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ORIGINAL ARTICLE

**Masao Hagihara · Balgansuren Gansuvd · Yoko Ueda
Takahide Tsuchiya · Aya Masui · Kei Tazume
Hiroyasu Inoue · Shunichi Kato · Tomomitsu Hotta**

Killing activity of human umbilical cord blood-derived TCRValpha24$^+$ NKT cells against normal and malignant hematological cells in vitro: a comparative study with NK cells or OKT3 activated T lymphocytes or with adult peripheral blood NKT cells

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Abstract **Purpose:** We aimed to determine the effects of human umbilical cord blood (UCB)-derived natural killer T (NKT) cells as immunological effectors against hematological malignancies, as well as auto- or allo-dendritic cells (DCs) or EB transformed cell lines (EBCLs). **Materials:** TCRVα24$^+$ Vβ11$^+$ UCB- or PB-NKT cells were isolated by sorting and activated by α-galactosylceramide-pulsed autologous DCs. UCB-NK cells were induced from CD34$^+$ cells by stem cell factor plus IL-15. UCB-T cells were primarily activated by anti-CD3 monoclonal antibody. All those effectors were cultured with IL-2 (100 U/ml), and their cytotoxic activities were evaluated by ^{51}Cr-release assay. UCB-NKT cells were cultured with IL-12, IL-18 or higher dose of IL-2 (1000 U/ml), and again tested for the cytotoxicity against selected targets. **Results:** UCB-NKT cells exhibited a pattern of killing activity against various hematological malignancies similar to that of UCB-NK cells, but could not kill K562, which was a vulnerable target for NK cells. The level of activity was quite similar to that of PB-NKT cells. In contrast, OKT-3-activated UCB-T lymphocytes showed a stronger and wider spectrum of killing compared with UCB-NK or NKT cells. IL-12, IL-18 or a higher dose of IL-2 upregulated the activity; however several targets, including fresh leukemic cells, still remained resistant. NKT cells killed auto- or allo-DCs at a level similar to that of T cells, but could not kill allo-EBCLs, which were efficiently killed by T cells. While NK cells showed only marginal or no killing against DC or EBCLs. **Discussion:** The anti-cancer activity of human NKT cells depends on the concentrations or the combination of Th1-cytokines. Basically, those cells might not be contributing to the immune surveillance of hematological malignancies, as shown by a relatively low cytotoxicity against malignant cells, together with the quite strong killing against auto-DCs.

Keywords Umbilical cord blood · Cytotoxicity · Vα24$^+$ NKT cells · Hematological malignancy · Dendritic cells

M. Hagihara and B. Gansuvd contributed equally to this manuscript

M. Hagihara (✉) · B. Gansuvd · Y. Ueda · T. Tsuchiya
K. Tazume · T. Hotta
Department of Hematology and Rheumatology,
Tokai University School of Medicine, Bohseidai,
Isehara, Kanagawa, Japan 259-1193
E-mail: masaoha@is.icc.u-tokai.ac.jp
Tel.: +81-463-931121
Fax: +81-463-965438

H. Inoue · S. Kato
Department of Pediatrics, Tokai University School of Medicine,
Bohseidai, Isehara, Kanagawa, Japan

A. Masui
Genetic Engineering and Cell Transplantation Research Center,
Tokai University School of Medicine, Kanagawa, Japan

Introduction

Murine NKT cells bearing Vα14 have strong anti-tumor activity, both in vitro and in vivo [14, 19]. Human NKT cells express invariant Vα24JαQ receptor, proliferate and show cytotoxic activity similar to their murine counterpart [2, 3, 15, 31]. Recently, Kawano et al. reported that NKT cells induced from peripheral blood (PB) of healthy volunteers kill malignant cell lines in a random fashion [16], and Nieda et al. stated that double negative (DN)-NKT cells kill U937, Molt-4, C1R and THP-1; however, NK-sensitive K562 or LAK-sensitive Daudi were resistant to killing [23]. Nieda et al. also reported that CD4$^+$ NKT cells have a similar pattern of killing activity and

produce much more IL-4 compared with DN-NKT cells after α-galactosylceramide (αGalCer) stimulation [25].

Umbilical cord blood (UCB) has recently been utilized as a source for stem cell transplantation [9, 21]. $CD34^+$ progenitors circulating in UCB have markedly higher proliferating potential than those in bone marrow (BM), whereas the immunological immaturity [34], such as lower NK cell activity [11] and impaired defense against viral targets, is a serious handicap after the engraftment of donor cells. Recently, it was also reported that UCB-NKT cells already acquired a memory activated phenotype of surface phenotype, such as CD45RO or CD25, which indicates that they already encountered a natural ligand during fetal life [6, 37]. The number of NKT cells in UCB is twice as high as that in PB [16]. Therefore, there is a possibility that NKT cells can compensate for the functional immaturity of UCB lymphocytes, and can be utilized as an alternative anti-cancer or viral effector in complications after UCB transplantation.

