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan.
E-mail: ytakaue@ncc.go.jp
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depleted-HSCT between February 2002 and May 2005 were included in this study. Patients were eligible with the availability for 160 days of follow-up. The study was approved by the Ethics Committee and a written informed consent was given by all patients. Peripheral blood samples were obtained at days 30 ± 7 and 60 ± 7 after transplantation. When patients agreed to additional sampling, additional samples were obtained every 2–3 weeks. The median age of studied patients was 52 (21–68). The genotype for HLA-A02 in 37 eligible patients was HLA-A*0201 in 20 patients, HLA-A*0206 in 16 patients and both the HLA-A*0201 and HLA-A*0206 genotypes in one patient. Nine patients received BMT from an unrelated donor, two received BMT from a related donor and the remaining 26 received peripheral blood HSCT from a related donor. With regard to the conditioning regimen, 11 patients received a conventional regimen that included 120 mg/kg CY plus 16 mg/kg BU or 120 mg/kg CY plus 12 Gy of TBI, whereas 26 received a reduced-intensity regimen with 0.66 mg/kg cladribine (2-chlorodeoxyadenosine) plus 8 mg/kg BU or 180 mg/m^2 fludarabine plus 8 mg/kg BU. For patients who received a graft from an unrelated donor or DNA-mismatched donor, 4 Gy of TBI or 5 mg/kg of rabbit antithymocyte globulin (ATG) were added to reduced-intensity conditioning.

Diagnostic tests for CMV infection and CMV disease
CMV seropositivity was assessed by the detection of IgG antibodies to CMV late antigen. All patients and 31 donors (84%) were seropositive for CMV. CMV antigenemia was monitored weekly after engraftment to day 60, and at longer intervals thereafter, by using the immunocytochemical detection of pp65 antigen in leukocytes. Test results were considered to be positive when more than one cell per 50 000 leukocytes was positively stained. CMV disease was diagnosed clinically, with confirmation by biopsy of the involved organ. Pre-emptive antiviral therapy was given with an antigenemia of more than 10 positive cells per 50 000 leukocytes, which we defined as high antigenemia. The initial therapy was ganciclovir 5 mg/kg once per day, which was adjusted according to the follow-up CMV antigenemia value.

Peptide and CMV antigen
A >80% pure HLA-A02-binding peptide NLVPMVATV (AA 495–503, referred to as NLV peptide) from the CMV pp65 phosphoprotein was obtained using high-performance liquid chromatography (Qiagen, Tokyo, Japan).

Tetramer staining
Tetramer staining was performed as recently described.[13] Briefly, 5 µl CD8-FITC, CD4-PC5, CD19-PC5, CD13-PC5 and 2 µl PE-conjugated tetrameric HLA-A*0201 NLV peptide complex (CMV-tetramer), purchased from Beckman Coulter Inc. (Fullerton, CA, USA), were added to 100 µl heparinized blood and incubated for 30 min. After RBC were lysed and washed twice, the cells were fixed and acquired on a flow cytometer (FACS Calibur, Becton Dickinson, Franklin Lakes, NJ, USA). More than 20 000 cells in the lymphocyte gate were acquired and analyzed using Cellquest software. The CD4−, CD19−, CD13− and CD8+ CMV-tetramer-positive fraction of the lymphocyte gate was defined as CMV-specific CTL.

Intracellular cytokine assay
Intracellular cytokine staining was performed as recently described[14] with the following modifications. Peripheral whole blood (1 ml) was stimulated for 6 h at 37 °C with 10 µg/ml NLV peptide or 1 µg/ml CMV lysate (Advanced Biotechnologies, Colombia, MD, USA), in the presence of costimulatory monoclonal antibodies, CD28 and CD49d (Becton Dickinson, 1 µg/ml each). Breferdin A (Sigma, St Louis, MO, USA; 10 µg/ml) was added for the last 4 h of incubation. Positive and negative controls were obtained by stimulating the cells with 10 µg/ml staphylococcal enterotoxin B or phosphate-buffered saline. Samples were lysed, permeabilized and stained with 2.5 µl CD69-FITC, 20 µl IFN-γ-PE, 0.6 µl CD3-APC and 10 µl CD8− or CD4− PerCP. More than 10 000 cells in the lymphocyte gate were acquired and analyzed using an FACS Calibur. The cells were gated on the CD3+ fraction of the lymphocyte gate and the proportion of IFN-γ and CD8 or CD4 was analyzed. CD69 was used as a marker for activated T-cells.

Statistical analysis
The difference between groups was compared with the Wilcoxon–Mann–Whitney *U*-test and the probabilities of $P<0.05$ were defined as statistically significant.

Results

Tetramer staining
CMV antigenemia was observed in 27 patients (73%) between day 23 and day 56 (median, day 34) after transplantation; 13 (35%) of them had a peak antigenemia level of >10/50 000 leukocytes (high antigenemia) which required ganciclovir therapy and four (11%) subsequently developed CMV disease. The median number of leukocytes and lymphocytes were 3500 (1300–17 200)/µl and 576 (228–3333)/µl at day 30 and 3900 (1400–9700)/µl and 1018 (192–6790)/µl at day 60, respectively. The median percentages of CD4+ and CD8+/lymphocytes were 35% (7–64%) and 38% (20–83%) at day 30 and 25% (6–37%) and 52% (27–83%) at day 60, respectively.

The tetramer analysis showed that the mean and median number of CMV-specific CTL at day 30 was, respectively, 11 and 1.9/µl for patients without CMV antigenemia, 23 and 7.8/µl for those with antigenemia, 33 and 15/µl for those with peak antigenemia <10/50 000, 12 and 3.7/µl for those with high antigenemia, and 21 and 2.4/µl for those who developed CMV disease. There was no significant correlation between the number of CMV-specific CTL and the incidence or severity of CMV antigenemia ($P>0.05$) (Figure 1).

