

# Influence of cell isolation method on the optimization of CD4+ T cell expansion using anti-CD3/CD28 coated beads

Nattawat Onlamoon,<sup>1,2</sup> Michittra Boonchan,<sup>1,3</sup> Petai Unpol,<sup>1,3</sup> Narakorn Khunweeraphong,<sup>1,2</sup> Kasama Sukapirom,<sup>1</sup> Palanee Ammaranond<sup>4</sup> and Kovit Pattanapanyasat<sup>1,2</sup>

## Summary

**Backgrounds:** Activation of CD4+ T lymphocytes with anti-CD3/CD28 coated magnetic beads promotes intrinsic resistance to HIV as well as cell expansion. The propose of this study is to define the optimal cell isolation protocol for expansion of CD4+ T lymphocytes by using anti-CD3/CD28 coated bead stimulation with an ultimate goal of using these cells for adoptive immunotherapy.

**Methods:** CD4+ T cells were isolated from healthy donor blood samples using three different methods including immunorosette formation, negative selection and CD8 depletion using immunomagnetic beads. These cells were activated with anti-CD3/CD28 coated beads at a bead to cell ratio of 1:1 and cell expansion was carried for 3 weeks. Cell numbers, cell viability and phenotypic characterization were determined by trypan blue exclusion and flow cytometry.

**Results:** Purified CD4+ T lymphocytes which were isolated via immunorosette formation can be expanded up to 1000-fold within 3 weeks with high viability (90%) and high purity of CD4+ T lymphocytes (>95%). However, cell expansion from purified CD4+ T lymphocytes which were isolated by negative selection and CD8-depletion provided approximately 300-fold expansion.

**Conclusions:** The results demonstrate that purified CD4+ T lymphocytes from immunorosette formation provided the highest CD4+ T lymphocyte expansion when stimulated with anti-CD3/CD28 coated beads. This method can be used to obtain a large number of expanded CD4+ T cells for adoptive immunotherapy. (*Asian Pac J Allergy Immunol 2013;31:99-105*)

**Key words:** CD4+ T lymphocyte, in vitro expansion, anti-CD3/CD28 coated beads, adoptive transfer, immunotherapy

## Introduction

Human immunodeficiency virus (HIV) infection causes a progressive decrease in CD4+ T lymphocytes which increases susceptibility to opportunistic infection.<sup>1</sup> However, long-term non-progressor patients (LTNPs) maintain a stable CD4+ T lymphocyte count within the normal range and undetectable HIV-1 viral load for many years with asymptomatic and slowly progressive disease in the absence of antiretroviral therapy (ART).<sup>2</sup> Although ART results in an increased number of CD4+ T lymphocyte, CD4+ T lymphocyte recovery is still limited in some patients.<sup>3</sup> Many alternative strategies to induce immune reconstitution have been introduced, such as transfusion of autologous CD8+ T lymphocyte into HIV-infected patients, but the response is limited due to a rapid exhaustion of these cells, since CD4+ T lymphocytes are required to maintain CD8+ T lymphocyte functions via cytokine production and CD40L expression on the surface of CD4+ T lymphocytes.<sup>2,4</sup> Adoptive T lymphocyte immunotherapy has been proposed as a treatment of many diseases,

From 1. Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

2. Center for Emerging and Neglected Infectious Diseases, Mahidol University, Nakhon Pathom, Thailand.

3. Graduate program in Immunology, Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.

4. Innovation Center for Research and Development of Medical Diagnostic Technology Project and Department of Transfusion Medicine, Faculty of Allied Health Sciences, Chulalongkorn University, Bangkok, Thailand.

Corresponding author: Nattawat Onlamoon and Kovit Pattanapanyasat

E-mail: nattawat.onl@mahidol.ac.th and kovit.pat@mahidol.ac.th

Submitted date: 9/4/2012

Accepted date: 24/9/2012



including HIV infection, due to its ability to reconstitute the immune response of patients leading to long-lasting immunity *in vivo*.<sup>5,6</sup>

