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**Increased number of CD16<sup>+</sup>CD56<sup>dim</sup> NK cells in peripheral blood mononuclear cells after allogeneic cord blood transplantation**

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Running Title: Increased CD16<sup>+</sup>CD56<sup>dim</sup> NK cells after CBT

Key word: NK cell; Cord blood transplantation; Graft-versus-leukemia effect

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## ABSTRACT

In the present study, we investigated subpopulations of NK cells and the expression of stimulatory and inhibitory NK receptors after adult blood and bone marrow transplantation (BBMT) and cord blood transplantation (CBT). There were significant increases in CD16<sup>+</sup>CD56<sup>dim</sup> cell proportion and absolute number in peripheral blood mononuclear cells (PBMC) during a period of 4 months to 9 months after CBT compared with these in normal PBMC, cord blood (CB) and in PBMC after BBMT. Also, increased number of CD16<sup>+</sup>CD56<sup>dim</sup> NK cell sustained in some patients until 4 years after CBT. This CD16<sup>+</sup>CD56<sup>dim</sup> cell subset after CBT exhibited decreased expression of NKG2A compared with that in CB and increased expression of NKG2C. Purified CD16<sup>+</sup>CD56<sup>dim</sup> cells from patients 8-9 months after CBT exhibited significantly higher level of cytolytic activity against K562 than did purified CD16<sup>+</sup>CD56<sup>bright</sup> cells and also whole PBMC. The CD16<sup>+</sup>CD56<sup>dim</sup> cell subset with a high level of cytolytic activity significantly increased after CBT, and these cells may be responsible for NK cell-mediated immunity after CBT.

## 1. Introduction

Human natural killer (NK) cells are large granular lymphoid cells defined as being membrane CD3<sup>-</sup>, CD16<sup>+</sup> and/or CD56<sup>+</sup> and account for approximately 10-15% of cells in lymphocytes [1-4]. The majority of adult peripheral blood NK cells are CD16<sup>+</sup> CD56<sup>+</sup> with a minor population of cells that are CD16<sup>-</sup> CD56<sup>+</sup>. NK cells can attack target cells such as tumor cells and pathogen-infected cells without prior sensitization and without major histocompatibility restriction. It has been proposed that there are 5 stages of development of human NK cells from BM-derived human stem cells [4]. From stage 1 to 3, NK cells become committed to the NK cell lineage, and stage 4 CD56<sup>bright</sup> NK cells may preferentially produce IFN- $\gamma$  and then stage 5 CD56<sup>dim</sup> NK cells may preferentially mediate cellular cytotoxicity.

Neonatal cord blood (CB) cells have been demonstrated to contain a higher percentage of NK cells [5] but immature with low level of cytolytic activity [6]. It has also been reported that cord blood contains CD16<sup>+</sup>56<sup>-</sup> cells with low level of cytolytic activity and that these cells are possible precursors of mature NK cells [7]. However, expression level of perforin and granzyme B have been reported be higher in CB NK cells, and it has been suggested that CB NK cells are phenotypically and functionally mature [8].

Cord blood transplantation (CBT) has been increasingly used for the treatment of hematological malignancies in adults [9-11]. It has been suggested that cord blood is a source of

stem cells that is as safe and effective as bone marrow or mobilized peripheral blood.

Overall results for CBT recipients have been shown to be better than those for BMT recipients in terms of graft-versus-host disease (GVHD) due to the immaturity of T cells in cord blood [12], and results for CBT recipients are also potentially better in terms of transplantation-related mortality (TRM) and disease-free survival (DFS). Therefore, it was suggested that immunocompetent cells other than T cells may mediate the graft-versus-leukemia (GVL) effect after CBT. NK cells have an important role in the GVL effect after HLA-mismatched stem cell transplantation [13,14]. CBT is often carried out from HLA-mismatched donors, and NK cells may therefore contribute to the development of GVL after CBT. However, there is little knowledge about subpopulations of peripheral blood NK cells after allogeneic CBT.

In this study, we tried to analyze subpopulations of NK cells after adult blood and bone marrow transplantation (BBMT) and allogeneic CBT in order to characterize the development of NK cells after transplantation.

