

Purification of Human Hemopoietic Stem Cells for Transplantation in Thailand

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In the past several decades, stem cell transplantation (SCT) has been shown to be the most effective and curative therapeutic modality for many patients suffering from tragic hematologic and immunologic disorders.^{1,2} The success of SCT depends on the presence of a unique, yet rare, cell in the bone marrow, the hemopoietic stem cell (HSC).³ HSC is the most primitive cell in the hemopoietic hierarchy capable of self-renewal of its very extensive proliferation and differentiation potentialities.^{4,5} HSC and their progeny are also known to be the target of several hematologic and immunologic disorders, including non-malignant diseases such as aplastic anemia and severe combined immunodeficiency disease (SCID) or malignant diseases such as acute and chronic leukemia. SCT has led to cure and better quality of life in most patients transplanted. It is considered the most important and remarkable discovery in medicine and led to the Nobel Prize being awarded to

SUMMARY Stem cell transplantation (SCT) has become the therapy of choice for many hematologic and immunologic disorders. At present, only 25% of patients have suitable HLA-identical donors. In an attempt to increase the donor pool for SCT in Thailand and Southeast Asia, we developed a program whereby parents and mismatched siblings can be used as donors. In this preliminary study, after granulocyte-colony-stimulating factor (G-CSF) was given to adult donors, peripheral blood stem cells (PBSC) were collected and CD34⁺ cells purified using a ClinIMACS immunomagnetic device (Miltenyi Biotec, Germany). In seven experiments, purified CD34⁺ cells could be obtained from G-CSF-stimulated PBSC in large numbers ($1.71 \pm 0.19 \times 10^6$), with high purity ($93 \pm 2.4\%$) and excellent recovery (64.28% - 85.62%). Immune reactive T and NK cells were adequately depleted to less than 0.2%. The purification procedure can be completed within 3 hours. In conclusion, a clinical stem cell purification program using this novel device is now established in Thailand and for the first time in Southeast Asia. This should allow further development of advanced SCT therapy including haploidentical and mismatched CD34⁺ SCT for patients lacking HLA-identical donors in this region.

the physician who pioneered the field in 1990.⁶

The identification of hemopoietic stem cell (HSC), however, has been elusive given its lymphocyte-like morphology that is indistinguishable from other mononuclear cells in the bone marrow (BM).⁷ Thus, HSC cannot be identified or isolated using simple morphological methods. Recently,

a new way to select and isolate HSC by the discovery of the monoclonal antibody, CD34 antibody that identifies the CD34 antigen on the surface of stem cells was

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developed.⁸⁻⁹ Subsequently, transplantation using CD34⁺ stem cells was shown to lead to BM engraftment and cure of many lethally irradiated recipients.¹⁰⁻¹¹

The population of Thailand has various hematologic and immunologic disorders that can be cured by SCT.¹²⁻¹³ The current SCT strategy at Siriraj Hospital, which is the first hospital in the country to perform SCT, requires infusion of BM or growth factor-stimulated peripheral blood stem cells (PBSC)¹⁴ harvested from the best HLA-matched sibling donors into patients in order to lessen the risk and severity of graft versus host disease (GVHD).^{15,16} However, only 25% of patients have available HLA-matched sibling donors given the pattern of inheritance of HLA alleles on chromosome 6 from each parent.¹⁷ The majority of patients, therefore, cannot undergo SCT and will be left with no alternatives and death. In an attempt to expand the donor pool in Thailand, we initiated a program for mismatched or haploidentical SCT in Thailand by using purified CD34⁺ stem cells from HLA-disparate donors such as parents and haploidentical or mismatched siblings. This study is preliminary research determined to evaluate whether stem cell purification is feasible for Thai patients using the immunomagnetic device, CliniMACS (Miltenyi Biotec, Bergisch-Gladbach, Germany).¹⁸⁻²⁰ The recovery and purity of CD34⁺ stem cells and the percentages of T cells, B cells and NK cells in the purified products were analyzed.