Since adoptive transfer of T lymphocyte requires a large number of cells, there are several cell stimulating agents used in cell expansion protocols including phytohaemagglutinin (PHA)<sup>7</sup>, autologous antigen presenting cells with specific peptide<sup>8</sup>, and anti-CD3/CD28 monoclonal antibodies immobilized on magnetic beads.<sup>9</sup> With regard to its application in HIV infection, activation of CD4+ T lymphocytes with anti-CD3/CD28 coated magnetic beads promotes intrinsic resistance to HIV infection which is specific for macrophage-tropic isolates of HIV-1.<sup>9</sup> Studies in both human and nonhuman primates have shown that activation of CD4+ T lymphocytes with anti-CD3/CD28 coated beads induced cell expansion, increased expression of RANTES, MIP-1 $\alpha$ , as well as MIP-1 $\beta$  and decreased expression of CCR5.<sup>9-12</sup> Moreover, expanded cells predominantly exhibited cytokine secretion associated with T helper cell type 1 function, increasing telomerase activity and increasing the diversity of TCR V $\beta$  repertoires.<sup>10,11</sup>

Adoptive transfer of anti-CD3/28 expanded CD4+ T cells is successful in both SIV-infected nonhuman primates and HIV-infected patients and is safe and effective in improving their immune system.<sup>13-14</sup> However, previous methods used for expansion of CD4+ T lymphocyte exhibited a low expansion rate and also required a large number of starting cells, which is a limitation for clinical application, especially in HIV infected patients, since most patients have low CD4+ T lymphocyte counts. Since the process of cell expansion is labor intensive and time consuming, development of an efficient method is required in order to get an appropriate number of cells for adoptive immunotherapy. Therefore, the propose of this study was to define the optimal cell isolation protocol for expansion of CD4+ T lymphocyte by using anti-CD3/CD28 coated bead stimulation with the ultimate goal of using these cells for adoptive immunotherapy.

## Methods

### Subjects

Three normal healthy volunteers were recruited for this study. All subjects signed an informed consent form that was approved by the Institutional Review Board of the Faculty of Medicine Siriraj Hospital, Mahidol University. Fifteen ml of blood was collected in sodium heparin-containing vacutainer tubes. An aliquot of a blood sample was separated

on Ficoll-Hypaque gradients to obtain peripheral blood mononuclear cells (PBMCs).

### CD4+ T lymphocyte enrichment

Isolation of CD4+ T lymphocyte enrichment was performed by 3 different cell isolation methods. In the first method, enriched CD4+ T lymphocytes were directly isolated from whole blood with immunorosette formation by mixing 5 ml of whole blood sample with RosetteSep® Human CD4+T cell enrichment cocktail (STEMCELL Technologies, Vancouver, BC, Canada) following the manufacturer's instructions. Purified CD4+ T lymphocytes were isolated by a standard Ficoll-Hypaque gradient centrifugation according to the manufacturer's protocol. In the second method, an aliquot of PBMC was mixed with Dynabeads® untouched™ Human CD4 T cells (Invitrogen Dnal, Oslo, Norway) according to manufacturer's instructions. Enriched CD4+ T lymphocytes were isolated by negative selection using a magnet to remove non-CD4+ T cells. In the third method, another aliquot of PBMC was mixed with Dynabeads® CD8 (Invitrogen Dnal, Oslo, Norway) according to manufacturer's instructions. Enriched CD4+ T lymphocytes were isolated by CD8 depletion using a magnet to remove CD8+ cells.

### Antibodies

The following monoclonal antibodies (mAb) and their conjugated fluorochromes were commercially obtained from BD Bioscience (BDB, San Jose, CA): anti-CD3 conjugated with fluorescein isothiocyanate (FITC), anti-CD4 conjugated with phycoerythrin (PE), anti-CD19 PE, anti-CD45 conjugated with peridinin chlorophyll protein (PerCP), anti-CD8 conjugated with allophycocyanin (APC), anti-CD16 APC and anti-CD56 APC.

### Immunofluorescence staining and analysis

Whole blood samples were stained with fluorochrome conjugated mAb for 15 min followed by the addition of FACS lysing solution (BDB) in order to lyse the red blood cells and fix the sample. After cell washing in wash buffer (Phosphate buffered saline (PBS) containing 2% fetal bovine serum), stained cells were maintained in PBS with 1% paraformaldehyde. Samples were acquired using a FACSCalibur flow cytometer (BDB) and analyzed by FlowJo software (Tree Star, San Carlos, CA). Data are presented as mean  $\pm$  S.D. of samples for the frequency of each cell subset.