## 2. Materials and methods

### *2.1. Patients and blood samples*

Fourteen healthy peripheral blood samples and 14 cord blood samples were obtained from Hokkaido Red Cross Blood Bank, Sapporo, Japan. Thirty peripheral blood samples from 11 patients after peripheral blood stem cell transplantation (PBSCT) or bone marrow transplantation (BMT) from serological HLA-matched donors and 21 samples from 9 patients after CBT (Table 1) were obtained. 8 CBT patients were transplanted serological HLA 4 of 6 loci matching CB and one patient was transplanted 5 of 6 loci matching CB. We obtained HLA-C serological typing as it possible. There were no group 1 HLA-C among cord blood units and no GVHD direction mismatch concerning about HLA-C group between cord blood units and recipients. Only one patient had relapsed of leukemia 8 months after CBT among our 9 patients with hematological malignancy who underwent CBT (relapse-free survival period of 8 to more than 61 months with a mean period of more than 38 months). Among these 9 patients who underwent CBT, acute GVHD of grade I, grade II and grade III developed in 2, 2 and 2 patients, respectively, and chronic GVHD of limited type and of extensive type developed in 4 and 2 patients, respectively. All patients achieved complete donor type chimerism (more than 97% of donor type) within 3 months after CBT. Informed consent for the analysis of blood cells was obtained from all patients.

## *2.2. Immunofluorescent staining for flow cytometric analysis and monoclonal antibodies*

The following antibodies were used in this study: the phycoerythrin (PE)-conjugated monoclonal antibody (mAb) HP-3D9 (anti-CD94), obtained from Ancell (Bayport, MN, USA); Z199 (anti-NKG2A), ON72 (anti-NKG2D), EB6 (anti-CD158a), GL183 (anti-CD158b), Z27.3.7 (anti-CD158e1, NKB1), Z25 (anti NKp30), Z231 (anti-NKp44) and BAB281 (anti-NKp46), obtained from Immunotec (Marseilles, France); 134591 (anti-NKG2C), obtained from R & D Systems (Minneapolis, MN, USA); and FITC-conjugated anti-CD16 and Cy5-conjugated anti-CD56, obtained from Becton Dickinson (BD, San Jose, CA, USA). Lymphocytes were gated using FSC and SSC. The three-colors fluorescence intensity of the cells was analyzed using a FACS Calibur. CD16<sup>+</sup>56<sup>bright</sup> cells and CD16<sup>+</sup>CD56<sup>dim</sup> cells were sorted using JSAN (Japan-made sort analyzer, Bay bioscience, Kobe, Japan). Statistical analysis was performed using Student's t-test.

## *2.3 Evaluation of cytolytic activity of CD16<sup>+</sup>56<sup>bright</sup> cells and CD16<sup>+</sup>CD56<sup>dim</sup> cells*

The cytolytic activities of unfractionated PBMC, purified CD16<sup>+</sup>56<sup>bright</sup> cells and CD16<sup>+</sup>CD56<sup>dim</sup> cells were tested against <sup>51</sup>Cr-labeled human erythroleukemic K562 cells (5 x 10<sup>3</sup>) using a 4-hour standard <sup>51</sup>Cr release assay (effector-to-target ratio is 10:1).

## *2.4. Long-term observation of CD16<sup>+</sup>CD56<sup>dim</sup> NK cells after CBT*

Additional 13 PBMC samples from 6 patients were analyzed during 12 months to 50months after CBT. All these patients were alive 20 to 50 months after CBT.



### 3. Results

#### *3.1. Subpopulations and absolute number of NK cells after stem cell transplantation at engraftment phase*

PBMC within 2 months after CBT contained higher proportion of CD16<sup>dim</sup>CD56<sup>bright</sup> cells than those after BBMT and contained more CD16<sup>+</sup>CD56<sup>bright</sup> cells than those in normal adult blood and cord blood and more CD16<sup>+</sup>CD56<sup>dim</sup> cells than those in other blood (data not shown). However, absolute numbers of these cells are almost same between after BBMT and CBT (Table 2).

#### *3.2. Subpopulations and absolute number of NK cells 4 to 9 months after stem cell transplantation*

PBMC 4 to 9 months after CBT contained higher proportion of CD16<sup>+</sup>CD56<sup>dim</sup> cells than those in normal adult blood and cord blood and also after BBMT (25.7±12.7% vs 2.2±0.9%, 2.4±2.3% and 4.0±2.5%, p<0.01, mean±standard deviation, respectively). Also, PBMC after CBT at chronic phase contained significantly more absolute number of CD16<sup>low</sup>CD56<sup>bright</sup>, CD16<sup>+</sup>CD56<sup>bright</sup> and CD16<sup>+</sup>CD56<sup>dim</sup> cells than those after BBMT (Table 2).