MATERIALS AND METHODS

Donors

After informed consent,

normal healthy adult donors were given G-CSF (Neupogen[®], Roche, Basel, Switzerland) at 16 µg/kg subcutaneously for 5 days. On days 5 and 6, a three-to-six hour session of large volume (15-litre) leukapheresis was performed using a standard COBE leukapheresis machine.²¹

Stem cell purification with CliniMACS

Methods for magnetic activated cell sorter (MACS) separation were performed according to standard protocols supplied by the company (Miltenyi Biotec, Bergisch-Gladbach, Germany). In principle, BM was labeled with CD34 antibody that was linked to superparamagnetic iron-dextran particles.¹⁸ The labeled BM suspension was passed through the MACS separation column and the magnetic fields applied. The magnetically-labeled CD34⁺ cells were retained in the column under the strong magnetic fields and later eluted from the column after removal of the magnetic fields. Other unwanted cells, including mature cells and reactive immune cells, are CD34-negative and therefore would flow through the column and be discarded.

The detailed procedures are as follows a: Up to 4 x 10¹⁰ mononucleated cells (MNC) of the leukapheresis products were transferred into a 600 ml transfer bag (Baxter, USA). MNC were diluted with a double-volume of CliniMACS PBS/EDTA buffer (Miltenyi Biotec, Bergisch-Gladbach, Germany) with 5% human serum albumin (Alpha Therapeutic Corporation, USA) and centrifuged at ambient temperature, 200 x g for 15 minutes without break. The supernatant was discarded to a volume of

95 ± 10 ml. A volume (7.5 ml) of CliniMACS CD34⁺ reagent was then added to the cells and incubated in the same bag at ambient temperature for 30 minutes on a rotator and then washed twice with the same buffer. After another centrifugation at 200 x g for 15 minutes, cells were resuspended to give a final volume of approximately 100 ml with the same buffer at a concentration of less than 4 x 10⁸ cells/ml. The bag then was connected to the CliniMACS instrument. The machine was run following the manufacturer's protocol. Aliquots from leukapheresis products before and after CD34⁺ cell separation were taken for cell counts and FACS analysis.

Blood cell count and FACS analysis

Blood samples from unprocessed leukapheresis products before CD34⁺ cell separation were diluted 1:5 with PBS/EDTA buffer before cell counts and FACS analysis. Cell counts were done by a cell counter (Sysmex K800). Phenotypic staining of CD34⁺ cells and lymphocyte subpopulations were done on a FACScan flow cytometer (Becton Dickinson, California, USA) equipped with 15 mw argon ion laser and standard filter set-up. All monoclonal antibodies were obtained from Becton Dickinson. Expression of CD34 antigen on CD45⁺ lymphocytes was measured on whole blood using monoclonal anti-CD34 with phycoerythrin (PE) and anti-CD45 conjugated with fluorescein isothiocyanate (FITC). Lymphocyte subpopulations were stained by two-color monoclonal antibodies as following: CD3FITC/CD19PE, CD4FITC/CD8PE and CD57-FITC.²² In brief, 1 x 10⁶ MNC

were incubated with 20 μ l of each monoclonal antibody in the dark at 4°C for 30 minutes. After incubation, the red blood cells were lysed by 2 ml of FACS lysing solution at room temperature for 10 minutes. The cells were washed twice with PBS buffer and fixed with 0.5% paraformaldehyde. A total of 10,000 cells were acquired for each sample and data were analyzed using Cell Quest Software (Becton Dickinson, USA).

RESULTS

Seven experiments were performed from blood of adult donors to evaluate the feasibility of using the CliniMACS system of stem cell purification to be used for hematologic and immunologic patients in Thailand. Donors received G-CSF (Neupogen^R) at high dose (16 μ g/kg for 5 days) had no major complications and only mild headache, bone pain and nausea were reported. This G-CSF regimen is the current standard regimen at Siriraj Hospital which is the biggest hospital in Bangkok, Thailand and has been used for PBSC mobilization for over 50 Thai donors without serious problems.

Peripheral blood stem cell collections were performed on days 5 and 6 of G-CSF injections via central or peripheral venous line without difficulties. Each session lasted from 3-6 hours. In this study, apheresis products contained a volume ranging from 163 ml to 600 ml (263 ± 58 ml) with the total number of MNC ranging from 3.28×10^{10} to 4.30×10^{10} cells ($3.9 \pm 0.15 \times 10^{10}$ cells). Our previous studies showed comparable numbers of MNC per each apheresis.¹⁵

Table 1 summarizes the

purification results of seven experiments. The total number and percentage of recovery of CD34⁺ cells, CD3⁺ T cells, CD19⁺ B cells and CD57⁺ NK cells before and after purification were analyzed. Figs. 1 and 2 show representative diagrams of flow cytometric analysis of hemopoietic cells before and after purification. The mean \pm standard error of mean (SEM) of total CD34⁺ cells before purification was $1.99 \pm 0.45 \times 10^8$ (0.78 to 4.20×10^8 cells) with the mean percentage of CD34⁺ cells in the apheresis product of $0.64 \pm 0.12\%$ (ranging from 0.34 to 1.08%). The percentage of CD3⁺ cells, CD19⁺ cells and NK cells in the apheresis product was $60.68 \pm 3.29\%$, $15.61 \pm 1.54\%$ and $9.34 \pm 1.92\%$, respectively.