### Cell Stimulation

One million cells isolated using each isolation method were stimulated with anti- CD3/CD28 mAb immobilized on magnetic beads (Invitrogen Dynal, Oslo, Norway) at a 1:1 bead to cell ratio. The cells were expanded in complete media (RPMI1640 with 10% fetal calf serum, 50 µg/ml Penicillin-Streptomycin and 2 mM L-glutamine) and incubated at 37°C in a humidified 5%CO<sub>2</sub>. The expanded cell culture was re-stimulated on day 7 and maintained by the addition of fresh media on day 4, 7, 11, 14, 17 and 21. Cell numbers and viability were determined at each time point by trypan blue exclusion. Data are presented as mean ± S.D. of samples for fold of expansion and %viability. The expanded cells were transferred to appropriate container as needed to maintain a cell concentration of 0.5x10<sup>6</sup> cells/ml.

### Results

#### Comparisons of cell recovery by different cell isolation methods

To determine whether a cell isolation method has an effect on the number of cell recovered and the phenotype of the purified cell population, CD4<sup>+</sup> T lymphocytes were isolated from 5 ml of whole blood which was collected from healthy volunteers. Three different cell isolation methods were used, namely immunorosette formation (Method 1), negative selection using immunomagnetic beads (Method 2) and CD8 depletion using immunomagnetic beads (Method 3).

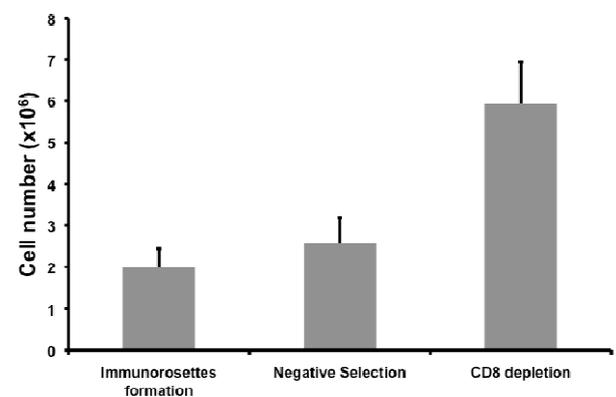
In method 1, Purified CD4<sup>+</sup> T lymphocytes were directly isolated from whole blood via bispecific antibody tetrameric complexes that crosslink between non-CD4<sup>+</sup> T lymphocytes and red blood cells. These crosslink complexes were removed by density gradient centrifugation which allows collection of purified CD4<sup>+</sup> T cells from the interphase between cell separating medium and plasma. In contrast, methods 2 and 3 involved isolation of CD4<sup>+</sup> T cells from PBMC by using cell-lineage-specific antibody conjugated with magnetic beads to deplete non-CD4<sup>+</sup>T cells from PBMC. While method 2 used an antibody specific to every cell type in PBMC except CD4<sup>+</sup> T cells, method 3 used antibody that was specific to CD8<sup>+</sup> cells.

The recovery of isolated cells using different methods is compared in Figure 1. The results show that the highest number of isolated cells were provided by method 3, whereas method 1 provided the lowest number of isolated cells. The average