Considering those backgrounds, in the present study NKT cells were enriched and expanded using auto-dendritic cells (DCs) pulsed with αGalCer, and their anti-tumor activity against various hematological malignancies, including fresh leukemic cells, was examined in comparison with the other main effectors, NK cells and T cells activated by OKT-3 from the same source. In the next, the activity of UCB-NKT cells was compared with that of adult PB-NKT cells.

NKT cells are activated not only by αGalCer-pulsed DCs, but also synergistically by IL-12, as shown by Nishimura et al [18]. IL-18 also synergizes with IL-12, facilitating human T cells to produce IFN-γ [36]. Those cytokines, or higher amounts of IL-2, were added and tested for their enhancing effects of target cell killing.

NKT cells also recognize and attack auto- or allo-DCs in the context of CD1d restriction, and play an important role in the regulation of the hyperimmune status related to autoimmune disorders, such as systemic sclerosis in humans [32] or in non-obese diabetes (NOD) in mice [1, 10]. This indicates that these cells might have important roles in the suppression of the graft versus host (GVH) reaction by depleting strong APCs from either the donor or recipient. Then, in the present study, the killing activity against DCs or EBV-transformed cell lines (EBCLs) was also examined.

Materials and methods

UCB and PB

Human UCB samples were obtained from normal full-term deliveries in accordance with the Tokai University Committee on Clinical Investigation. The cells were stored at room temperature and processed within 24 h of collection. PB samples were collected from normal healthy volunteers.

DCs

Mononuclear cells (MNCs) were separated from UCB by Ficoll-Hypaque density gradient (G = 1.077 g/dl). $CD34^+$ cells were isolated from MNCs using the MACS immunomagnetic separation system and cultured in 2 different culture systems. For the first culture system, $CD34^+$ cells (1×10^4) were directly induced to DCs by a standard method in 2 ml of αMEM (Gibco-BRL, Grand Island, N.Y.) plus 10% fetal bovine serum (FBS; Gibco-BRL), supplemented with 100 ng/ml human GM-CSF (kindly provided from Kirin Brewery, Gumma, Japan), 10 ng/ml human TNF-α (Dako Japan Co., Kyoto, Japan), 50 ng/ml human SCF (Kirin Brewery) from day 0, and 10 ng/ml human IL-4 (Dako Japan) from day 5. For the second culture system, $CD34^+$ cells were firstly incubated in complete medium containing 50 ng/ml SCF, 50 ng/ml of human Flt-3 (Pepro Tech, London, UK), 50 ng/ml human TPO (Kirin Brewery), and then exposed to a secondary conditioning medium containing 100 ng/ml of GM-CSF, 10 ng/ml of IL-4 plus 10 ng/ml TNF-α to obtain mature DCs (MDCs).

Enrichment and expansion of UCB- or PB-derived NKT cells

The $TCRV\alpha24^+$ cells were isolated from MNCs using a magnetic cell sorting system (MACS, Miltenyl Biotec, Gladbach, Germany). Briefly, $V\alpha24^+$ cells were enriched by staining with PE-conjugated mouse anti-Vα24 mAb and PE-MACS beads and then applied to a MACS column.

Malignant cells of leukemia or lymphoma cell lines were purchased from RIKEN Cell Bank (Tsukuba Life Science City, Ibaragi, Japan), for use as targets. The lineage or the origin of each line is listed in Table 1. KY and KU were kindly provided by Dr. K. Kishi (Tokai Univ., Kanagawa, Japan), and Taka and Ozawa were lines recently established in our laboratory.

Fresh leukemia or lymphoma cells were harvested from bone marrow (BM), PB, or affected lymphoid organs and frozen at –170 °C until use. Informed consent was obtained from each subject.

Epstein-Barr virus (EBV)-transformed cell lines (EBCLs)

MNCs were depleted of T/NK cells by anti-CD2 mAb conjugated Dynabeads (Dynal), and incubated with supernatant obtained from B95-8 cells. These MNCs became blastoid 2–3 weeks later.