To further evaluate the accurate number of CMV-specific CTL at the onset of CMV antigenemia, serial analysis of CMV-specific CTL was performed weekly in 14 patients (Figures 2 and 3). Patient's characteristics are shown in Table 1. CMV antigenemia was observed in 12 patients, and five of them (UPN1-5) developed high

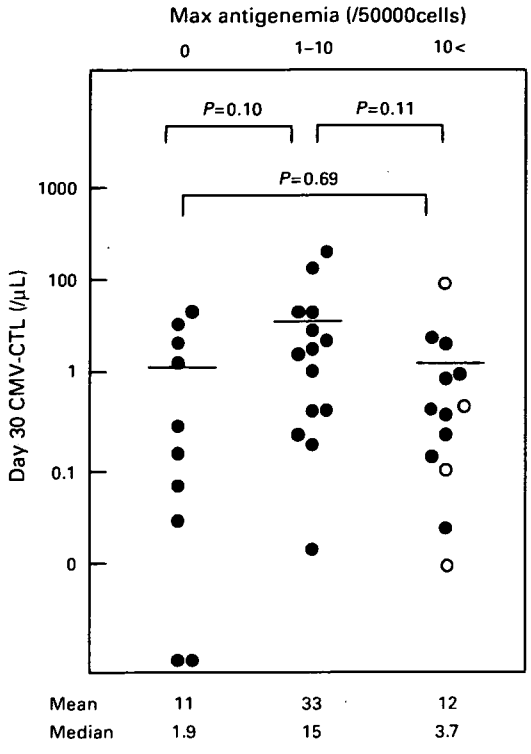

Figure 1 The number of CMV-specific CTL as evaluated by tetramer assay on day 30 post transplantation. The number of CMV-specific CTL did not differ between patients who did not develop CMV antigenemia, who had antigenemia below 10/50000, who had antigenemia of >10/50000. The outlined circle ○ indicates patients who developed CMV colitis.

antigenemia, including three (UPN1-3) with CMV colitis. The mean and median number of CMV-specific CTL at the first detection of CMV antigenemia was 21/μl and 4.7 (0–100)/μl in the 12 patients, and three (UPN2, 13, 14) showed >10/μl. For those who did not require antiviral therapy (UPN6-14), the number of CMV-specific CTL was widely ranged. While UPN6-8 showed <10/μl throughout the observation time, the maximum CTL count was >200/μl for UPN12-14. The number of CMV-specific CTL for UPN1 and UPN2 who developed CMV colitis showed >10/μl, which was 14 and 80/μl when diarrhea occurred, and 88 and 63/μl, respectively at the time of colon biopsy which proved CMV colitis.

It has been demonstrated that in patients coexpressing HLA-A02 and HLA-B07, CMV-specific cellular immune responses restricted by HLA-B07 dominate those restricted by HLA-A02, possibly because CD8+ T cells specific for dominant epitopes are able to suppress immune responses to less favored epitopes.[3] The allele frequency of HLA-B07 is low (5.2%) among Japanese[15] and only one patient coexpressed HLA-B07 in this study. We did not exclude this patient (UPN14) from the analysis because the number of HLA-A02-restricted CMV-specific CTL in this patient was 9.5/μl on day 30 and the maximum value reached 243/μl on day 128 suggesting that the coexpression of HLA-B07 seems not to have affected the immunoresponse of HLA-A2 in this patient.

Intracellular cytokine assay

Upon stimulation with CMV lysate, intracellular IFN-γ staining among five patients (UPN1–5) who developed high

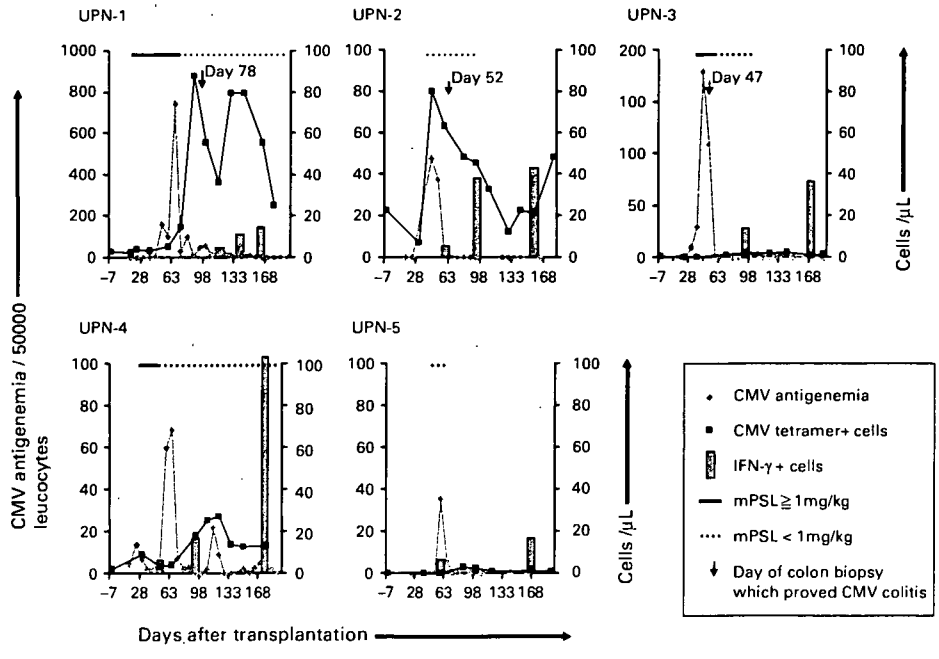

Figure 2 Serial analysis of patients who had high antigenemia of >10/50000. ■ indicates CMV-specific CTL as evaluated by tetramer assay, ♦ indicates CMV antigenemia, gray bar indicates the number of IFN-γ+cells/μl peripheral blood when stimulated with CMV lysate, the solid line indicates methylprednisolone administration of 1 mg/kg/day or more, the dashed line indicates corticosteroid administration less than 1 mg/kg/day and ↓ indicates the day of colon biopsy which CMV disease was diagnosed. UPN1, 2, 3 developed CMV disease. Intracellular IFN-γ was undetectable on day 60 and day 90 for UPN1 and on day 60 for UPN3.