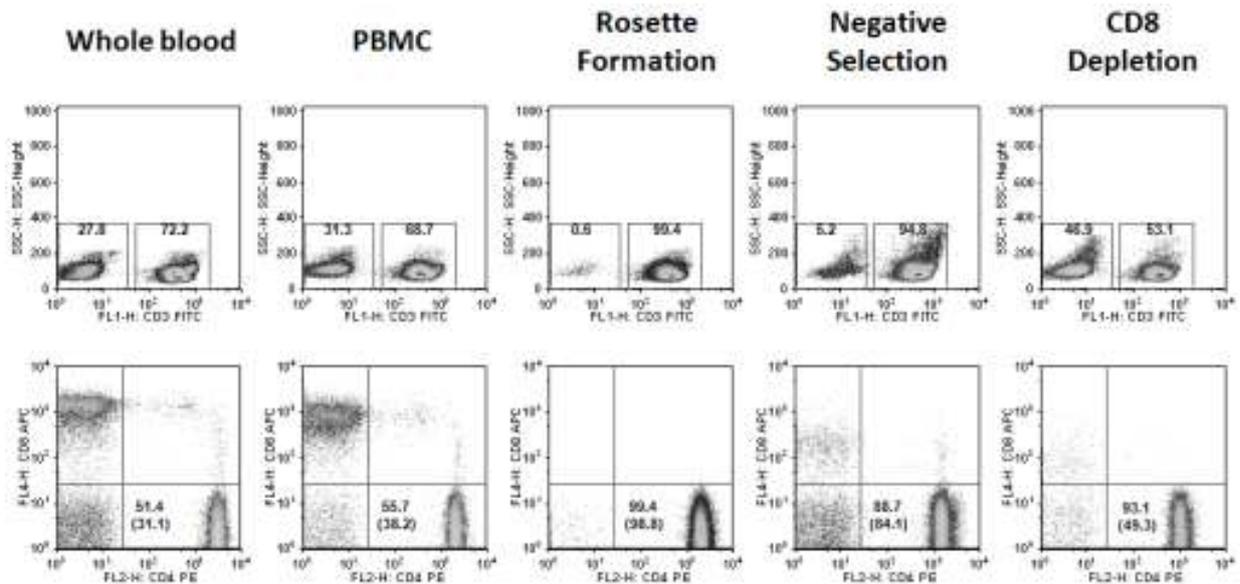
number of isolated cells from method 1 was 2.0 ± 0.5 million cells. Since cell isolation by method 2 and 3 was performed on PBMC, we found that approximately 9x10<sup>6</sup> cells of PBMC were isolated from 5 ml of whole blood. The average number of isolated cells using method 2 was 2.6 ± 0.6 million cells, whereas 5.9 ± 1.0 million cells were collected using method 3. No difference in the viability of freshly isolated cells was observed between these three methods (data not shown).

#### Phenotypic characterization of isolated CD4<sup>+</sup> T lymphocytes

To determine phenotypic characteristic of isolated cells, the expression of CD45, CD3, CD4 and CD8 were analyzed by the flow cytometry technique. The flow cytometric data analysis comparing cells isolated using the 3 different methods is shown in Figure 2. Interestingly, while the majority of isolated cells from all methods were CD4<sup>+</sup> T lymphocytes, method 1 showed the lowest frequency of non-CD4<sup>+</sup> T lymphocytes. Method 2 showed a higher frequency of CD3+CD4<sup>-</sup> cells when compare to method 1. Furthermore, since method 3 depleted only CD8<sup>+</sup> cells from PBMC sample, it provided the highest CD3<sup>-</sup> population.



**Figure 1.** Comparison of cell recovery using three different isolation methods. Trypan blue exclusion was used to determine a total number of viable cells for enriched CD4<sup>+</sup> T lymphocytes using each isolation protocols. Data are presented as a mean value of three healthy individuals with standard deviations.



**Figure 2.** Phenotypic characterization of cells isolated using three different methods. Flow cytometric two-color dot plots for CD3 versus side scatter and CD4 versus CD8 are shown. The top panel shows identifications and percentages of CD3+ and CD3- lymphocytes among the total lymphocyte population. The lower panel shows identifications and percentages of T lymphocyte subsets among CD3+ T lymphocytes. The percentage of CD4+ T lymphocyte among a total lymphocyte population is indicated in parenthesis.

Based on this analysis, the percentages of T cell subsets among the lymphocyte populations isolated using different methods are shown in Table 1. The result shows that method 1 gave the highest percentage of CD4+ T cells ( $98.2 \pm 1.0$ ) when compared with methods 2 and 3 ( $86.6 \pm 5.6$  and  $52.5 \pm 9.9$  respectively). Interestingly, while method 3 gave the highest number of isolated cells, approximately half of these cells are non-T cells. The percentage of total T lymphocytes from this method was lowest ( $60.9 \pm 11.3$ ) when compared with method 1 and 2 ( $99.4 \pm 0.3$  and  $96.1 \pm 3.0$ , respectively). In order to determine the percentage yield for each method, the actual number of isolated CD4+ T lymphocytes was calculated relative to the number of CD4+ T lymphocytes in whole blood. The results show that the highest percentage yield was obtained using method 3 ( $34.8 \pm 10.2$ ) when compared with method 1 and 2 ( $22.3 \pm 6.8$  and  $25.2 \pm 6.5$ , respectively).