After purification, the mean \pm SEM of the total number of CD34⁺ cells in the final suspension was $1.71 \pm 0.18 \times 10^8$ (ranging from 1.16 to 2.70×10^8 cells). The purified fraction had on average $93.33 \pm 2.44\%$ CD34⁺ cells (80.63% to 99.10%) with 0.09% CD3⁺ cells, 3.89% CD19⁺ B cells and 0.11% NK cells (Table 1). Recovery of CD34⁺ cells ranged from 65% to 86% ($75.82 \pm 4.02\%$). On average, 1.5×10^5 CD3⁺ cells, 6.15×10^9 CD19⁺ cells and 2×10^5 NK cells were left in the purified CD34⁺ cell suspension. Thus, T cells and NK cells were mostly depleted by the purification system (Fig. 2). The number of B cells in the final suspension was reduced by 3 logs from the apheresis product. The purified CD34⁺ products obtained from day 6 (experiments 2, 5 and 7) seemed to have an increased number of B cells as compared to day 5 (experiments 1, 3, 4 and 6) although the total number of B cells in the final products was still

minimal. In one experiment (experiment 2), the apheresis product was kept overnight at 4°C by dilution with two volumes of autoplasm before CD34⁺ cell purification on the next day. The recovery and purity of CD34⁺ cells were not different from other experiments.

DISCUSSION

In the last decade, several strategies have been tried to increase the donor pool for patients who desperately need to be transplanted for cure. In North America, a National Bone Marrow Registry has been established to recruit as many normal donors as possible into the registry.²³ Until now, more than 3 million people with various HLA types have been registered and many of them have been used for another type of BMT, the matched unrelated donor (MUD) transplantation²⁴ for which the BM is matched from unrelated (non-family member) individuals. This procedure has led to cure but is complicated by GVHD resulting in serious morbidity and mortality. GVHD reaction is the result of serious disparity between the donor and recipient outside the HLA-A, -B and -DR antigens and thought to be related to the number of donor reactive immune cells contaminated in the BM or PBSC inoculum.²⁵ Given the inherent problems associated with it, MUD transplant is considered the last resource for patients who need BMT but have no identical or matched sibling donors. In addition, setting up the National BM Registry by performing HLA typing of 3 million donors are very costly and is unlikely to be applicable to the countries where financial resources are limited.

Table 1 Results of CliniMACS separation

Exp. No.	Total No. of MNC in initial PBSC product (x 10 ¹⁰)	CD34 ⁺ cells in initial PBSC product (%)	Total No. of CD34 ⁺ cells in final purified fraction (x 10 ⁸)	Recovery of CD34 ⁺ cells in final purified fraction (%)	Purity of CD34 ⁺ cells in final purified fraction (%)	CD3 ⁺ T cells in initial PBSC product (%)	CD3 ⁺ T cells in final fraction (%)	CD19 ⁺ B cells in initial PBSC product (%)	CD19 ⁺ B cells in final fraction (%)	CD57 ⁺ NK cells in initial PBSC product (%)	CD57 ⁺ NK cells in final fraction (%)
1	3.28	ND*	1.66	ND*	97.67	78.58	0.09	11.63	2.40	8.11	0.07
2	3.80	ND*	1.16	ND	80.63	64.18	0.18	11.74	11.20	7.43	0.11
3	4.00	0.54	1.80	83.33	96.33	58.84	0.07	13.78	1.86	3.58	0.03
4	4.20	0.54	1.73	76.21	96.46	59.84	0.06	13.78	1.57	3.60	0.04
5	4.30	0.34	1.25	85.62	89.33	54.68	0.04	16.28	6.01	16.21	0.20
6	3.89	1.08	2.70	64.28	99.10	53.59	0.07	22.11	0.58	11.43	0.19
7	3.38	0.72	1.69	69.64	93.85	55.06	0.14	20.01	3.67	15.06	0.15
Mean	3.83	0.64	1.7	75.82	93	60.68	0.09	15.61	3.89	9.34	0.11
± SEM	± 0.15	± 0.12	± 0.18	± 4.02	± 2.43	± 3.29	± 0.01	± 1.53	± 1.38	± 1.92	± 0.02