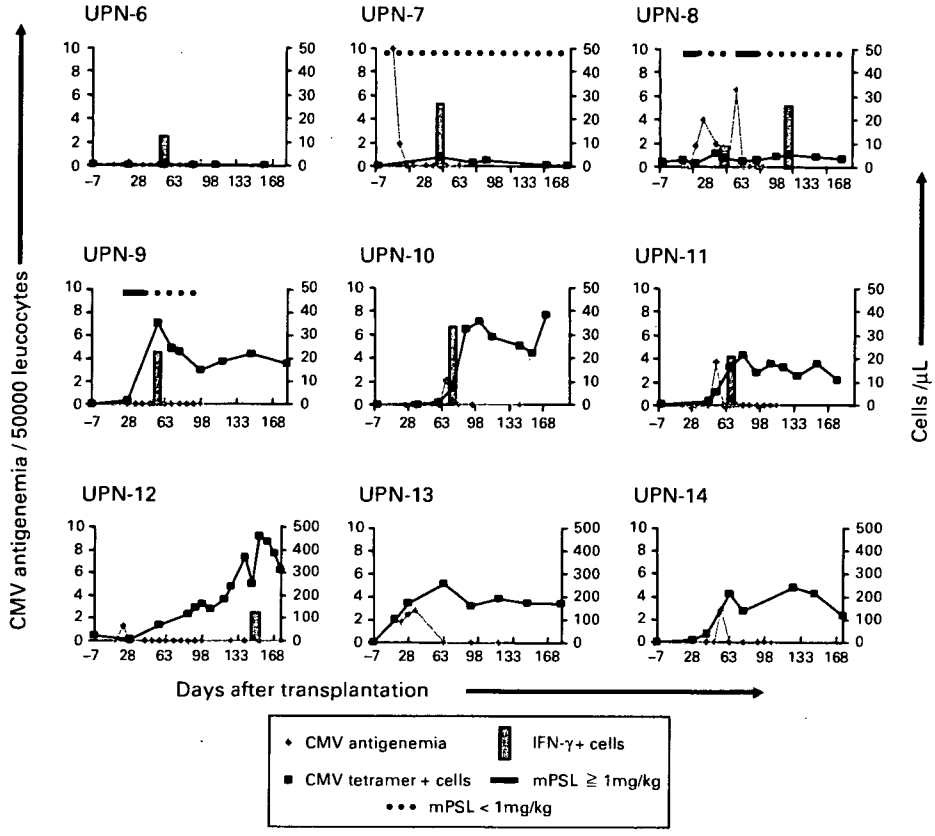

Figure 3 Serial analysis in patients with CMV antigenemia of < 10/50 000 or patients without CMV antigenemia. The legends are the same as Figure 2. Intracellular cytokine was not assessed for UPN13 and UPN14.

Table 1 Patients' characteristics

ID	*Age*	*HLA-A locus*	*Primary disease*	*Conditioning regimen*	*GVHD prophylaxis*	*Stem cell source*	*CMV serology*		*Max CMV-Ag*	*CMV disease*
							Recipient	*Donor*		
UPN-01	63	0201, 0206	CML (AP)	CdA/BU	CSP→TAC	PB	+	+	740	+
UPN-02	57	0201	NHL (DLBCL)	CdA/BU	CSP	PB	+	+	48	+
UPN-03	49	0201	NHL (low grade)	CdA/BU	CSP→TAC	PB	+	+	178	+
UPN-04	54	0206	MCL	CdA/BU/ ATG	CSP + sMTX	PB	+	+	68	–
UPN-05	59	0206	AML	CdA/BU/TBI	CSP + sMTX	UBM	+	+	35	–
UPN-06	66	0206	MDS (RA)	Flu/BU	CSP + sMTX	PB	+	–	0	–
UPN-07	61	0201	NHL (low grade)	Flu/BU/ATG	CSP	UBM	+	+	10	–
UPN-08	62	0201	AML	CdA/BU	TAC	PB	+	+	6.5	–
UPN-09	43	0201	MDS (RA)	BU/CY	CSP + sMTX	UBM	+	–	0	–
UPN-10	41	0206	AML	BU/CY	CSP + sMTX	RBM	+	+	2.1	–
UPN-11	54	0201	NHL (low grade)	Flu/BU	CSP + sMTX	PB	+	+	3.7	–
UPN-12	32	0206	RCC	CdA/BU	CSP	PB	+	+	2.8	–
UPN-13	42	0206	PCL	CdA/BU/ ATG	CSP + sMTX	PB	+	+	2.8	–
UPN-14	43	0206	RCC	CdA/BU/ ATG	CSP	PB	+	+	1.3	–

Abbreviations: ATG = antithymocyte globulin; CdA = cladribine; CML (AP) = CML (accelerated phase); CSP = cyclosporine; DLBCL = diffuse large B-cell lymphoma; Flu = fludarabine; MCL = mantle cell lymphoma; MDS (RA) = myelodysplastic syndrome (refractory anemia); NHL = non-Hodgkin lymphoma; PB = peripheral blood; PCL = plasma cell leukemia; RBM = related bone marrow; RCC = renal cell carcinoma; sMTX = short term methotrexate; TAC = tacrolimus; UBM = unrelated bone marrow.

antigenemia and required antiviral therapy showed that the mean number of IFN-γ-producing cells was 3.6 (0–6.7)/μl at day 60, which subsequently increased to 72 (15–250)/μl at day 160. As for three patients with CMV colitis (UPN1–3), only one patient (UPN2) had detectable level of IFN-γ-producing cells (4.8/μl) at the time of disease

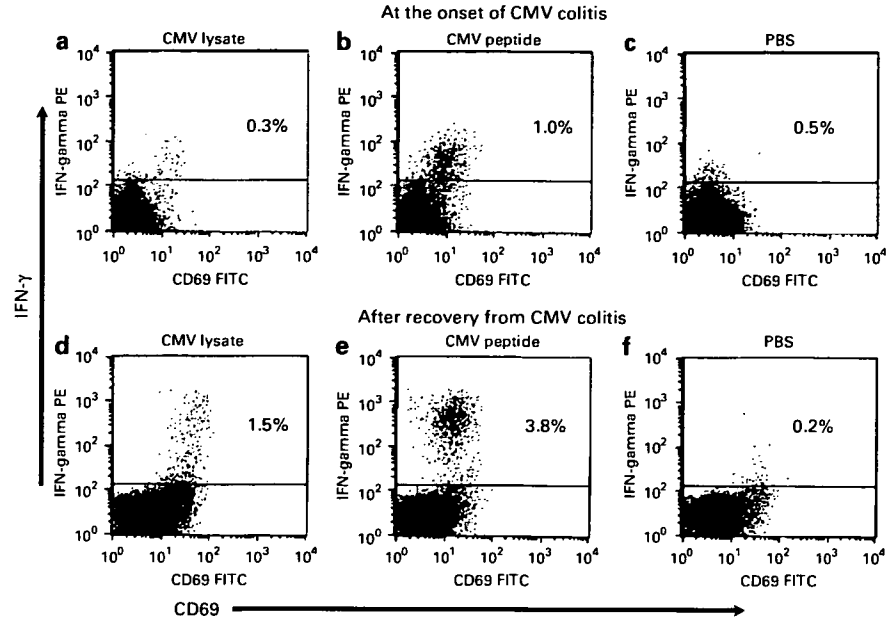

Figure 4 Intracellular cytokine assay in a patient with CMV colitis (UPN2). The samples were taken at the onset of CMV colitis (**a–c**) and after recovery from CMV colitis (**d–f**). The numbers of IFN-γ-producing cells on lysate stimulation (**a**, **d**) and peptide stimulation (**b**, **e**) both increased after recovery from CMV colitis. (**c**) and (**f**) are negative controls.

onset and were undetectable for the other two patients, which remained negative until day 90 for UPN1. The mean number of IFN-γ+ cells subsequently increased to 19 (5–38)/μl after recovery from CMV disease (Figures 2, 4a and d). Among the patients who did not require antiviral therapy, the IFN-γ-producing cells were all >10/μl at day 60.