**Expansion rate and phenotypic characterization of isolated CD4+ T lymphocytes**

To determine the expansion rate of CD4+ T lymphocytes from isolated cells by three isolation methods, one million cells isolated using each method was stimulated by anti-CD3/CD28- coated

beads at a 1:1 bead to cell ratio. Cell culture was performed in the absence of exogenous cytokine or feeder cells. As shown in Figure 3, the results indicate no difference in the expansion rate of cells isolated using the three different methods during the early phase of cell expansion. After bead stimulation for 14 days, expansion of isolated cells using method 1 showed a marked increase in the expansion rate when compared with the cells using methods 2 and 3. Moreover, within 3 weeks of cell expansion, the expansion rate of cells isolated using method 1 was  $1055.3 \pm 158.7$  -fold, whereas it was  $390.3 \pm 297.1$  and  $445.0 \pm 262.5$  -fold in cells isolated using method 2 and 3, respectively.

Determination of cell viability was performed on days 0, 4, 7, 11, 14, 17 and 21. As shown in Table 2, cell viability was maintained at approximately 90% throughout the expansion period in all three populations. At the end of cell expansion on day 21, the percentage of cell viability in those isolated using method 1 was  $90.4 \pm 3.8$ , compared to  $87.7 \pm 6.2$  and  $91.3 \pm 4.4$  for those isolated using methods 2 and 3, respectively. Furthermore, determination of cell size and complexity by flow cytometry showed that a population of blast cells was maintained until day 21 of cell expansion (data not shown).

**Table 1.** The percentages of T lymphocyte subsets among lymphocyte populations obtained from whole blood, PBMC and isolated cells using three different methods.

Cell population	Whole blood	PBMC	Immunorosette formation	Negative selection	CD8 depletion
CD3+	67.1±4.9	61.0±8.1	99.4±0.3	96.1±3.0	60.9±11.3
CD3+CD4+	40.1±4.0	42.3±6.5	98.2±1.0	86.6±5.6	52.5±9.9
CD3+CD8+	19.4±2.6	13.3±7.8	0	1.1±1.2	1.4±1.6
CD3+CD4+CD8+	0.4±0.3	1.0±0.8	0	0.1±0.1	0.1±0.1

Results are shown as mean percentages ± standard deviation.

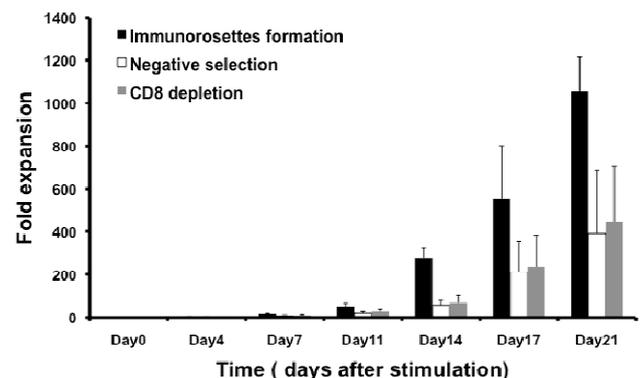
The phenotypic characterizations of expanded cells were determined on day 14 and day 21 of cell expansion. As shown in Table 3, the results indicate no difference in the percentages of CD3+ T cells among the lymphocyte populations on day 14 and day 21 for all isolation methods. Although more than 90% of expanded cells from all isolation methods were CD4+ T lymphocytes, cells isolated using method 1 had the highest frequencies of CD4+ T lymphocytes after three weeks of cell expansion (99.3±0.2), when compared with those isolated using methods 2 and 3 (96.0±3.7 and 92.6±14.9, respectively). Phenotypic characterization of expanded cells showed a purified CD4+ T lymphocyte phenotype without any significant expression of CD8, CD19, CD16 and CD56 (data not shown).

## Discussion

Although several studies showed many parameters have an effect on the proliferation of T lymphocytes, the influence of different cell isolation methods has not been previously reported. The activation of cell during the isolation procedure might lead to a reduction of T lymphocyte functions, including proliferation, cytokine production and alteration of the phenotypic characterization of the cells.<sup>15-17</sup> In this study, the effect of the cell isolation method on the expansion rate of cells and their phenotypic characterization were determined. The results demonstrate that the CD8 depletion method gave the highest cell recovery compared with CD4 enrichment by negative selection and immunorosette formation. However, the lowest percentages of CD4+ T lymphocyte were obtained by CD8 depletion since this method removes only CD8+ T cells.