#### *3.3. Inhibitory and stimulatory NK cell receptors on CD16<sup>+</sup>CD56<sup>dim</sup> cells after 4 to 9 months after stem cell transplantation*

Expression level of inhibitory and stimulatory NK cell receptors on CD16<sup>+</sup>CD56<sup>dim</sup> cells in normal PBMC, CB and also PBMC 4 to 9 months after BBMT vary in each blood cell sources (Tables

3, 4). However, the CD16<sup>+</sup>CD56<sup>dim</sup> cell subset after CBT exhibited decreased expression of NKG2A compared with CB and after BBMT (16.1 ± 15.3% vs 49.4 ± 10.4% and 29.2 ± 18.1%, p<0.01, 0.05, respectively) and increased expression of NKG2C compared with CB and after BBMT (32.5 ± 12.2% vs 8.3 ± 6.1% and 18.5 ± 14.9%, p<0.01, respectively).

#### *3.4. Cytolytic activity of subpopulations of NK cells after CBT*

Purified CD16<sup>+</sup>CD56<sup>dim</sup> cells (Fig. 1) from PBMC obtained from a AML (M4) patient 9 months after CBT (Case 1) exhibited a significantly higher level of cytolytic activity against K562 than the activity of purified CD16<sup>+</sup>CD56<sup>bright</sup> cells and whole PBMC (Table 5). Purified CD16<sup>+</sup>CD56<sup>dim</sup> cells obtained from a PhALL patient 8 months after CBT (Case 2) also exhibited a significantly higher level of cytolytic activity than that of whole PBMC. These two patients were alive more than 34 months after CBT.

#### *3.5. Long-term observation of CD16<sup>+</sup>CD56<sup>dim</sup> NK cells after CBT*

Increased proportion and absolute number of CD16<sup>+</sup>CD56<sup>dim</sup> NK cells (about 10-20% and more than 200/μl) after CBT sustained during 2 to 4 years. Also, all these CBT patients were alive without relapse (Fig. 2).

#### **4. Discussion**

Immune recovery early after transplantation is thought to be thymus-independent [15] and following this early recovery, naïve lymphocytes derived from the differentiation of donor hematopoietic stem cells colonize lymphoid tissues and sustains the late immune recovery. The second recovery involves selection of donor-derived precursor cells in the thymus and peripheral selection sites [16].

Although a much smaller number of lymphocytes is transferred with CBT, recovery of lymphocyte number and function has been reported to be rapid and comparable to that after BMT [17]. On the other hand, Hamza et al. reported that donor-derived lymphocyte recovery was slower in CBT patients at early phase, however, surpassed from day 60 to 365 [18]. Concerning lymphocyte subset reconstitution after CBT, it has been reported that recovery of NK cell, CD19<sup>+</sup> cell, CD8<sup>+</sup> cell and CD4<sup>+</sup> cell was achieved at a median of 2-3, 6, 8-9 and 12 months, respectively [19,20]. This prompt immune recovery may be favored by the reduction of incidence and severity of GVHD after CBT. While innate immunity reconstitute quickly, T-cell lymphopoiesis may be compromised for years due to the decrease of thymopoiesis following transplantation. Early arising and persisting NK-cells would suggest the possibility of an alternative and safer GVL effect after CBT than after conventional transplantation from adult donors, where T-cells are predominant [21-23].

Cord blood cells themselves before transplantation have been

demonstrated to contain a higher percentage of NK cells [5] and CD16<sup>+</sup>CD56<sup>dim</sup> cells with low level of cytolytic activity, and these cells are possible precursors of mature NK cells [7]. The results of our previous preliminary study showed a significant increase in the CD16<sup>+</sup>CD56<sup>dim</sup> cell subset during a period of 4 months to 9 months after CBT [24]. Lu et al. reported that an increase in the CD16<sup>+</sup>CD56<sup>dim</sup> NK cell count in PB was observed in 7 (64%) of 11 CBT patients [25]. However, dynamic changes in subpopulations of NK cells after CBT have not been clarified. In this study, we demonstrated a marked increase of proportion and absolute number of CD16<sup>+</sup>CD56<sup>dim</sup> cells 4 to 9 months after CBT. Also, increased proportion and absolute number of CD16<sup>+</sup>CD56<sup>dim</sup> NK cells (about 10-20% and more than 200/ $\mu$ l) after CBT sustained for more than 2 years. On the other hand, CD16<sup>+</sup>CD56<sup>dim</sup> cell number was 138 $\pm$ 33 (n=6, mean 40 months) after adult stem cell transplantation. Although CD16<sup>+</sup>CD56<sup>dim</sup> cells comprise monocytes, the CD16<sup>+</sup>CD56<sup>dim</sup> cells had a high level of cytolytic activity against HLA class I-negative NK cell target K562 cells. The CD16<sup>+</sup>CD56<sup>dim</sup> cells expressed inhibitory and stimulatory NK cell receptors and exhibited decreased expression of inhibitory NKG2A and increased expression of stimulatory NKG2C compared with cord blood and also PBMC after BBMT. Therefore, stage 4 CD16<sup>-</sup>CD56<sup>bright</sup> NK cells may be differentiated into stage 5 CD16<sup>+</sup>CD56<sup>dim</sup> NK cells with cytotoxicity [4] after CBT at chronic phase. CD16<sup>+</sup>CD56<sup>dim</sup> cells have been reported to exist in CB with low level of cytolytic activity as possible precursors of mature NK cells [7]. However, CD16<sup>+</sup>CD56<sup>dim</sup> cells 8 to 9 months after