When stimulated with CMV peptide, IFN-γ-producing cells numbered 8 (0–16)/μl at the time of disease onset with a subsequent increase to 47 (15–95)/μl after recovery from CMV disease (Figures 4b and e).

Regarding the phenotype of IFN-γ-producing cells, median of 81% (76–100) were CD4+ and <20% were CD8+ upon stimulation by CMV lysate. The staining of IFN-γ was brighter in CD4+ than in CD8+ cells and CD69 was positive for both CD4+ and CD8+ fraction. IFN-γ-producing cells were CD69 low positive and median of 42% (25–68) were CD8+, while the rest were CD8−/CD4− phenotype upon CMV peptide stimulation.

Discussion

Our results showed that it is difficult to predict CMV infection by the number of CMV-specific CTL alone as this did not correlate with the incidence and severity of CMV infection. While UPN1 and UPN2 developed CMV colitis after the recovery of sufficient number of CTL, UPN6, UPN7 and UPN8 did not require antiviral therapy despite low CMV-specific CTL. These results showed that CMV disease could occur after HSCT even in patients with >10/μl CMV-specific CTL as evaluated by tetramer assay, which has been considered to be sufficient to protect against CMV infection.[5,7]

CMV-specific CTL emerged immediately following the detection of antigenemia in most patients, suggesting that CMV infection can be a trigger for the recovery of CMV-specific immunity. However, UPN9 had recovery of CMV-specific CTL at day 60 even though his CMV antigenemia and CMV DNA as evaluated by PCR were negative throughout the course.

On the other hand, intracellular analysis revealed that IFN-γ production in both CD4+ and CD8+ T lymphocytes was depressed in patients with high antigenemia or CMV disease and this had subsequently recovered at disease resolution. Functional analysis methods for CMV-specific immune response by flow cytometry have been established,[16] and it was reported that patients who developed CMV disease after SCT had no detectable IFN-γ production by CD3+/4+ T-cells upon CMV AD-169 antigen stimulation.[17] It has also been demonstrated that levels of IFN-γ-producing CD4+ cells less than one cell/μl and CD8+ less than three cells/μl upon stimulation by CMV-infected autologous dendritic cells are not protective against recurrent infection.[18] As assessed by IFN-γ ELISPOT assay, the threshold level for protection against CMV reactivation was estimated as over one cell/μl peripheral blood upon CMV pp65 peptide stimulation.[19] The number of IFN-γ-producing cells upon CMV lysate stimulation were above ten cells/μl among patients whose antigenemia was <10/50 000 cells in our study, which may be sufficient for protection against CMV reactivation. It is difficult to determine the exact threshold level for protection against CMV since IFN-γ production differs among various stimulating agents. Also the magnitude of response is higher in the cytokine flow cytometry assay while the cytokine flow cytometry assay was less likely than the ELISPOT assay to detect low-level responses.[20]

Several studies on HIV-infected patients have shown the availability of analyzing the phenotype and other cytokine production of virus-specific T-cells such as IL-2, TNF-α.[21,23] It has been demonstrated that virus-specific T-cells, which produce both IFN-γ and IL-2 are important in virus-specific immunity, and that IFN-γ/IL-2 secreting CD8+ T-cells were CD45RA−/CCR7− phenotype and correlated with that of proliferating T-cells, whereas single IFN-γ-secreting cells were either CD45RA−/CCR7− or CD45RA+/CCR7−.[22] Another study has shown that immunorestored patients had increased levels of circulating CMV-specific CD8+ T-cells with 'early' (CD27+/CD28+/CD45RA+, CD27+/CD28+/CD45RA−) and 'intermediate' (CD27−/CD28+/CD45RA−) phenotype.[23] Only IFN-γ production was assessed in our study, however higher-order flow cytometry might have added more discriminatory value. Foster *et al*.[24] demonstrated that CMV-specific CD4+ T-helper cells show the same reconstitution kinetics as CD8+ CTL. Thus, functional analysis of lymphocytes upon lysate stimulation that can be used to assess both CD4+ and CD8+ cells is a useful tool for monitoring T cell immunity against CMV in patients after HSCT. This method is more widely applicable than peptide stimulation or tetramer assay, since it is not restricted to HLA or a single epitope. However, peptide stimulation and tetramer assay may still be a major procedure in the analysis of CD8+ T-cells, since tetramers are widely applied to adoptive immunotherapy of CMV[25] and the dominant population of IFN-γ-producing cells upon lysate stimulation was CD4+. Previous study has demonstrated that flow cytometry following stimulation of PBMC with pp65 and immediate early (IE)-1 peptide pools consisted of 15-aa peptides was highly sensitive and specific in predicting the presence of recognized epitope in the respective proteins.[26] Furthermore, it has been shown that IE-1-specific responses were more important in protective immunity than pp65-specific responses in heart and lung transplant recipients.[27] The stimulation with comprehensive peptide pools might have better assessed both functional CD4+ and CD8+ T-cell responses. Further study is needed to identify whether IE-1 is more important than pp65 in allogeneic HSCT patients, and the significance of IE-1 in Japanese population with low allele frequency of HLA-A1 (1.8%), -B7 (5.2%) or -B8 (<1%),[15] which is known to present IE-1 epitopes.