*Not determined

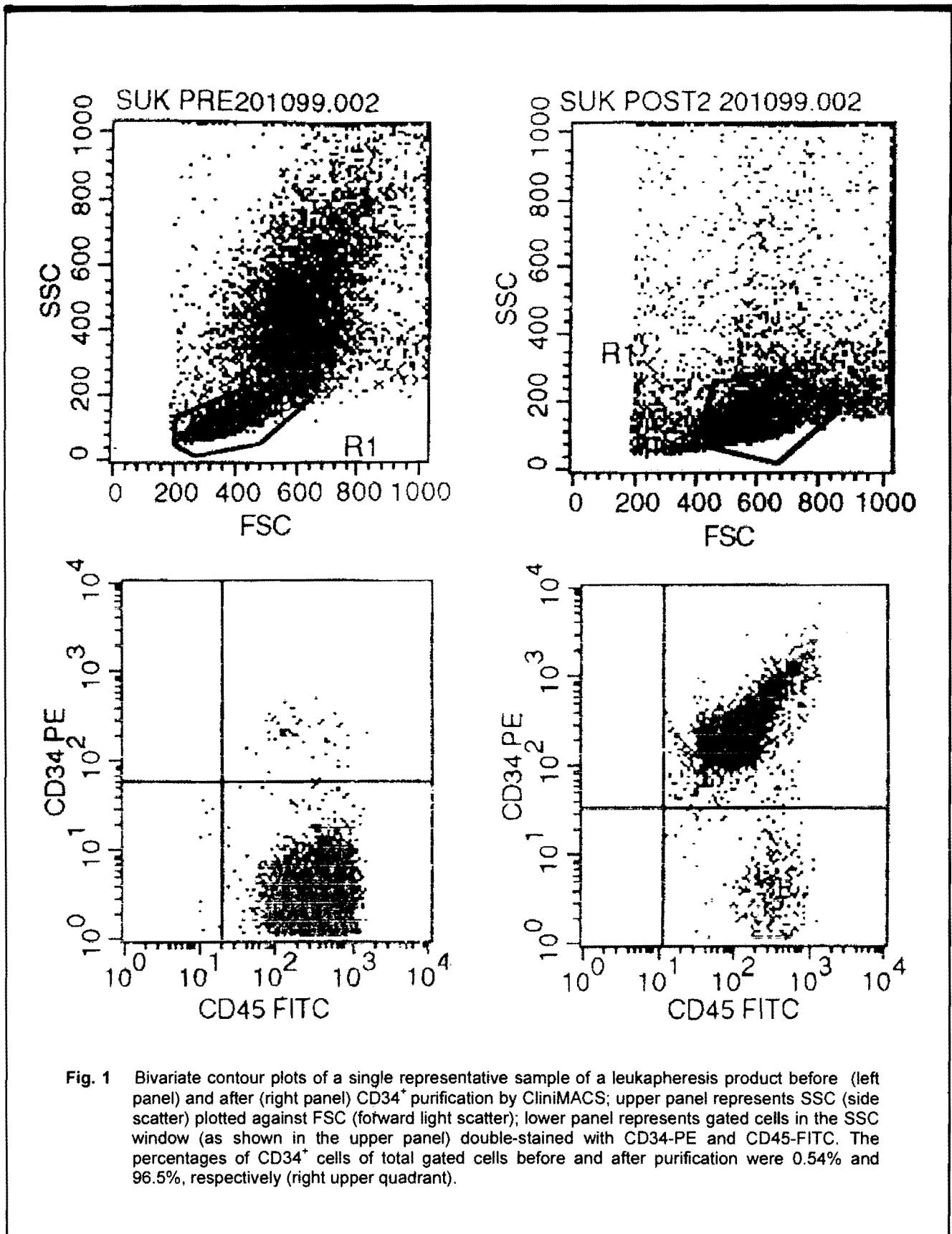


Fig. 1 Bivariate contour plots of a single representative sample of a leukapheresis product before (left panel) and after (right panel) CD34⁺ purification by CliniMACS; upper panel represents SSC (side scatter) plotted against FSC (forward light scatter); lower panel represents gated cells in the SSC window (as shown in the upper panel) double-stained with CD34-PE and CD45-FITC. The percentages of CD34⁺ cells of total gated cells before and after purification were 0.54% and 96.5%, respectively (right upper quadrant).

