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The Effects of Anti-CD3/CD28 Coated Beads and IL-2 on Expanded T Cell for Immunotherapy

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article

Abstract

Background. The activation of peripheral blood mononucleated cells (PBMCs) with anti-CD3/CD28-coated magnetic beads promotes intrinsic resistance to HIV as well as cell expansion.

Objectives. The aim of this study was to define an optimal cell isolation protocol for the expansion of PBMCs using anti-CD3/CD28-coated bead stimulation, with the ultimate goal of using these cells for adoptive therapy.

Material and Methods. PBMCs were isolated from healthy donor blood samples. To determine the effect of varying the bead-to-cell ratios on the expansion rate and phenotypic characterization of the expanded cells, one million PBMCs were stimulated by anti-CD3/CD28-coated beads at bead-to-cell ratios of 0.1 : 1, 0.5 : 1 and 1.0 : 1 in the presence of 100 U/mL exogenous IL-2; also, one million PBMCs were stimulated by anti-CD3/CD28-coated beads at a bead-to-cell ratio of 0.5 : 1 in the presence of varying concentrations of IL-2 (20, 100 and 1000 U/mL). Cell expansion was carried out for three weeks. The cell numbers, cell viability and phenotypic characterization were determined by trypan blue exclusion and flow cytometry.

Results. The initial experiments showed no difference in the expansion rate from cells grown with the three different bead-to-cell ratios. PBMCs can be expanded up to 1000-fold at a 0.5 : 1 bead-to-cell ratio after three weeks of cell expansion with a high viability (90%). Furthermore, in the presence of 100 U/mL IL-2, the percentages of CD3-CD16+CD56+ cells showed marked increases.

Conclusions. The results demonstrate that PBMCs were stimulated with anti-CD3/CD28-coated beads. This method may provide an alternative for driving T cell expansion, which may be very useful in adoptive immunotherapy (*Adv Clin Exp Med* 2016, 25, 5, 821–828).

Key words: PBMCs, IL-2, anti-CD3/CD28, cell expansion.

T lymphocyte activation requires two signals from professional antigen presenting cells (APCs) [1]. Signal 1 is generated by the interaction of the T cell receptor (TCR) and the peptide-MHC complex on professional APCs, and Signal 2 is generated by the interaction of CD28 on T lymphocytes and CD80 (B7.1) or CD86 (B7.2) on professional APCs [1]. Although the interaction of the TCR-CD3 complex induces signaling during T lymphocyte activation, TCR signaling is

not sufficient to induce clonal proliferation [2]. In addition, the CD28-B7 interaction promotes the up-regulation of transcriptional IL-2 cytokine production and the expression of the anti-apoptotic gene (*bcl-x_L*), which leads to an increase in cell proliferation and a decrease in apoptotic death [3]. Since the survival and functional development of T lymphocytes are associated with the stimulation strength *via* the TCR, various factors that control the rate of TCR triggering (antigen affinity and

density or the degree of signal amplification) and incubation time (co-stimulation *via* CD28 and the duration of the DC-T cell interaction) are used to determine the overall strength of T cell stimulation [4]. A study by Kalamasz et al. demonstrated that naive T cells are activated and proliferate through high levels of CD3/TCR receptor engagement, whereas the same signal causes activation-induced cell death (AICD) of memory T cells [4]. This suggests that different levels of signaling can result in different outcomes.

Previous studies by the present authors and others have demonstrated that magnetic beads coated with anti-CD3 and anti-CD28, which mimic professional APCs, can be a convenient reagent for the expansion of T lymphocytes *in vitro* [5, 6]. Moreover, stimulating purified CD4+ T lymphocytes from HIV-infected donors induces an approx. 37-fold expansion within two weeks, whereas PBMC stimulation from healthy donors induces an approx. 56-fold expansion within three weeks [7]. In order to identify the optimal conditions for the expansion of lymphocytes with anti-CD3/CD28-coated beads, several studies have demonstrated the effects of cytokine supplementation on proliferation rates. The results showed that IL-12 supplementation supports the function of T lymphocytes; however, this cytokine does not induce an increase in the number of these cells [8]. It has also been shown that supplementation with any single or possible combination of exogenous IL-2, IL-7, IL-15 and IL-21 does not increase the proliferation rate in healthy *rhesus macaques* [5]. In contrast, the treatment of cells in the range of 0.2–20 units/mL of IL-2 shows a positive correlation with T lymphocyte proliferation [6]. Although many cytokines affect T lymphocyte proliferation and differentiation *in vivo*, these cytokines are not sufficient to expand T lymphocytes *in vitro*.