Flowcytometry

The effectors and targets were analyzed by flowcytometry. The following mAbs were used: FITC-anti-CD3 (Dako Japan Co., Kyoto, Japan), FITC- or APC-anti-CD4 (Pharmingen, San Diego, Calif.), PE-anti-CD8 (Pharmingen), PE-anti-CD56 (Pharmingen), FITC-anti-TCRVβ11 (Immunotech, Marseille, France), PE-anti-TCRVα24 (Immunotech). Anti-CD1d mAb was a gift from Prof. SA. Porcelli (Department of Microbiology and Immunology, Albert Einstein College of Medicine, Bronx, N.Y.).

The cytoplasmic expression of perforin was stained by anti-perforin mAb (Immunotech), followed by detection with FITC-conjugated goat-derived anti-mouse immunoglobulin (Becton Dickinson).

In vitro activation by cytokines

NKT cells were cultured with human IL-12 (10 ng/ml; Pepro Tech) or human IL-18 (10 ng/ml; provided by Dr. Tadao Ohno, M.D. from RIKEN Cell Bank), or both, or a high dose of IL-2 (1000 U/ml) for 5 days together with αGalCer stimulation.

Cytotoxic activity

The killing activity was analyzed by a standard ^{51}Cr-release assay. The targets were labeled with $Na^{51}CrO_4$ (2 MBq/ml; Amersham, Bucks, UK), and added at 5×10^3/well of a 96-well V-bottomed plate. The effector was adjusted to a 20:1 E/T ratio and applied in triplicate.

Table 1 The expression of CD1d molecule in various hematological malignancies. Results are given as percentages and MFI of $CD1d^+$ cells, which were estimated by flowcytometry (*MFI* mean fluorescence intensity, *n.d.* not done)

Name	Definition	CD1d (%)	CD1d (MFI)
Kasumi	M2	17	12
NB4	M3	12	18
HL-60	M3	18	22
KY	M4	79	60
KU	CML	17	23
YS-1	CML	0	2
K562	CML	2	3
Taka	ALL	7	8
Ozawa	ALL	20	16
Molt4	T-ALL	99	56
Jurkat	T-lymphoma	62	51
Daudi	B-lymphoma	35	29
Raji	B-lymphoma	4	5
U937	lymphoma	0	3
	AML-M1	n.d.	n.d.
	AML-M2 case1	24	12
	AML-M2 case2	0	3
	AML-M4	20	21
	AML-M5	21	20
	CMMoL case1	n.d.	n.d.
	CMMoL case2	0	3
	CMMoL case3	21	18
	ALL	2	3
	CML	15	20
	T-lymphoma	47	21
	B cell	3	4
	IMDC	75	31
	MDC	85	95
	EBCL	18	33

Results

Phenotypes of human UCB-NKT, NK and activated T cells

Vα24-positive cells were preferentially expanded at 3 weeks from the standard culture, and Vβ11 Vα24 double-positive cells were sorted (Fig. 1A). The rate of expansion was similar between UCB and adult PB (data not shown). Most cells showed a $CD4^+$ phenotype (data not shown). NK cells appeared as the major fraction at 4–8 weeks after $CD34^+$ separation, and their phenotype was $CD3^-$ $CD56^+$, as shown in Fig. 1A. T lymphocytes proliferated at 2–3 weeks of OKT3 activation, and all cells exhibited a $CD3^+$ phenotype, and neither NKT nor NK cells were included (Fig. 1A). All the effectors expressed perforin molecules at almost a similar level (Fig. 1B).

CD1d expression of targets

CD1d was expressed strongly (in more than 50% of total cells) by KY, Molt-4, and Jurkat cells, and also DCs, and moderately (25–49%) by Daudi, and one of the fresh T lymphoma cell isolates. In other targets, CD1d was either weakly expressed (<20%) or not detectable, as shown in Table 1.

Killing activity against hematological malignancies (cell lines)

As shown in Fig. 2A, among the cell lines Molt-4 was the most sensitive to killing; Jurkat was also significantly killed (>20%); and several targets, such as Kasumi,