It is likely that the patients who did not have CMV reactivation despite low CMV-specific CTL had sufficient T-cell immune-recovery against CMV since the number of intracellular IFN-γ positive cells upon CMV lysate stimulation was as high as that in patients who had recovered from CMV reactivation. As for CD8+ T cells in these patients, CTL against other CMV-epitopes besides NLV might have helped to protect against CMV. It is reported that the recovery of CMV specific T-cells is earlier in patients who received reduced-intensity conditioning compared to conventional regimen and this was delayed by the use of ATG.[19,28] Additionally, the graft source and CD3+ T-cell dose significantly influence the recovery of CMV-specific immunity.[28] The difference of immune recovery according to the conditioning regimen and graft source was not demonstrated in this study, probably due to heterogeneous patients and small sample size. Functional depression of the lymphocytes due to corticosteroid for GVHD seems to be the major cause of CMV infection as documented in all patients with high antigenemia. Moreover, 75% of the patients with CMV disease were receiving more than 1 mg/kg/day of methylprednisolone (mPSL), while among those who did not require antiviral therapy, only 13% had received 1 mg/kg/day or more mPSL. The influence of corticosteroid on the number of CMV-specific CTL is controversial. Some studies have reported that a significant reduction of CMV-specific CTL occurred with corticosteroid therapy.[6,8] Others have shown that the frequency and the absolute number of CMV-specific CD8+ T cells were similar in patients receiving corticosteroids and those who didn't, while the CMV-specific CD8+ T cells showed decreased cytokine production.[10,11] Our result was consistent with the latter observation that while the number of CMV-specific CTL does not decrease significantly with corticosteroid therapy, IFN-γ production of CMV-specific CTL is severely suppressed. Therefore, concomitant assessment of T-cell function is essential in patients after HSCT, especially in those who are receiving corticosteroid therapy.

References

1 Boeckh M, Nichols WG, Papanicolaou G, Rubin R, Wingard JR, Zaia J. Cytomegalovirus in hematopoietic stem cell transplant recipients: current status, known challenges, and future strategies. *Biol Blood Marrow Transplant* 2003; **9**: 543–558.

2 Zaia JA, Sissons JG, Riddell S, Diamond DJ, Wills MR, Carmichael AJ *et al*. Status of Cytomegalovirus Prevention and Treatment in 2000. *Hematology (Am Soc Hematol Educ Program)* 2000, 339–355.

3 Lacey SF, Villacres MC, La Rosa C, Wang Z, Longmate J, Martinez J *et al*. Relative dominance of HLA-B*07 restricted CD8+ T-lymphocyte immune responses to human cytomegalovirus pp65 in persons sharing HLA-A*02 and HLA-B*07 alleles. *Hum Immunol* 2003; **64**: 440–452.

4 Singhal S, Shaw JC, Ainsworth J, Hathaway M, Gillespie GM, Paris H *et al*. Direct visualization and quantitation of cytomegalovirus-specific CD8+ cytotoxic T-lymphocytes in liver transplant patients. *Transplantation* 2000; **69**: 2251–2259.

5 Gratama JW, van Esser JW, Lamers CH, Tournay C, Lowenberg B, Bolhuis RL *et al*. Tetramer-based quantification of cytomegalovirus (CMV)-specific CD8+ T lymphocytes in T-cell-depleted stem cell grafts and after transplantation may identify patients at risk for progressive CMV infection. *Blood* 2001; **98**: 1358–1364.

6 Aubert G, Hassan-Walker AF, Madrigal JA, Emery VC, Morte C, Grace S *et al*. Cytomegalovirus-specific cellular immune responses and viremia in recipients of allogeneic stem cell transplants. *J Infect Dis* 2001; **184**: 955–963.

7 Cwynarski K, Ainsworth J, Cobbold M, Wagner S, Mahendra P, Apperley J *et al*. Direct visualization of cytomegalovirus-specific T-cell reconstitution after allogeneic stem cell transplantation. *Blood* 2001; **97**: 1232–1240.

8 Engstrand M, Tournay C, Peyrat MA, Eriksson BM, Wadstrom J, Wirgart BZ *et al*. Characterization of CMVpp65-specific CD8+ T lymphocytes using MHC tetramers in kidney transplant patients and healthy participants. *Transplantation* 2000; **69**: 2243–2250.