A previous study showed that unfractionated cells in culture are more useful than purified populations of T cells for cell proliferation due to autocrine and paracrine action, such as IL-1 production from monocytes and IL-12 production from macrophages, as well as B-lymphoblast cells, that are presented to

co-stimulate T lymphocytes.<sup>18</sup> However, we showed that the highest expansion rate was obtained from expanded cell cultures that use enriched CD4+ T lymphocytes from immunorosette formation, which provides highly purified CD4+T lymphocytes. This result might occur because this method involves the least manipulation of cells during isolation and the majority of isolated cells that are used in the expansion procedure are CD4+ T lymphocytes, when compared with other methods. However, although CD4 enrichment by negative selection provided a similar purity of CD4+ T lymphocyte, a cell expansion culture of these cells provided a lower-fold expansion when compared to immunorosette formation. CD4 enrichment by negative selection used PBMC for CD4+ T cell isolation, whereas immunorosette formation isolated cells directly from whole blood. It is possible that a longer period of cell isolation has an effect on isolated cells. Other



**Figure 3.** Expansion rate of anti-CD3/28-expanded CD4+ T cells during 3 weeks of culture. Enriched CD4+ T lymphocytes obtained using different isolation method were stimulated with anti-CD3/28 coated beads. The expanded cells were counted on day 4, 7, 11, 14, 17 and 21. The fold expansion was calculated relative to the starting cell number and represents a mean value for three healthy individuals.

**Table 2.** Cell viability of anti-CD3/28 expanded cells collected using three isolation methods on day 0, 4, 7, 11, 14, 17 and 21.

Method	Cell viability						
	Day 0	Day 4	Day 7	Day 11	Day 14	Day 17	Day 21
Immunorosette formation	100.0 ± 0.0	98.4 ± 1.5	93.2 ± 2.3	92.1 ± 4.9	94.9 ± 3.2	92.0 ± 6.3	90.4 ± 3.8
Negative selection	100.0 ± 0.0	97.2 ± 3.3	92.7 ± 2.5	89.6 ± 3.8	91.7 ± 2.6	94.4 ± 5.0	87.7 ± 6.2
CD8 depletion	100.0 ± 0.0	94.8 ± 4.1	95.9 ± 2.4	86.0 ± 7.7	89.4 ± 2.9	93.5 ± 2.8	91.3 ± 4.4

Results are shown as mean percentages ± standard deviation.

factors that have an effect on the cell growth of anti-CD3/28 activated T lymphocytes, such as IL-2 and serum supplementation, have been reported.<sup>12,18</sup> Although cell expansion in this study was carried for three weeks, our results also confirmed that autocrine cytokines were sufficient to support proliferation of anti-CD3/28 expanded CD4+ T lymphocyte without any requirement for exogenous feeder cells or IL-2 supplementation.<sup>19</sup>

According to our previous studies, CD4+ T lymphocytes isolated from SIV-infected monkeys and expanded with anti-CD3/CD28 coated beads provide 100-fold expansion within 2 weeks.<sup>12</sup> Levine BL et al also reported that stimulation of purified CD4+ T lymphocytes from HIV-infected donors expanded approximately 37-fold within 2 weeks.<sup>20</sup> Furthermore, cell expansion using PBMC provided 10,000-fold expansion but the proliferation of CD8+ T lymphocytes was higher than CD4+ T lymphocytes.<sup>18</sup> In this study, purified CD4+ T lymphocytes isolated using immunorosette formation exhibited more than 1000-fold expansion in three weeks with high cell viability and high purity of CD4+ T lymphocytes. This protocol might be useful for expansion of CD4+ T lymphocyte from HIV-infected patients since the cellular dysfunctions that are associated with several markers including CD57, CTLA-4 or PD-1, which lead to impairment of proliferation, cytokine production or cell exhaustion, occur during the chronic phase of HIV infection.<sup>21</sup>