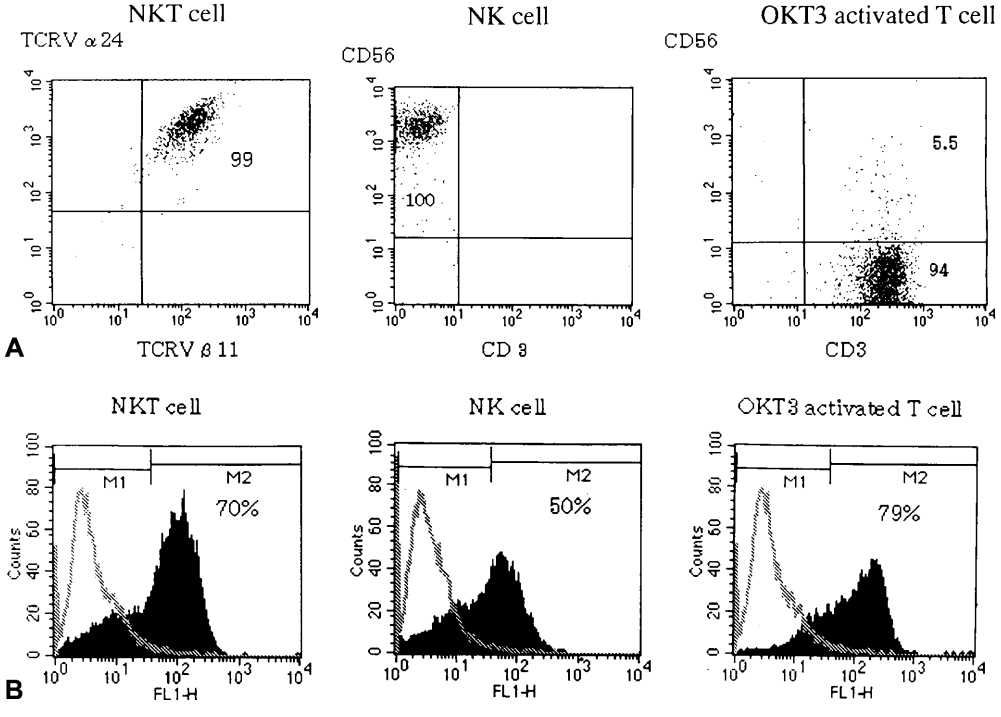

Fig. 1A, B FACS analysis of UCB-NKT, NK and T cells. **A** The phenotypes of NKT, NK and OKT-3 activated T cells. NKT cells expressed both Vβ11 (FL1; *horizontal side*) and Vα24 (FL2; *vertical side*). NK and T cells showed CD3 $(FL1)^-$ and CD56 $(FL2)^+$, and $CD3^+$ $CD56^-$, respectively. **B** Intracellular expression of perforin. The perforin was expressed almost at the same level. All of these effectors were induced or expanded from the same source of UCB, and the representative of 3 different samples is shown here