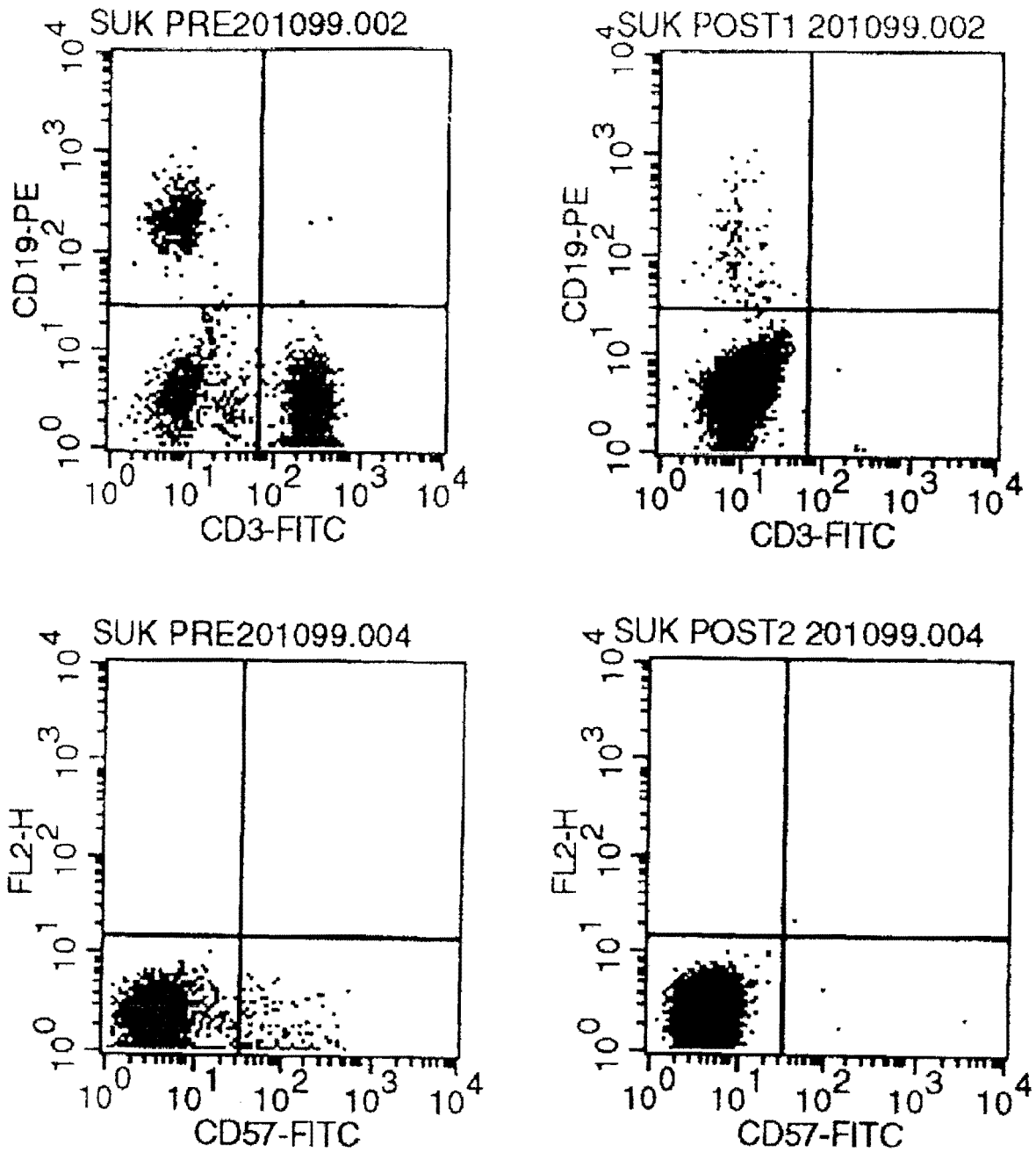


Fig. 2 Bivariate contour plots of a single representative sample of a leukapheresis product before (left panel) and after (right panel) CliniMACS separation; upper panel represents cells double-stained with CD19-PE and CD3-FITC; lower panel represents cells stained with CD57-FITC. The percentages of CD19⁺ cells of total gated cells before and after CD34⁺ purification were 13.8% and 1.6%, respectively (left upper quadrant). The percentages of CD3⁺ cells of total gated cells before and after CD34⁺ purification were 60% and 0.06%, respectively (right lower quadrant).