Moreover, since the number of CD4+ T lymphocyte that can be obtained in patients are low when compared to healthy donors<sup>22</sup>, this cell isolation and expansion procedure reduces the amount of isolated cells that are required to generate large scale expansion of CD4+ T lymphocyte for HIV infected patients. The ultimate goal of CD4+ T lymphocyte expansion is to explore a potential novel treatment by autologous transfer of expanded CD4+ T lymphocytes to HIV infected patients. However, phenotypic characteristic and the function of CD4+ T lymphocytes are different in each stage of HIV disease progression, therefore CD4+ T lymphocyte expansion using anti-CD3/28 coated beads at different stages of the disease progression need further investigations before clinical testing of this protocol.

CD4+ T lymphocytes contain many subtypes of helper T cells that play a role in protection against viral infection, such as type 1 helper T cells that respond to intracellular infection and support cytotoxic T cell function, type-2 helper T cells that support the generation of an antibody response against pathogens, and regulatory T cells that play a role in suppression of immune responses.<sup>2,23</sup> Although more than 99% of expanded cells reported in this study are CD4+ T lymphocytes, a further study is needed to determine the phenotypic characterizations and functions of these cells.

**Table 3.** The percentages of T lymphocyte subsets among lymphocyte populations obtained from anti-CD3/28 expanded CD4+ T cells using three different cell isolation methods.

Cell population	Day14			Day21		
	Immunorosette formation	Negative selection	CD8 depletion	Immunorosette formation	Negative selection	CD8 depletion
CD3+	99.9 ± 0.1	99.9 ± 0.1	99.7 ± 0.1	99.8 ± 0.2	99.8 ± 0.1	99.9 ± 0.1
CD3+CD4+	99.8 ± 0.1	96.6 ± 2.0	93.5 ± 12.1	99.3 ± 0.3	96.0 ± 3.7	92.6 ± 14.9
CD3+CD8+	0	0.4 ± 0.5	0.2 ± 0.4	0.0 ± 0.1	2.0 ± 2.1	0.1 ± 0.1
CD3+CD4+CD8+	0	1.5 ± 1.9	0.1 ± 0.1	0	0.7 ± 0.6	0.5 ± 0.9

Results are shown as mean percentages ± standard deviation.

In conclusion, CD4+ T lymphocyte enrichment via immunorosette formation provides an optimal isolation method for expansion of CD4+ T lymphocytes. High number of anti-CD3/28 expanded CD4+ T lymphocytes can be generated within three weeks. Taken together, a combination of this cell isolation method with anti-CD3/28 coated bead expansion provided high viability and high purity of CD4+ T lymphocytes.

### Acknowledgment

This project is supported by the Office of the Higher Education Commission and Mahidol University under the National Research Universities Initiative, Siriraj Graduate Thesis Scholarship (to MB) as well as the Senior Research Scholar grant from the Thailand Research Fund (to KP). KP and NO are supported by Chalermphrakiat Grant from Faculty of Medicine Siriraj Hospital. PA is supported by Chulalongkorn University Centenary Academic Development Project.