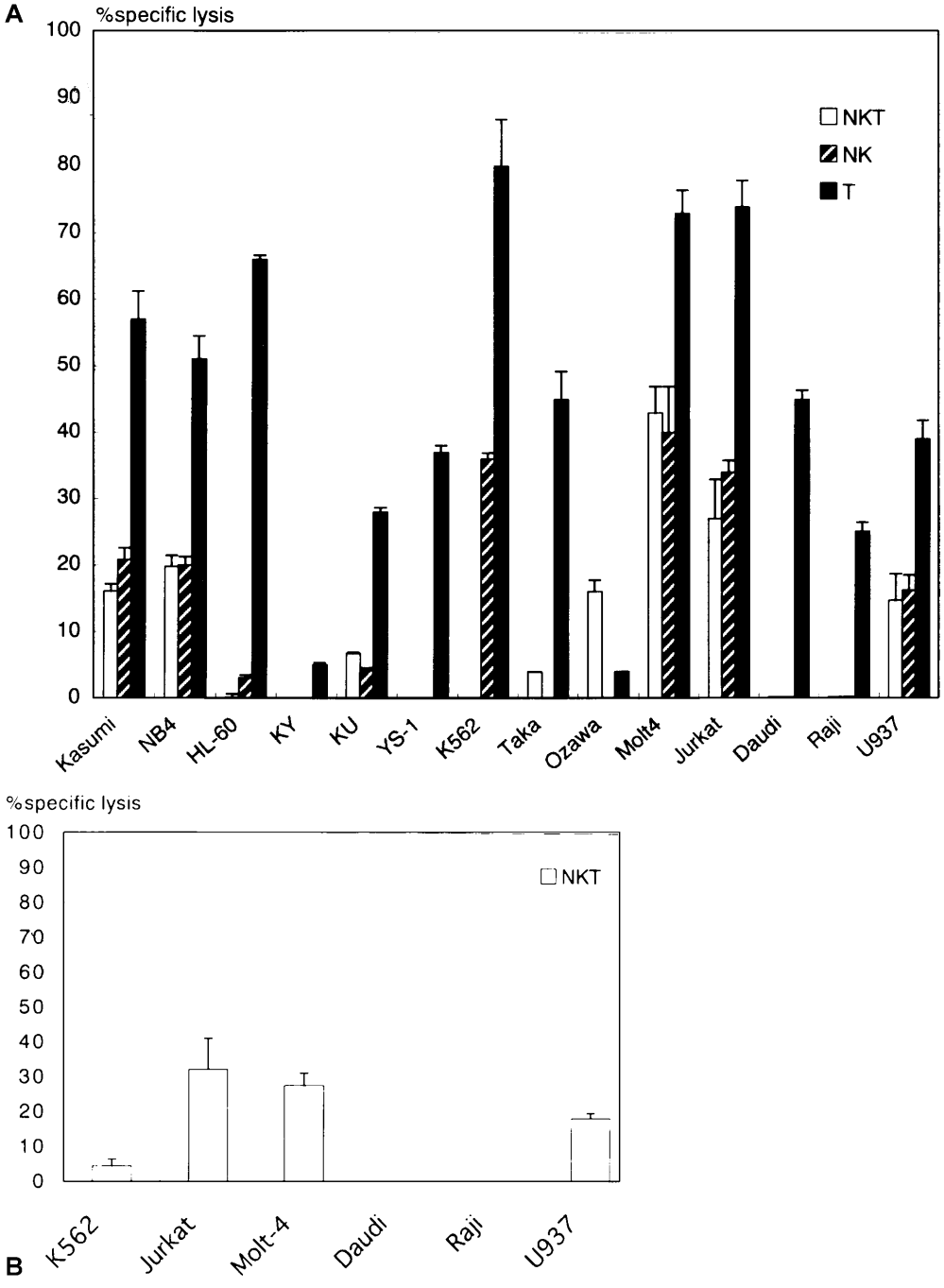

Fig. 2A, B The killing activity of UCB- and PB-NKT cells against hematological malignancy (cell lines). **A** UCB-NKT cells and NK cells showed a similar pattern of killing activity against the targets, except for K562. OKT-3 activated T cells showed the highest killing activity. Each value shown represents the mean ± SD of specific lysis performed in triplicate at an E/T ratio of 20:1. All the effectors were derived from the same source of UCB, and representative data of 3 different sources are shown here, as there was no significant difference between them. **B** PB-NKT cells showed a similar pattern and level of killing activity as that shown by UCB-NKT cells. Representative data of 3 repeated experiments are shown

NB4, Ozawa, and U937, were moderately killed (10–20%). The NK cells showed a quite similar pattern of killing to NKT cells, and the only striking difference was the killing against K562. OKT-3-activated T cells randomly killed malignant cell targets, except KY and Ozawa. PB-NKT cells showed a similar pattern and level of killing activity as shown by UCB-NKT cells (Fig. 2B).

Killing activity against hematological malignancy (fresh leukemia and lymphoma cells)

NKT cells significantly killed fresh chronic myeloid leukemia (CML) cells, which were taken from a patient with blastic crisis, and moderately killed acute myeloid leukemia (AML) target cells, but showed no, or marginal, killing against one the other targets; whereas activated T cells showed a wide spectrum of killing (Fig. 3). NK cells could not be tested because of insufficient cell numbers.

Killing activity against auto- or allo-DCs and EBCLs

UCB-NKT cells exhibited quite high killing activity against auto- or allo-DCs, but could not kill EBCLs at all. OKT-3-activated T cells killed auto/allo- DCs, and allo-EBCLs. Whereas, NK cells showed no, or only marginal, killing against those targets (Fig. 4).