IL-2 is the predominant factor responsible for supporting T cell proliferation *in vivo*, and T cell growth and is routinely used for T lymphocyte expansion *in vitro* [9]. IL-2 promotes activation and differentiation signals through a specific receptor on the cell surface, IL-2 receptor (IL-2R) [9]. Studies have shown that activated CD4+ T lymphocytes mainly produce IL-2 at the initiation of the priming stage, within 0–2.5 h after stimulation, which contributes to the activation and differentiation of CD8+ T lymphocytes, natural killer (NK) cells and B lymphocytes [9, 10]. However, the range of 20–7200 U/mL of IL-2 is used for cell expansion [6].

Based on these results, the effects of the IL-2 concentration and the strength of T cell stimulation on the proliferation rate and phenotypic characterization of stimulated cells have not been fully

characterized. The aim of this study was to define the optimal conditions for expanding T lymphocytes by varying the bead-to-cell ratio and IL-2 concentration, with the ultimate goal of using this method for cell preparation for future clinical applications.

Material and Methods

The Subjects

Three healthy volunteers were recruited for this study. All of the subjects signed an informed consent form that was approved by the Ethics Review Committee for Research Involving Human Research Subjects of the Health Science Group at Chulalongkorn University (Bangkok, Thailand). From each volunteer, 10 mL of blood was collected in sodium heparin-containing vacutainer tubes. An aliquot of each blood sample was separated on Ficoll-Hypaque gradients to obtain peripheral blood mononuclear cells (PBMCs).

Antibodies

The following monoclonal antibodies (mAbs) and their conjugated fluorochromes were commercially obtained (BD Biosciences, San Jose, USA): 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 mAbs for 15 min followed by the addition of FACS lysing solution (BD Biosciences) to lyse the red blood cells and fix the samples. After washing in wash buffer (phosphate buffered saline [PBS] containing 2% fetal bovine serum), the stained cells were maintained in PBS with 1% paraformaldehyde. The samples were acquired by a FACSCalibur flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star Inc., San Carlos, USA).

Cell Stimulation

One million cells were stimulated with anti-CD3/CD28 mAbs immobilized on magnetic beads (Invitrogen Dynal AS, Oslo, Norway) with various

bead-to-cell ratios (0.1 : 1, 0.5 : 1 and 1.0 : 1) in the presence of 100 U/mL IL-2 (ProSpec-Tany TechnoGene Ltd., Rehovot, Israel). The effects of different IL-2 concentrations (20 U/mL, 100 U/mL and 1000 U/mL) with a 0.5 : 1 bead-to-cell ratio stimulation were also evaluated. The cells were expanded in complete media (RPMI 1640 with 10% fetal calf serum, 50 µg/mL penicillin-streptomycin and 2 mM L-glutamine) and were incubated at 37°C in humidified 5% CO₂. The expanded cell cultures were re-stimulated on day 7 and maintained by the addition of fresh media on days 4, 7, 11, 14, 17 and 21. The expanded cells were transferred to appropriate containers as needed to maintain a cell concentration of 0.5×10^6 cells/mL. The cell numbers and viability were determined at each point in time by trypan blue exclusion. The fold expansion was calculated using the viable cell number at each indicated time point divided by the viable cell number at the beginning of cell expansion (1×10^6 cells).

Statistical Analysis

The data are presented as the mean \pm standard deviation of the samples for fold expansion, the percentage of cell viability and the percentage of each cell subset. The statistical analysis was performed using GraphPad Prism[®] software v. 5 (GraphPad Software Inc., La Jolla, USA). The t-test was used to determine statistical differences between the mean values of fold expansion in each group of samples. P-values < 0.05 were considered statistically significant.