9 Lacey SF, Gallez-Hawkins G, Crooks M, Martinez J, Senitzer D, Forman SJ *et al.* Characterization of cytotoxic function of CMV-pp65-specific CD8+ T-lymphocytes identified by HLA tetramers in recipients and donors of stem-cell transplants. *Transplantation* 2002; **74**: 722–732.
10 Engstrand M, Lidehall AK, Totterman TH, Herrman B, Eriksson BM, Korsgren O. Cellular responses to cytomegalovirus in immunosuppressed patients: circulating CD8+ T cells recognizing CMVpp65 are present but display functional impairment. *Clin Exp Immunol* 2003; **132**: 96–104.
11 Ozdemir E, St John LS, Gillespie G, Rowland-Jones S, Champlin RE, Molldrem JJ *et al.* Cytomegalovirus reactivation following allogeneic stem cell transplantation is associated with the presence of dysfunctional antigen-specific CD8+ T cells. *Blood* 2002; **100**: 3690–3697.
12 Morita Y, Hosokawa M, Ebisawa M, Sugita T, Miura O, Takaue Y *et al.* Evaluation of cytomegalovirus-specific cytotoxic T-lymphocytes in patients with the HLA-A*02 or HLA-A*24 phenotype undergoing hematopoietic stem cell transplantation. *Bone Marrow Transplant* 2005; **36**: 803–811.
13 Morita Y, Heike Y, Kawakami M, Miura O, Nakatsuka S, Ebisawa M *et al.* Monitoring of WT1-specific cytotoxic T lymphocytes after allogeneic hematopoietic stem cell transplantation. *Int J Cancer* 2006; **119**: 1360–1367.
14 Rauser G, Einsele H, Sinzger C, Wernet D, Kuntz G, Assenmacher M *et al.* Rapid generation of combined CMV-specific CD4+ and CD8+ T-cell lines for adoptive transfer into recipients of allogeneic stem cell transplants. *Blood* 2004; **103**: 3565–3572.
15 Tokunaga K, Ishikawa Y, Ogawa A, Wang H, Mitsunaga S, Moriyama S *et al.* Sequence-based association analysis of HLA class I and II alleles in Japanese supports conservation of common haplotypes. *Immunogenetics* 1997; **46**: 199–205.
16 Waldrop SL, Pitcher CJ, Peterson DM, Maino VC, Picker LJ. Determination of antigen-specific memory/effector CD4+ T cell frequencies by flow cytometry: evidence for a novel, antigen-specific homeostatic mechanism in HIV-associated immunodeficiency. *J Clin Invest* 1997; **99**: 1739–1750.
17 Avetisyan G, Larsson K, Aschan J, Nilsson C, Hassan M, Ljungman P. Impact on the cytomegalovirus (CMV) viral load by CMV-specific T-cell immunity in recipients of allogeneic stem cell transplantation. *Bone Marrow Transplant* 2006; **38**: 687–692.
18 Lilleri D, Gerna G, Fornara C, Lozza L, Maccario R, Locatelli F. Prospective simultaneous quantification of human cytomegalovirus-specific CD4+ and CD8+ T-cell reconstitution in young recipients of allogeneic hematopoietic stem cell transplants. *Blood* 2006; **108**: 1406–1412.
19 Ohnishi M, Sakurai T, Heike Y, Yamazaki R, Kanda Y, Takaue Y *et al.* Evaluation of cytomegalovirus-specific T-cell reconstitution in patients after various allogeneic haematopoietic stem cell transplantation using interferon-gamma-enzyme-linked immunospot and human leucocyte antigen tetramer assays with an immunodominant T-cell epitope. *Br J Haematol* 2005; **131**: 472–479.
20 Karlsson AC, Martin JN, Younger SR, Bredt BM, Epling L, Ronquillo R *et al.* Comparison of the ELISPOT and cytokine flow cytometry assays for the enumeration of antigen-specific T cells. *J Immunol Methods* 2003; **283**: 141–153.
21 Betts MR, Nason MC, West SM, De Rosa SC, Migueles SA, Abraham J *et al.* HIV nonprogressors preferentially maintain highly functional HIV-specific CD8+ T cells. *Blood* 2006; **107**: 4781–4789.
22 Zimmerli SC, Harari A, Cellerai C, Vallelian F, Bart PA, Pantaleo G. HIV-1-specific IFN-gamma/IL-2-secreting CD8 T cells support CD4-independent proliferation of HIV-1-specific CD8 T cells. *Proc Natl Acad Sci USA* 2005; **102**: 7239–7244.
23 Sinclair E, Tan QX, Sharp M, Girling V, Poon C, Natta MV *et al.* Protective immunity to cytomegalovirus (CMV) retinitis in AIDS is associated with CMV-specific T cells that express interferon- gamma and interleukin-2 and have a CD8+ cell early maturational phenotype. *J Infect Dis* 2006; **194**: 1537–1546.
24 Foster AE, Gottlieb DJ, Sartor M, Hertzberg MS, Bradstock KF. Cytomegalovirus-specific CD4+ and CD8+ T-cells follow a similar reconstitution pattern after allogeneic stem cell transplantation. *Biol Blood Marrow Transplant* 2002; **8**: 501–511.
25 Cobbold M, Khan N, Pourgheysari B, Tauro S, McDonald D, Osman H *et al.* Adoptive transfer of cytomegalovirus-specific CTL to stem cell transplant patients after selection by HLA-peptide tetramers. *J Exp Med* 2005; **202**: 379–386.
26 Kern F, Faulhaber N, Frommel C, Khatamzas E, Prosch S, Schonemann C *et al.* Analysis of CD8 T cell reactivity to cytomegalovirus using protein-spanning pools of overlapping pentadecapeptides. *Eur J Immunol* 2000; **30**: 1676–1682.
27 Bunde T, Kirchner A, Hoffmeister B, Habedank D, Hetzer R, Cherepnev G *et al.* Protection from cytomegalovirus after transplantation is correlated with immediate early 1-specific CD8 T cells. *J Exp Med* 2005; **201**: 1031–1036.
28 Mohty M, Mohty AM, Blaise D, Faucher C, Bilger K, Isnardon D *et al.* Cytomegalovirus-specific immune recovery following allogeneic HLA-identical sibling transplantation with reduced-intensity preparative regimen. *Bone Marrow Transplant* 2004; **33**: 839–846.

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Infectious complications in chronic graft-versus-host disease: a retrospective study of 145 recipients of allogeneic hematopoietic stem cell transplantation with reduced- and conventional-intensity conditioning regimens

S. Yamasaki, Y. Heike, S. Mori, T. Fukuda, D. Maruyama, R. Kato, E. Usui, K. Koido, S. Kim, R. Tanosaki, K. Tobinai, T. Teshima, Y. Takaue. Infectious complications in chronic graft-versus-host disease: a retrospective study of 145 recipients of allogeneic hematopoietic stem cell transplantation with reduced- and conventional-intensity conditioning regimens.
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Abstract: To assess infectious complications associated with chronic graft-versus-host disease (cGVHD) after allogeneic hematopoietic stem cell transplantation (HSCT) with reduced- and conventional-intensity conditioning regimens (RIC, $n = 91$; CIC, $n = 54$, respectively), we retrospectively analyzed data from 145 consecutive patients with cGVHD after allogeneic HSCT from a human leukocyte antigen-matched related or unrelated donor. In the present retrospective analysis, 57% (83/145) of patients with cGVHD developed infections, with a mortality rate of 27% (22/83). The incidences of bacteremia ($n = 28$), central venous catheter-related infections ($n = 11$), bacterial pneumonia ($n = 4$), invasive aspergillosis ($n = 7$), and adenoviral hemorrhagic cystitis ($n = 8$) were significantly higher in patients with prednisolone dose ≥ 1 mg/kg at the time of diagnosis of cGVHD. The present results suggest that infections associated with cGVHD, especially after high-dose prednisolone, are predictive of poor outcome regardless of whether the patient received RIC or CIC.

S. Yamasaki[1], Y. Heike[1], S. Mori[1], T. Fukuda[1], D. Maruyama[1], R. Kato[1], E. Usui[1], K. Koido[1], S. Kim[1], R. Tanosaki[1], K. Tobinai[1], T. Teshima[2], Y. Takaue[1]

[1]Division of Hematology/Hematopoietic Stem Cell Transplantation, National Cancer Center Hospital, Tokyo, Japan, [2]Center for Cellular and Molecular Medicine, Kyushu University Hospital, Fukuoka, Japan

Key words: infectious complication; chronic graft-versus-host disease; allogeneic hematopoietic stem cell transplantation; reduced-intensity conditioning; HLA-matched donor

Correspondence to:
Yuji Heike, MD, PhD, Division of Hematology/Hematopoietic Stem Cell Transplantation, National Cancer Center Hospital, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan
Tel: + 81 3 3542 2511
Fax: + 81 3 3545 3567
E-mail: yheike@ncc.go.jp

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Infectious complications contribute to morbidity and mortality following allogeneic hematopoietic stem cell transplantation (HSCT). Well-known factors affecting susceptibility to infections include donor type, conditioning regimen, development of graft-versus-host disease (GVHD), and environmental factors. Reduced-intensity conditioning (RIC) regimens are thought to lower the risk of infections because they involve relatively little damage to vital organs (1). However, our experience indicates that with both RIC and conventional-intensity conditioning (CIC) regimens, the incidence of bacterial infections during neutropenia and *Aspergillus* infections is high after allogeneic HSCT (2, 3). Thus, it appears that RIC alone is not sufficient to improve the safety of allogeneic HSCT.