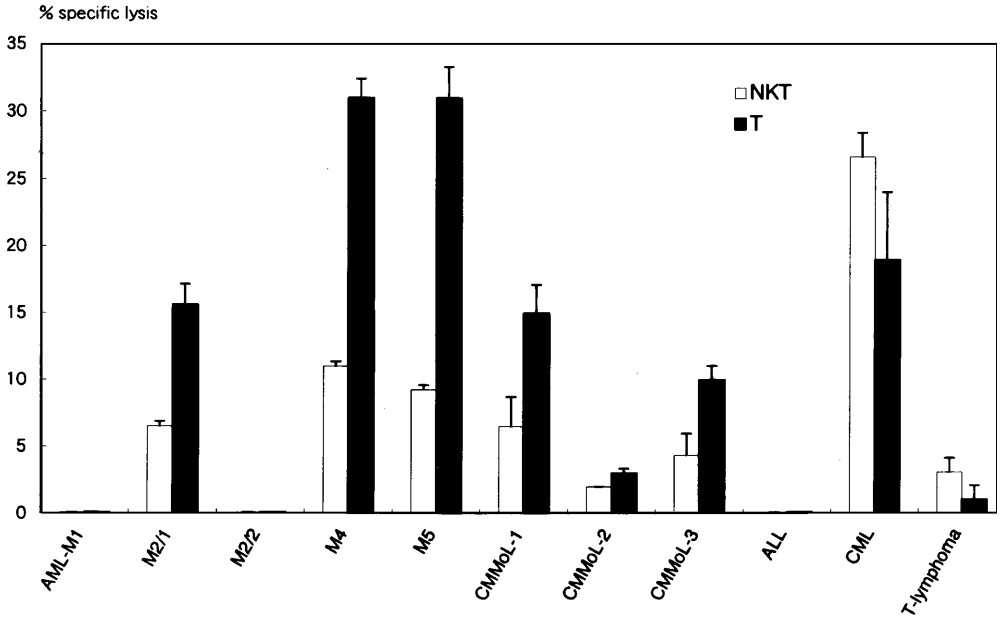

Fig. 3 The killing activity against fresh leukemia and lymphoma cells. UCB-NKT cells showed limited (less than 10%) killing activity against the targets, except for CML, whereas T cells showed a higher killing activity, with a broad spectrum. Each value shown represents the mean ± SD of specific lysis performed in triplicate at an E/T ratio of 20:1. Both NKT and T cells were derived from the same source of UCB and representative data of 3 different sources are shown here, as there was no significant difference between them

Cytokine upreguration

IL-12 and IL-18 additively promoted a killing activity against several targets, such as Jurkat, U937, K562 and 2 out of 3 fresh leukemic cell isolates. A higher dose of IL-2 similarly upregulated the killing against those targets. In contrast, there were several targets, such as Daudi and a fresh leukemic cells, which still remained resistant (Fig. 5).

Discussion

The present study has showed that human Vα24 Vβ11 double-positive NKT cells can be expanded from UCB after repeated stimulation by auto-DCs pulsed with αGalCer. Almost all cells showed a CD4 phenotype, and few cells expressed DN or CD8 molecules. Takahashi et al. reported that there is no clear difference between DN and $CD4^+$ NKT cells derived from human PB, except for the higher production of IL-4 by $CD4^+$ NKT cells [23, 24, 25]. We also tried to induce DN-NKT cells from PB-MNCs; however, again only $CD4^+$ NKT cells proliferated as a major component. The culture condition with our protocol contains a higher (100 U/ml) IL-2 concentration than the previous report (5–50 U/ml), which might have induced a preferential proliferation of $CD4^+$ NKT cells from both UCB and PB. Considering the clinical application, much easier protocols for cell manipulation or culture should be contemplated. $CD4^+$ NKT cells continued to proliferate for over 3 months after the separation, and could also be safely stored frozen. Therefore, we considered that $CD4^+$ NKT cells, but not DN-NKT cells, could be candidates for future immune cell therapy, if the significance of their activity is proven.

NKT cells, which bear Vα14 as a specific receptor for αGalCer, have a quite strong killing activity against malignant cells, not only in vitro but also in vivo [14, 18]. In humans, Kawano et al. proved that Vα24-positive cells killed various targets and succeeded in inhibiting the growth of human esophageal cancer cells inoculated in nude mice [16]. In contrast, Nieda et al. showed that human PB-NKT cells killed U937, Molt-4 and THP-1 cells, but did not kill K562 and Daudi cells [23, 24, 25]. The major difference between the two studies is that Nieda et al. separated NKT cells by sorting, whereas Kawano et al. did not. We also found that the direct effect of human NKT cells was not so strong and they killed only limited targets; therefore, the results of Kawano et al. might be due to other contaminating effectors, such as NK cells or conventional T cells. Ishihara et al. reported that hepatic $CD3^-$ $CD56^+$ NK cells are activated by αGalCer or α-glucosylceramide in vitro, although the enhancing effect was not necessarily attributed to the existence of $V\alpha24^+$ NKT cells [13]. Among $V\alpha24^+$ cells, not all cells express the invariant JαQ chain [17, 29]. And the pairing with Vβ11 is critical for constructing such a NKT-specific receptor. Therefore, in the present study, the strict separation of the Vβ11 Vα24 double-positive fraction was performed by FACS sorting, which should have provided the identical type of effectors analyzed by Nieda et al.

Human NKT cells show a proliferative response to αGalCer in the context of CD1d restriction [5, 31]. However, the expression of CD1d is not necessarily a sufficient factor to exhibit target cell killing, as shown in the present study. CD1d is expressed in a wide range of normal tissues, including the intestine, liver, pancreas, etc. In contrast, the expression of CD1d on hematopoietic lineage is restricted only to DC and B cells [4] The type or lineage of leukemia, as shown by FAB classification, did not predict the level of CD1d