CBT had a higher level of cytolytic activity against K562 cells than did CD16<sup>+</sup>CD56<sup>bright</sup> cells in this study. Therefore, CD16<sup>+</sup>CD56<sup>dim</sup> cells after CBT may comprise a mature NK cell subset.

In our 9 patients who underwent CBT, only one patient with MDS overt leukemia in non-CR state had relapsed of leukemia 8 months after CBT during a mean observation period of 38 months (range: 11-61 months). There seemed to be no definitive correlation between GVHD and CD16<sup>+</sup>CD56<sup>dim</sup> cells.

The present results suggest that NK cell subsets and expression of NK cell receptors after SCT may vary depending on stem cell source. Also, the increase in CD16<sup>+</sup>CD56<sup>dim</sup> NK cells with strong cytolytic activity after CBT may have an important role in the GVL effect after CBT. However, we need confirmation for short and long term reconstitution of NK cells after CBT with higher number of patients.

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## Figure Legends

**Fig.1.** Surface expression of CD16 and CD56 on PBMC from a patient after CBT and sorting of CD16<sup>+</sup>CD56<sup>dim</sup> and CD16<sup>+</sup>CD56<sup>bright</sup> cells.

(A) Lymphocytes were gated using FSC and SSC. (B) PBMC were stained with anti-CD56 (PE) and anti-CD16 (FITC). (C) After sorting of CD16<sup>+</sup>CD56<sup>dim</sup> cells. (D) After sorting of CD16<sup>+</sup>CD56<sup>bright</sup> cells. The percentage of cells is shown in each quadrant.

**Fig.2.** Long-term observation of absolute number of CD16<sup>+</sup>CD56<sup>dim</sup> NK cells after CBT.

Increased absolute number of CD16<sup>+</sup>CD56<sup>dim</sup> NK cells (about 200-400/ $\mu$ l) after CBT sustained during 2 to 4 years.

**Table 1. Patient characteristics and outcome after allo-CBT**

Age/Sex	Diagnosis	Conditioning	GVHD prophylaxis	aGVHD	cGVHD	Months/16 <sup>+</sup> 56 <sup>dim</sup> (%)	Months	Outcome
33/F	MDSover nonCR	Ara-C+CY+TBI (12)	FK+sMTX	III	0	4M/23.5 9M/15.2	61	Alive
65/M	MM PR	Flu+Bu+TBI (2)	CsA+sMTX	II	limited	4M/19.6	57	Alive
48/M	AML (M4) CR2	Flu+Bu+TBI (4)	CsA+sMTX	III	0	7M/12.3	55	Alive
28/F	AML (M3) CR3 Second CBT	Flu+Bu+TBI (2) CY+TBI (6)	CsA+sMTX FK	NE I	NE 0	NE 5M/4.0	2 55	Rejection Alive
52/M	AML(M5b) CR1	Ara-C+CY+TBI (12)	FK+sMTX	0	limited	5M/29.4	42	Alive
51/F	MDSover nonCR	Flu+Bu+TBI (8)	CsA+sMTX	0	extensive	4M/39.3 8M/36.4	11	Dead (Relapse)
26/F	PhALL CR1	Ara-C+CY+TBI (12)	FK+sMTX	0	limited	5M/33.9 8M/44.5	23	Alive
29/F	AML(M4) CR2	Ara-C+CY+TBI (12)	FK+sMTX	I	limited	6M/38.5 9M/26.6	21	Alive
40/F	ALL(L2) CR1	Ara-C+CY+TBI (12)	FK+sMTX	II	extensive	4M/10.8	16	Alive