GVHD and the treatment of GVHD with immunosuppressive drugs are also well-known predominant risk factors for the development of opportunistic infections (4–6). In the case of acute GVHD, inpatients can be given comprehensive prophylaxis, including environmental control, to prevent infections over the short term. In contrast, chronic GVHD (cGVHD) is most often a late complication of allogeneic HSCT, and is usually treated on an outpatient basis. Consequently, the resources that can be used to control infections in patients with cGVHD are limited, and prophylaxis should be considered as a long-term approach, taking into account the safety and emergence of drug-resistant pathogens. In Japanese patients, the incidence of cGVHD after allogeneic HSCT is reportedly as high as 50%, with 20% of those who develop cGVHD contracting concurrent infections (7). At present, more transplantation procedures are being performed with peripheral blood stem cell (PBSC) products, in older patients, and with

unrelated donors. The available evidence suggests that all of these factors would result in greater numbers of patients with cGVHD. Thus, management of cGVHD is one of the greatest challenges to physicians practicing HSCT.

In the present study, we evaluated infectious complications associated with cGVHD in patients who received an RIC or a CIC regimen before undergoing PBSC transplantation (PBSCT) from a human leukocyte antigen (HLA)-matched relative (related PBSCT) or bone marrow transplantation (BMT) from an HLA-matched unrelated volunteer (unrelated BMT).

Patients and methods

Patient characteristics

We retrospectively analyzed data from 145 consecutive adult patients with hematologic malignancies who had received allogeneic HSCT with an RIC ($n = 91$) or CIC ($n = 54$) regimen between January 2000 and December 2004 at our institution. All of these 145 patients had sustained engraftment, had survived for >100 days following transplantation, and had developed cGVHD. The following types of patients were excluded: patients who suffered from disease progression before the development of cGVHD and received donor lymphocyte infusion, and patients with a history of previous allogeneic HSCT. Significant differences were observed between the RIC and CIC groups in terms of the age of the patients and donors, the gender of the patients, diagnosis, disease risk (8), time from diagnosis to transplantation, donor type and source of stem cells, and GVHD prophylaxis. The patient characteristics are summarized in Table 1. Typing for HLA-A, -B, and -DR antigens of the donor and recipient was performed using low-resolution DNA typing. The frequency with which allogeneic PBSCT is performed in Japan has been increasing since it became eligible for reimbursement from health insurance organizations in the year 2000, and our banking system only approves donation of bone marrow. The clinical characteristics of cGVHD, including use of immunosuppressive drugs at diagnosis and initial treatment, are summarized in Table 2. The present study was approved by the Ethics Committee of our institution, and all 145 subjects provided informed consent.

Conditioning regimen and supportive care

The CIC regimen consisted of cyclophosphamide (CY, 120 mg/kg), in combination with either 12 Gy total-body irradiation (TBI, $n = 25$) or busulfan (BU, 16 mg/kg; $n = 29$). The RIC regimen consisted of BU (8 mg/kg) in combination with either fludarabine (Flu, 180 mg/m^2; $n = 70$) or 2-chlorodeoxyadenosine (2-CdA, 0.66 mg/kg; $n = 21$); 14 patients received either anti-thymocyte globulin (ATG, 5–10 mg/kg; $n = 6$) or 4 Gy TBI ($n = 8$). All patients received cyclosporine (CSP, 3 mg/kg/day; $n = 137$) or tacrolimus (TAC, 0.03 mg/kg/day; $n = 8$), with ($n = 78$) or without ($n = 67$) short courses of methotrexate (MTX; related PBSCT, 10 mg/m^2 on day 1, and 7 mg/m^2 on days 3 and 6; unrelated BMT, 10 mg/m^2 on days 3, 6, and 11) as GVHD prophylaxis. All patients received prophylactic ciprofloxacin (200 mg orally 3 times daily) for prevention of infections until neutrophil recovery. Trimethoprim-sulfamethoxazole (80 mg of trimethoprim once daily) was administered for the prevention of *Pneumocystis* pneumonia and encapsulated bacterial infection, from the first day of the conditioning regimen until day 3, and from day +30 until 6 months after transplantation, or for prolonged periods in patients with cGVHD. Patients also received oral or intravenous fluconazole (100 mg once daily) for prevention of infection by *Candida* species, and low-dose acyclovir (600 mg until engraftment, and then 100 mg/day orally), starting at the same time as the conditioning regimens and continuing until cessation of administration of immunosuppressive drugs (9). Cytomegalovirus (CMV) antigenemia was monitored weekly until cessation of the administration of immunosuppressive drugs. Testing for CMV antigenemia consisted of direct immunoperoxidase staining of leukocytes with a peroxidase-labeled monoclonal antibody. Quantitative real-time polymerase chain reaction was not performed.

Definition of outcome

Patients with grades II–IV acute GVHD were treated with prednisolone (PSL) according to a standard regimen (10). Chronic GVHD was assessed and graded according to the standard criteria (11). The diagnosis and staging of cGVHD were also assessed according to the working report published by the National Institutes of Health Consensus Development Project (12). Relapse was defined either by morphologic evidence of the disease in the peripheral blood, marrow, or extramedullary sites, or by recurrence and persistence of pre-transplant chromosomal abnormalities in cytogenetic analysis of the marrow cells.

Infectious complications

A documented infection was defined as signs and symptoms associated with microbiological documentation of a pathogen from the site of infection. Culture-documented bacteremia, fungemia, or viremia was considered to be a definite infection, regardless of symptoms. On the other hand, clinical infection was defined as signs or symptoms consistent with an infection, but without microbiological confirmation. Central venous catheter (CVC)-related