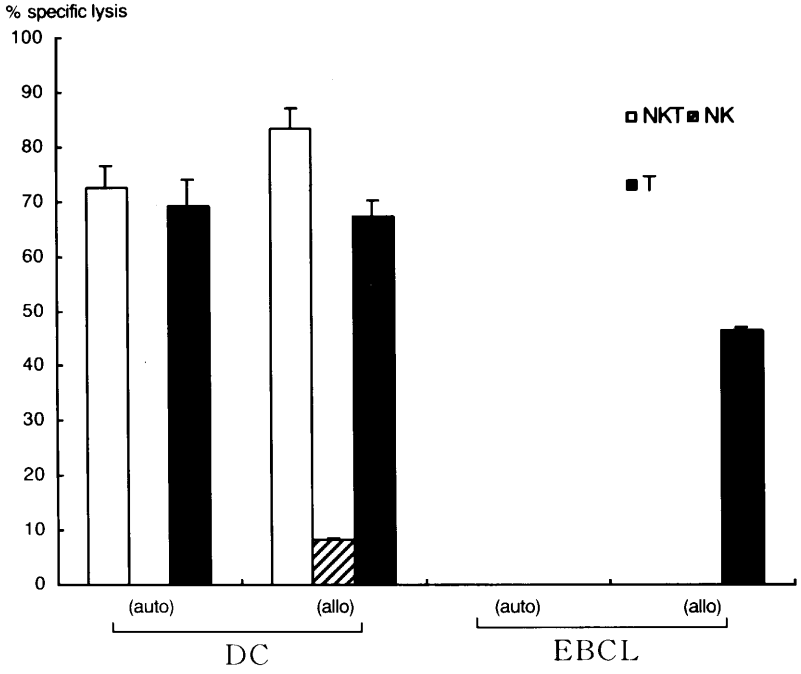

Fig. 4 The killing activity against auto- or allo-derived DCs and EBCLs. UCB-NKT cells exhibited the highest killing against auto- or allo-DCs; however, they could not kill EBCLs. OKT-3-activated T cells killed auto/allo-DCs and allo-EBCLs. Whereas, NK cells showed no or only marginal killing of these targets. Each value shown represents the mean ± SD of specific lysis performed in triplicate at an E/T ratio of 20:1

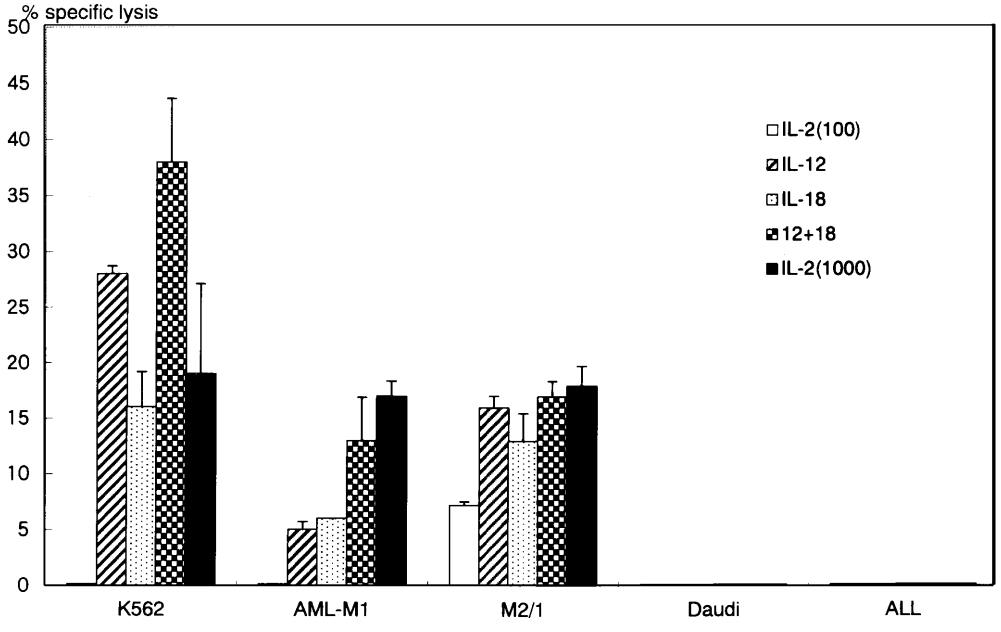

Fig. 5 Cytokine upregulation of NKT cell cytotoxicity against hematological malignancy. UCB-NKT cells were exposed to IL-12 (10 ng/ml), IL-18 (10 ng/ml), or both, or a high dose of IL-2 (1000 U/ml) for 5 days together with αGalCer stimulation. Each value shown represents the mean ± SD of specific lysis performed in triplicate at an E/T ratio of 20:1

expression. Two T-cell-lymphoma lines (Molt-4 and Jurkat), expressing CD1d at a significant level were sensitively killed by NKT cells, and the killing was dose-dependently neutralized by anti-CD1d mAb (manuscript submitted). While, several cell lines (Kasumi, NB4, Ozawa, U937) were moderately (10–20%) killed with even faint expression of CD1d. In contrast, there were two lines (KY and Daudi) and the cells of one fresh T lymphoma isolate that showed resistance, even if they significantly expressed CD1d. NKT cells could not kill these targets, even if they were pulsed with αGalCer (data not shown). These results indicate that the endogenous natural ligands bound to CD1d might be important for the recognition by NKT cells, or the cell lines could not catch up or bind αGalCer on their CD1d molecules.