NE: Not evaluated

**Table 2. Absolute number of NK cells after stem cell transplantation**

	(n)	CD16 <sup>dim</sup> CD56 <sup>bright</sup>	CD16 <sup>+</sup> CD56 <sup>bright</sup>	CD16 <sup>+</sup> CD56 <sup>dim</sup>
Within 2 months				
BBMT	(11)	88 ± 135	86 ± 151	14 ± 17
CBT	(8)	101 ± 65	81 ± 44	29 ± 23
4 to 9 months				
BBMT	(19)	55 ± 31	118 ± 62	59 ± 47
CBT	(11)	130 ± 128 <sup>b</sup>	345 ± 404 <sup>b</sup>	771 ± 455 <sup>a</sup>

Values indicate the absolute number of indicated markers-expressing cells (/μl). Significant differences were found in the values 4 to 9 months after CBT compared with the values after BBMT (blood and marrow stem cell transplantation) and CBT (P<0.01<sup>a</sup>, P<0.05<sup>b</sup>).

**Table 3. Inhibitory NK cell receptors on CD16<sup>+</sup>CD56<sup>dim</sup> cells 4 to 9 months after transplantation**

	(n)	CD94	NKG2A	CD158a	CD158b	CD158e1
Normal PB	(14)	64.5±12.8 <sup>b</sup>	23.2±15.4	9.8±8.5	22.4±15.3 <sup>b</sup>	12.3±8.0
Cord blood	(14)	73.0±11.0	49.4±10.4 <sup>a</sup>	16.5±10.0 <sup>b</sup>	23.6±9.2 <sup>a</sup>	7.8±6.1 <sup>a</sup>
BBMT	(19)	74.3±19.1	29.2±18.1 <sup>b</sup>	10.1±8.3	25.0±12.1 <sup>b</sup>	20.2±14.3
CBT	(13)	76.3±9.3	16.1±15.3	9.2±5.8	34.6±8.2	19.5±10.7

Values indicate the percentage of indicated markers-expressing cells. Significant differences were found in the values after CBT compared with normal PBMC and CB and with the values after BBMT (blood and marrow stem cell transplantation) and CBT (P<0.01<sup>a</sup>, <0.05<sup>b</sup>).

**Table 4. Stimulatory NK cell receptors on CD16<sup>+</sup>CD56<sup>dim</sup> cells 4 to 9 months after transplantation**

	(n)	NKG2C	NKG2D	NKp30	NKp44	NKp46
Normal PB	(14)	22.1±19.2	25.1±20.3 <sup>a</sup>	4.6±4.3	0.6±0.6	15.7±10.9
Cord blood	(14)	8.3±6.1 <sup>a</sup>	66.4±17.1 <sup>a</sup>	17.3±13.8 <sup>a</sup>	0.7±0.6	38.0±22.8
BBMT	(19)	18.5±14.9 <sup>a</sup>	69.1±14.3 <sup>a</sup>	4.0±3.4	0.6±0.6	26.1±16.1
CBT	(13)	32.5±12.2	50.8±9.1	1.8±3.3	0.3±0.7	27.2±17.9

Values indicate the percentage of indicated markers-expressing cells. Significant differences were found in the values after CBT compared with normal PB and CB and with the values after BBMT (blood and marrow stem cell transplantation) and CBT ( $P < 0.01^a$ ).

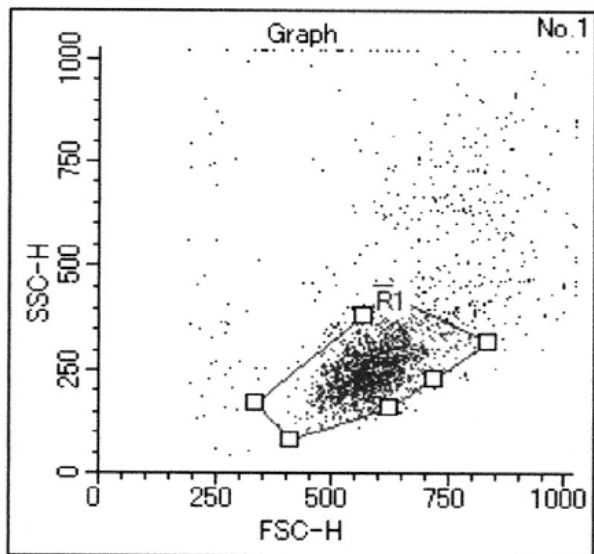
**Table 5. Cytolytic activity of CD16<sup>+</sup>CD56<sup>dim</sup> cells against K562 cell**