Results

The Influence of the Bead-to-Cell Ratio on the Expansion Rate and Phenotypic Characterization of Anti-CD3/CD28-Expanded Cells

To determine the effect of varying the bead-to-cell ratios on the expansion rate and phenotypic characterization of the expanded cells, one million PBMCs were stimulated by anti-CD3/CD28-coated beads at bead-to-cell ratios of 0.1 : 1, 0.5 : 1 and 1.0 : 1 in the presence of 100 U/mL exogenous IL-2. The initial experiments showed no difference between the expansion rates of cells grown with the three different bead-to-cell ratios (Fig. 1). A max 8.00-fold expansion rate was observed in stimulated cells at the 0.5 : 1 bead-to-cell ratio when bead re-stimulation was performed on day 7. After a 3^d-week culture period, a marked increase in the expansion rate of the stimulated cells at the 0.5 : 1 bead-to-cell ratio was observed when compare to stimulated cells at the 0.1 : 1 and 1 : 1 bead-to-cell ratios, although no statistically significant differences were observed. Thus, the expansion rate of the stimulated cells at the 0.5 : 1 bead-to-cell ratio was 1163.4 ± 1020.4 -fold, while 46.4 ± 45.8 and 83.0 ± 84.5 -fold expansion rates were observed in the stimulated cells at 0.1 : 1 and 1 : 1 bead-to-cell ratios, respectively. The cell viability at the end of the culture period was approximately 90% for all three bead-to-cell ratios (data not shown). Phenotypic characterizations of the expanded cells were determined on days 14 and 21 of the cell expansion. The results showed an increase in the percentages of CD3+ T lymphocytes (approx. 70–99%) among the lymphocyte population on

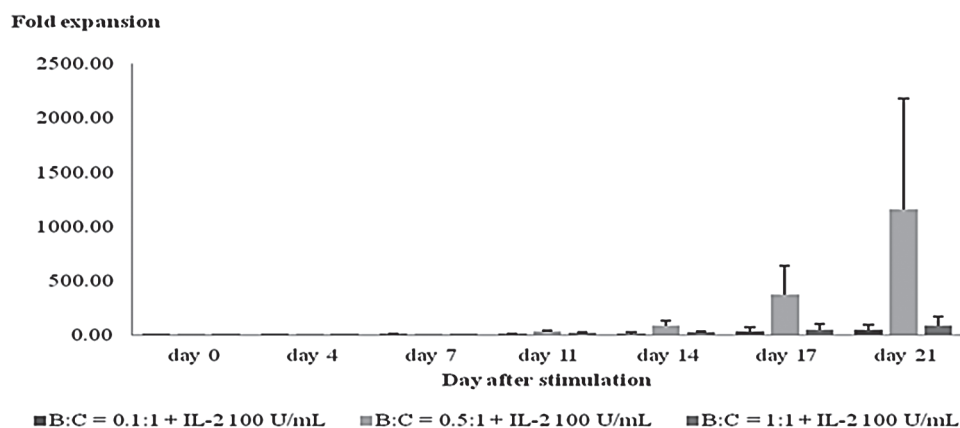


Fig. 1. The expansion rates of anti-CD3/28-expanded T lymphocytes during three weeks of culture. PBMCs were stimulated at three bead-to-cell ratios in the presence of 100 U/mL IL-2. The expanded cells were counted on days 4, 7, 11, 14, 17 and 21. The fold expansion was calculated relative to the starting cell number and represents the mean value from three healthy individuals

Table 1. The percentages of each T lymphocyte subset in the lymphocyte population obtained from anti-CD3/28-expanded cells at three bead-to-cell ratios

Cell population	Day 0	Day 14			Day 21		
		0.1 : 1	0.5 : 1	1 : 1	0.1 : 1	0.5 : 1	1 : 1
CD3+	58.27 ± 3.78	86.3 ± 9.1	97.1 ± 1.7	99.9 ± 0.1	71.8 ± 8.3	87.0 ± 18.3	99.9 ± 0.1
CD3+CD4+	22.7 ± 4.54	28.8 ± 13.8	54.4 ± 12.1	37.53 ± 5.2	12.1 ± 8.2	20.9 ± 10.1	59.8 ± 27.8
CD3+CD8+	32.43 ± 0.83	58.8 ± 13.4	45.6 ± 14.6	64.1 ± 7.2	57.7 ± 13.9	69.8 ± 14.3	41.3 ± 27.8
CD3+CD16+CD56+	4.36 ± 2.36	6.0 ± 2.4	2.2 ± 0.4	2.0 ± 0.4	12.9 ± 4.4	5.5 ± 2.1	8.0 ± 4.7
CD3- CD16+CD56+	18.87 ± 4.99	15.1 ± 10.3	2.2 ± 1.4	1.4 ± 1.9	26.4 ± 9.5	6.2 ± 6.4	5.9 ± 9.9