### References

1. Stevenson M. HIV-1 pathogenesis. *Nat Med.* 2003;9:853-60.
2. Altfeld M, Rosenberg ES. The role of CD4(+) T helper cells in the cytotoxic T lymphocyte response to HIV-1. *Curr Opin Immunol.* 2000;12:375-80.
3. Battegay M, Nuesch R, Hirschel B, Kaufmann GR. Immunological recovery and antiretroviral therapy in HIV-1 infection. *Lancet Infect Dis.* 2006;6:280-7.
4. Tan R, Xu X, Ogg GS, Hansasuta P, Dong T, Rostron T, et al. Rapid death of adoptively transferred T cells in acquired immunodeficiency syndrome. *Blood.* 1999;93:1506-10.
5. Paulos CM, Suhoski MM, Plesa G, Jiang T, Basu S, Golovina TN, et al. Adoptive immunotherapy: good habits instilled at youth have long-term benefits. *Immunol Res.* 2008;42:182-96.
6. Powell DJ Jr, Levine BL. Adoptive T-cell therapy for malignant disorders. *Haematologica.* 2008;93:1452-6.
7. Ho M, Armstrong J, McMahon D, Pazin G, Huang XL, Rinaldo C, et al. A phase 1 study of adoptive transfer of autologous CD8+ T lymphocytes in patients with acquired immunodeficiency syndrome (AIDS)-related complex or AIDS. *Blood.* 1993;81:2093-101.
8. Lutzky VP, Davis JE, Crooks P, Corban M, Smith MC, Elliott M, et al. Optimization of LMP-specific CTL expansion for potential adoptive immunotherapy in NPC patients. *Immunol Cell Biol.* 2009;87:481-8.
9. Levine BL, Mosca JD, Riley JL, Carroll RG, Vahey MT, Jagodzinski LL, et al. Antiviral effect and ex vivo CD4+ T cell proliferation in HIV-positive patients as a result of CD28 costimulation. *Science.* 1996;272:1939-43.
10. Onlamoon N, Plagman N, Rogers KA, Mayne AE, Bostik P, Pattanapanyasat K, et al. Anti-CD3/28 mediated expansion of macaque CD4+ T cells is polyclonal and provides extended survival after adoptive transfer. *J Med Primatol.* 2007;36:206-18.
11. Bernstein WB, Cox JH, Aronson NE, Tracy L, Schlienger K, Ratto-Kim S, et al. Immune reconstitution following autologous transfers of CD3/CD28 stimulated CD4(+) T cells to HIV-infected persons. *Clin Immunol.* 2004;111:262-74.
12. Onlamoon N, Hudson K, Bryan P, Mayne AE, Bonyhadi M, Berenson R, et al. Optimization of in vitro expansion of macaque CD4 T cells using anti-CD3 and co-stimulation for autotransfusion therapy. *J Med Primatol.* 2006;35:178-93.
13. Villinger F, Brice GT, Mayne AE, Bostik P, Mori K, June CH, Ansari AA. Adoptive transfer of simian immunodeficiency virus (SIV) naïve autologous CD4(+) cells to macaques chronically infected with SIV is sufficient to induce long-term nonprogressor status. *Blood.* 2002;99:590-9.
14. Levine BL, Bernstein WB, Aronson NE, Schlienger K, Cotte J, Perfetto S, et al. Adoptive transfer of costimulated CD4+ T cells induces expansion of peripheral T cells and decreased CCR5 expression in HIV infection. *Nat Med.* 2002;8:47-53.
15. Kikitenko AI, Zlobina GP, Shchurin MR, Kleshchinov VN, Brusov OS. Effect of different methods of isolation of platelets on surface structure and biochemical parameters on their serotonin system. *Biull Eksp Biol Med.* 1991;112:1579-82.
16. Berhanu D, Mortari F, De Rosa SC, Roederer M. Optimized lymphocyte isolation methods for analysis of chemokine receptor expression. *J Immunol Methods.* 2003;279:199-207.
17. De Paoli P, Reitano M, Battistin S, Castiglia C, Santini G. Enumeration of human lymphocyte subsets by monoclonal antibodies and flow cytometry: a comparative study using whole blood or mononuclear cells separated by density gradient centrifugation. *J Immunol Methods.* 1984;72:349-53.
18. Trickett AE, Kwan YL, Cameron B, Dwyer JM. Ex vivo expansion of functional T lymphocytes from HIV-infected individuals. *J Immunol Methods.* 2002;262:71-83.
19. Levine BL, Bernstein WB, Connors M, Craighead N, Lindsten T, Thompson CB, et al. Effects of CD28 costimulation on long-term proliferation of CD4+ T cells in the absence of exogenous feeder cells. *J Immunol.* 1997;159:5921-30.
20. Levine BL, Cotte J, Small CC, Carroll RG, Riley JL, Bernstein WB, et al. Large-scale production of CD4+ T cells from HIV-1-infected donors after CD3/CD28 costimulation. *J Hematother.* 1998;7:437-48.
21. El-Far M, Halwani R, Said E, Trautmann L, Doroudchi M, Janbazian L, et al. T-cell exhaustion in HIV infection. *Curr HIV/AIDS Rep.* 2008;5:13-9.
22. Paranjape RS. Immunopathogenesis of HIV infection. *Indian J Med Res.* 2005;121:240-55.
23. Norris PJ, Rosenberg ES. CD4(+) T helper cells and the role they play in viral control. *J Mol Med.* 2002;80:397-405.