Patient characteristics and transplant outcomes

	RIC ($n = 91$)	CIC ($n = 54$)	P
Median age of patients (range)	55 (26–68)	37 (18–53)	< 0.0001
Median age of donors (range)	50 (17–69)	34 (19–54)	< 0.0001
Male/female patient	57[1]/34	22/32	0.015
Female donor for male patient	19	10	0.83
Diagnosis AML (+ MDS)	27 (9)	17 (4)	0.0029
MDS	17	4	
CML	7	12	
ALL	1	8	
ML	36	13	
Others[2]	3	0	
Disease risk group (standard/advanced)[3]	14/77	21/33	0.0023
Median time interval[4] (range), (months)	19 (2–178)	10 (1–100)	0.014
KPS[5] ≤ 80%	10	5	0.41
HCT-SCI[6] ≥ 2	13	7	0.99
Prior infectious complications	6	3	0.99
Prior autologous transplantation	5	2	0.99
Donor type and source of stem cells			
Related PBSC/Unrelated BM	82/9	34/20	0.0002
GVHD prophylaxis			
CSP orTAC alone/MTX with CSP orTAC	66/25	1/53	< 0.0001
Acute GVHD grade II/III/IV	24/23/3	18/8/2	0.072
Median onset day (range) of grades II–IV acute GVHD[7]	39 (12–97)	32 (14–91)	0.48
Prior use of PSL for acute GVHD			
0.5– <1.0/1.0– <2.0/ ≥ 2.0 mg of PSL/kg	5/34/18	4/13/9	0.27
Relapse/progressive disease following cGVHD	16	10	0.99
Cause of death	30	20	0.27
Infection	15[8]	7[8]	
Chronic GVHD	9[8]	8[8]	
Lungs/gastrointestinal tract/MOF/Others[9]	3/1/3/2	3/3/2/0	
Others[10]	3	6	
Progression	8	2	
Median follow-up (range), (months)	39 (5–73)	45 (15–79)	0.20

[1]Number of patients, unless indicated otherwise.
[2]Others = myelofibrosis ($n = 1$), chronic lymphocytic leukemia ($n = 1$), and multiple myeloma ($n = 1$).
[3]Patients who were considered standard risk with a diagnosis of AML + MDS or AML, or ALL in first complete remission, CML in first chronic phase, or untreated refractory anemia in MDS. All other conditions were considered to indicate advanced risk.
[4]Time from diagnosis to transplantation.
[5]KPS was evaluated before the start of the conditioning regimen, and was graded according to Karnofsky performance status score.
[6]HCT-SCI was evaluated before the start of the conditioning regimen, and was graded according to hematopoietic cell transplantation-specific comorbidity index (ref. 8).
[7]Time from occurrence of grades II–IV acute GVHD to transplantation.
[8]Total number of patients differs because 8 patients (RIC, 5; CIC, 3) died of both infection and chronic GVHD.
[9]Others = renal ($n = 1$) and liver ($n = 1$).
[10]Others = RIC: cerebral infarction ($n = 1$), secondary hepatocellular carcinoma ($n = 1$), infection following secondary allogeneic cord blood stem cell transplantation; CIC: acute myocardial infarction ($n = 1$), cerebral infarction ($n = 1$), drug-induced interstitial pneumonia ($n = 1$), infection following chemotherapy ($n = 1$), and suicide ($n = 2$).
RIC, reduced-intensity regimen; CIC, conventional-intensity regimen; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; CML, chronic myeloid leukemia; ALL, acute lymphoblastic leukemia; ML, malignant lymphoma; PBSC, peripheral blood stem cell; BM, bone marrow; CSP, cyclosporine; TAC, tacrolimus; MTX, methotrexate; GVHD, graft-versus-host disease; PSL, prednisolone; cGVHD, chronic graft-versus-host disease; MOF, multiple organ failure.

Table 1

Clinical characteristics of cGVHD

	RIC	CIC	*P*
Median onset day (range)[1]	100 (79–479)	109 (93–348)	0.51
Limited/extensive	5/86	1/53	0.41
De novo/quiescent/progressive	27/38/26	24/9/21	0.16
KPS score 1/2/3	61/16/8	46/2/2	0.045
Skin score 1/2/3	27/33/8	17/12/3	0.15
Mouth score 1/2/3	40/29/3	23/9/1	0.87
Eyes score 1/2/3	30/14/7	15/9/1	0.38
Gastrointestinal tract score 1/2/3	28/4/12	19/1/7	0.84
Liver score 1/2/3	7/23/44	6/13/24	0.89
Lungs score 1/2/3	6/8/4	8/7/1	0.26
Joints and fascia score 1/2/3	13/2/0	8/5/1	0.13
Genital tract score 1/2/3	1/0/0	0/0/0	0.99
Eosinophilia $> 0.5 \times 10^9$/L	30	22	0.37
Platelets $< 100 \times 10^9$/L	26	20	0.36
Others[2]	5	2	0.39
Immunosuppressive drugs at diagnosis of cGVHD			
CSA/TAC	66/3	37/3	0.76
$< 0.5/0.5–< 1.0/1.0–< 2.0/ \geq 2.0$ mg of PSL/kg	17/9/11/2	9/2/3/1	0.57
Initial treatment for cGVHD			
Addition or increased dose of CSA/TAC	69/7	41/4	0.99
$< 0.5/0.5–< 1.0/1.0–< 2.0/ \geq 2.0$ mg of PSL/kg	18/14/15/4	10/7/6/2	0.74
Median follow-up from diagnosis of cGVHD (range) (months)	39 (5–73)	45 (15–79)	0.26

[1]Time from occurrence of cGVHD to transplantation.
[2]Others = pleural effusion ($n = 4$), pericardial effusion ($n = 3$), ascites ($n = 3$), and polymyositis ($n = 1$).
RIC, reduced-intensity regimen; CIC, conventional-intensity regimen; cGVHD, chronic graft-versus-host disease; KPS, Karnofsky performance status; CSP, cyclosporine; TAC, tacrolimus; PSL, prednisolone.

Table 2

infections consisted of exit site infections without bacteremia. Bacterial pneumonia was included in the category of definite infections, and was diagnosed by chest x-ray examination or computed tomography (CT) and identification of a bacterial pathogen on culture of sputum, bronchoalveolar lavage fluid, pleural fluid, or blood specimen. Fungal infections, including proven or probable invasive fungal infections, were diagnosed by identification of a fungal pathogen on culture or *Aspergillus* antigen and CT examination according to consensus criteria (13). Pneumonia of unknown origin was included in the category of undefined pneumoniae, which were diagnosed by chest x-ray and/or CT. There was no significant difference in CMV serostatus between the RIC and CIC groups (data not shown). A polymicrobial infection of 1 organ or several adjacent organs was considered to be a single infection. Death associated with a documented infection was defined as the death of a patient with findings consistent with an infection, or as detection of the pathogen in an autopsy specimen.

Statistical analysis

Comparisons of variables were performed using the 2-tailed Fisher exact test or the χ^2 test. Continuous variables were compared by the Mann–Whitney *U*-test. All *P*-values were 2-sided, and the type I error rate was fixed at $P < 0.05$.

Results

Transplant outcomes

The transplant outcomes are summarized in Table 1. Twenty-two patients (RIC, $n = 15$; CIC, $n = 7$) died of infections, of whom 8 patients (RIC, $n = 5$; CIC, $n = 3$) died of