	Whole	CD16 <sup>+</sup> CD56 <sup>bright</sup>	CD16 <sup>+</sup> CD56 <sup>dim</sup>
Case 1	20.3 ± 0.4 <sup>a</sup>	53.9 ± 3.7 <sup>a</sup>	74.1 ± 2.0
Case 2	38.0 ± 1.6 <sup>a</sup>	nd	78.0 ± 4.6

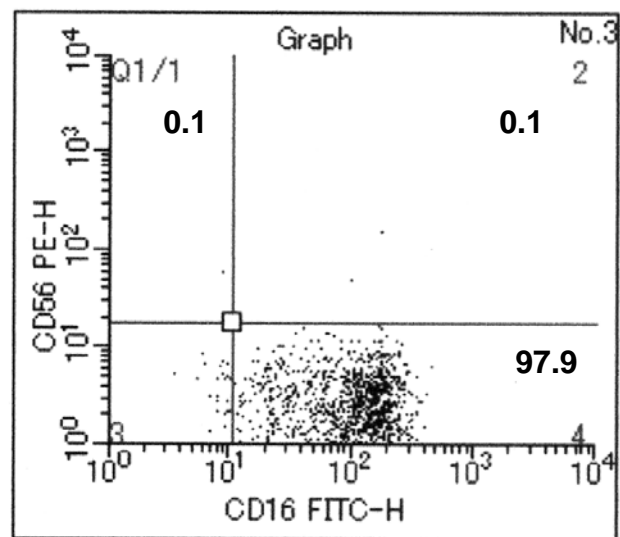
Values indicate the percentage <sup>51</sup>Cr release against K562 cells using 4-hour standard <sup>51</sup>Cr release assay. Significant differences were found in the values of CD16<sup>+</sup>CD56<sup>dim</sup> compared with whole cell and CD16<sup>+</sup>CD56<sup>bright</sup> cell (P<0.01<sup>a</sup>).



**Fig.1 A**

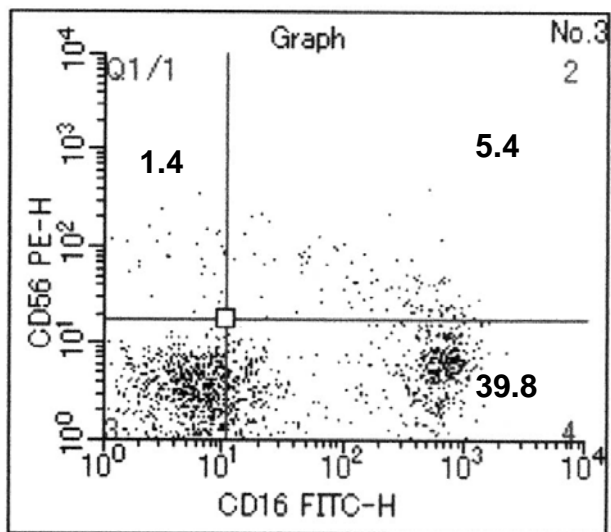


**C**



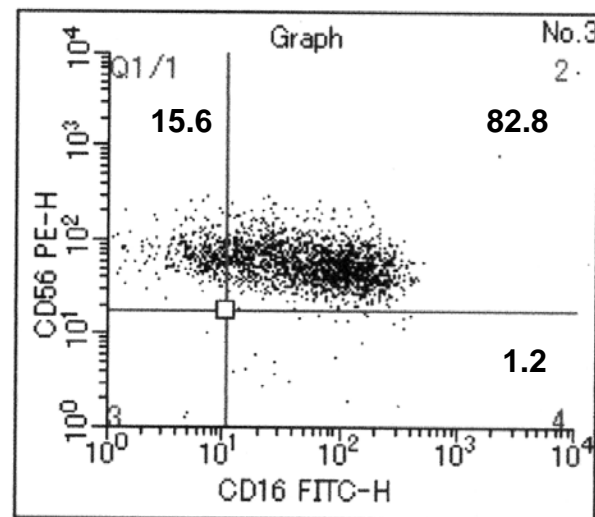
**After sorting (CD16<sup>+</sup>56<sup>low</sup>)**

**B**



**Before sorting**

**D**



**After sorting (CD16<sup>+</sup>56<sup>+</sup>)**

**Fig.2**