The cytotoxic activity of NKT cells is mediated mainly through the perforin pathway [16, 35]. Actually, NKT cells showed a similar intracellular expression of perforin to the other two effectors. In the present study, both NKT and NK cells, which also utilize perforin in their target cell killing, showed a limited repertoire of killing against hematological malignancies. In contrast, activated T cells showed the highest killing, in a broad spectrum, toward cell lines and fresh leukemia or lymphoma cells. It was then supposed that the amount of

perforin excreted from activated T cells far exceeded those from NK or NKT cells, or several receptor-ligand reactions might have inhibited the activity of NK and NKT cells. The NK cells express NK receptors (NKR), which deliver negative signals leading to target cell protection after interacting with HLA-class-I antigens [20]. In humans, NKR belong to 2 distinct families: an Ig superfamily, such as p58 or p70, and a CD94/NKG2 complex [20, 21]. NKT cells do not express both receptors. The most discriminating point is that K562 was killed not by NKT cells but by NK cells. Therefore, these 2 effectors must harbor different systems to regulate their killing activity.

Recently, Nishimura et al. proved that IL-12 and αGalCer synergistically promoted anti-cancer activity in an in vivo murine model [18]. Another immunostimulatory IFN-γ producing cytokine, IL-18, in a single addition increased the CTL activity or synergized with IL-12 to produce IFN-γ from human T cells [36]. In the present study, the enhancing effects of those 2 cytokines and also a higher dose of IL-2 against target cell killing were examined. The results showed upregulated killing, not synergistic but additive, by IL-12 and IL-18. A higher dose of IL-2 gave almost the same level of enhancing effect as IL-12 plus IL-18. The resistance of killing against K562 was first broken by such highly activated NKT cells; however, several targets, Daudi or a fresh leukemia, still showed a resistance. Nishi et al. reported that IL-15, -7, and -2 synergistically stimulated the expansion of $V\alpha24^+$ NKT cells from PB-MNCs, and IL-15 increased GrB expression. Wang et al. reported that IL-12 enhanced the response of NK cells through up-regulation of IL-12R [38]. In the future, the combined addition, or much higher doses, of those cytokines should be applied to maximally enhance their killing activity.

NKT cells might be expected to control GVH reaction through depleting the strongest APCs. In our hands, however, NKT cells killed both auto- and allo-DCs. Surprisingly, activated T cells also killed auto- as well as allo-DCs. This was not peculiar to UCB-T cells, as adult PB-T cells also killed both auto- and allo-DCs (data not shown). These findings might suggest that self-reacting T cells, as well as allo-reacting T cells, were activated by OKT3 stimulation. In contrast, NK cells never killed any targets, including DCs. It has been reported that short-term activated NK cells could lyse autologous DCs [8, 39]. The NK cells in their study were generated from PB-MNCs. The immature nature of UCB-NK cells, especially differentiated from $CD34^+$ cells, as shown by a $CD16^-$ $CD56^+$ marker, might have contributed to those discrepant results. In conclusion, NKT cells might not be beneficial in assisting host immune defense, as they specifically act to attack and delete the most important APCs, DCs.

Due to the immunological immaturity of T cells, UCB transplantation has a handicap in the mounting of antigen-specific responses compared with marrow transplantation. Especially for pediatric transplants, recipients are exposed to a high risk of infection; in particular, EBV-associated lymphoproliferative disorders [28, 30] as neonates generally have not yet been immunized with EBV. It is also quite difficult to induce such anti-EBV CTLs in vitro from UCB-MNCs [22, 33]. In the present study, all the effectors could not kill auto-EBCLs. An interaction between innate and acquired immunity has been discussed, and Nishimura et al. proved that NKT cells accelerated the development of tumor-specific generation in a murine model [27]. The selective activation of murine NK cells by αGalCer activated NKT cells was also reported by Eberl et al [7]. As the direct effect of NKT cells against various hematological malignancies is not that likely, an indirect activation through the interaction via other effectors (NK or T cells) should be considered in the near future.

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