Thailand has patients with various hematologic and immunologic disorders that can be cured by SCT. In the past, patients who lacked HLA-identical siblings had been denied SCT as unrelated BM registry does not exist in the country. This study, as part of a larger project determined to develop a new way for SCT for those patients lacking suitable donors, was undertaken to test if the CliniMACS immunomagnetic device is feasible for stem cell purification and transplantation in Thailand. We found that CliniMACS is easy to operate and able to yield very good recovery and purity of CD34⁺ stem cells (> 90%) with adequate depletion of immune reactive T and NK cells (< 0.2%). The procedure can be performed in one day and the apheresis product can be kept overnight before CD34⁺ cell purification without further loss of CD34⁺ cells. A large number of CD34⁺ cells, on average 1.7×10^8 CD34⁺ cells per session, can be obtained, and particularly for a recipient weighing less than 15 kg, this number is adequate for the so-called megadose SCT.²⁶⁻²⁷ However, it may require more than two sessions for adults or patients with higher body weights in order to perform adequate megadose SCT for them.

The purified CD34⁺ cells obtained through this method have been shown to have *in vitro* and *in vivo* qualities of stem cells and can result in normal engraftment in recipients.²⁷ We also tested the colony formation *in vitro* and found purified cells to be able to give normal BFU-E and CFU-GM colonies and good cloning efficiency (data not shown). The ability to purify and transplant CD34⁺ cells, in principle, should lead to no GVHD reaction, given that immune

reactive cells are CD34-negative and therefore should not be co-purified in the CD34⁺ stem cell suspension. Excellent depletion of immune cells in our studies was demonstrated. The total absolute numbers of T cells and NK cells were only 150,000 cells and 200,000 cells, respectively, in the final suspension. This will be very useful for transplantation in haploidentical and mismatched cases since these two procedures are related with higher GVHD than HLA-matched transplants. Although T-cell depletion may be associated with higher risk of rejection and relapse, the modification of grafts such as higher or megadose of stem cells and better conditioning of hosts may help to lessen that problem.²⁸

Transplantation using PBSC CD34⁺ cells was previously shown to lead to BM engraftment and cure of many lethally-irradiated recipients.¹⁴ PBSC, as another source of stem cells, is known to have similar characteristics to BM stem cells but gives more rapid engraftment.²⁹⁻³⁰ The ability to use mobilized PBSC CD34⁺ cells for transplantation is thus convenient as it does not require marrow harvest from the donor pelvic bone.³¹ In this study, our donors tolerated the procedure well. No operation was needed as no BM harvests were necessary. Only PBSC collections via the central or peripheral lines were performed in the transfusion unit. PBSC mobilization and collection are now being widely used in many countries,³² particularly Thailand where donors are reluctant to donate stem cells from bone marrow because of fear of the operation. The studies using immunomagnetically purified PBSC CD34⁺ cells for patients with leukemia and

immunodeficiency disorders are currently in phase I/II clinical trials in Europe.³³

In conclusion, clinical CD34⁺ cell purification program is feasible and now established for the first time in Thailand. Further clinical trials are ongoing to evaluate the applicability of this approach to Thai patients with hematologic and immunologic disorders. Our results will definitely set the stage for new therapy of patients with tragic disorders in Thailand and Southeast Asia, as from now, parents and mismatched siblings can be studied in phase I/II clinical trials as donors for patients who are desperately in need of BMT but lack HLA-identical sibling donors in this region. Purified autologous CD34⁺ stem cells can also be used to ensure regeneration of normal blood cells for patients with solid tumors such as breast cancer or ovarian cancer while undergoing lethal dose chemoradiotherapy.³⁴ In addition, purified stem cells can be used for further biological and molecular studies, e.g. as target for gene transfer and gene therapy.³⁵ *Ex vivo* generation of mature hemopoietic cells from CD34⁺ stem cells for transfusion donation may also be possible.³⁶ Future studies using CD34⁺ cells from unrelated donors should also be carried out.

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