Results are shown as mean percentages ± standard deviation.

day 14 and day 21 in cells grown in all three bead-to-cell ratios when compared with day 0 (Table 1). The results also showed that the stimulated cells at the 0.1 : 1 bead-to-cell ratio yielded the lowest percentages of CD3+ T lymphocytes among the lymphocyte population (71.8% ± 8.3) after three weeks of cell expansion when compared with the stimulated cells at the 0.5 : 1 and 1 : 1 bead-to-cell ratios (87.0% ± 18.3 and 99.9% ± 0.1, respectively). After 21 days of bead stimulation, the stimulated cells at the 1 : 1 bead-to-cell ratio yielded the highest percentage of CD4+ T lymphocytes (59.8% ± 27.8) when compared with the stimulated cells at 0.1 : 1 and 0.5 : 1 bead-to-cell ratios (12.1% ± 8.2 and 20.9% ± 10.1, respectively). Although the stimulated cells at the 1 : 1 bead-to-cell ratio showed the highest percentages of CD8+ T lymphocytes after two weeks of cell expansion, after three weeks of cell expansion the percentages of these lymphocytes in the stimulated cells were the highest at the 0.5 : 1 bead-to-cell ratio (69.8% ± 14.3) compared with stimulated cells at the 0.1 : 1 and 1 : 1 bead-to-cell ratios (57.7% ± 13.9 and 41.3% ± 27.8, respectively). The culture grown at the lowest bead-to-cell ratio (0.1 : 1) continually increased and yielded the highest percentage of NKT cells from day 14 to day 21 (6.0% ± 2.4 and 12.9% ± 4.4, respectively), whereas the higher bead-to-cell ratios (0.5 : 1 and 1 : 1) showed lower percentages of NKT cells after three weeks of cell expansion (5.5% ± 2.1 and 8.0% ± 4.7, respectively). Similarly, the culture at the lowest bead-to-cell ratio (0.1 : 1) yielded the highest percentages of NK cells (26.4% ± 9.5) compared with stimulated cells at the 0.5 : 1 and 1 : 1 bead-to-cell ratios after three weeks of cell expansion (6.2% ± 6.4 and 5.9% ± 9.9, respectively). There was no increase in the percentages of B lymphocytes after three weeks of bead stimulation.

The Influence of IL-2 Concentrations on the Expansion Rate and Phenotypic Characterization of Anti-CD3/CD28-Expanded Cells

The experiments described above showed that cell stimulation with anti-CD3/CD28-coated beads at a 0.5 : 1 bead-to-cell ratio resulted in the highest expansion rate. To evaluate the effect of the IL-2 concentration on the expansion rate and phenotypic characterization of the expanded cells, one million PBMCs were stimulated by anti-CD3/CD28-coated beads at a bead-to-cell ratio of 0.5 : 1 in the presence of varying concentrations of IL-2 (20, 100 and 1000 U/mL). The results showed that there was no difference between the expansion rates during the first seven days of cell expansion in the different IL-2 concentrations (Fig. 2). After bead re-stimulation on day 7, however, the stimulated cells in the presence of 20 and 100 U/mL IL-2 continually increased their expansion rate from day 11 to day 17 compared with cells stimulated in the presence of 1000 U/mL IL-2. After three weeks of cell expansion, the highest expansion rate was observed in cells stimulated with 100 U/mL IL-2 (632.3 ± 277.4) compared with cells stimulated with 20 and 1000 U/mL IL-2 (340.6 ± 108.3 and 82.9 ± 107.6, respectively). Although no statistically significant difference was observed when compared to cells stimulated with 20 U/mL IL-2, the results showed a significantly higher expansion when compared to cells stimulated with 1000 U/mL IL-2 ($p = 0.0246$). Furthermore, cells stimulated with 100 U/mL IL-2 had a higher expansion rate when compared to cells stimulated with 1000 U/mL IL-2 ($p = 0.0266$). Cell viability was maintained at approx. 90% throughout the expansion period at all of the different IL-2 concentrations. The anti-CD3/CD28-expanded cells were phenotypically characterized on day 14 and day 21 of the cell expansion. The results showed an

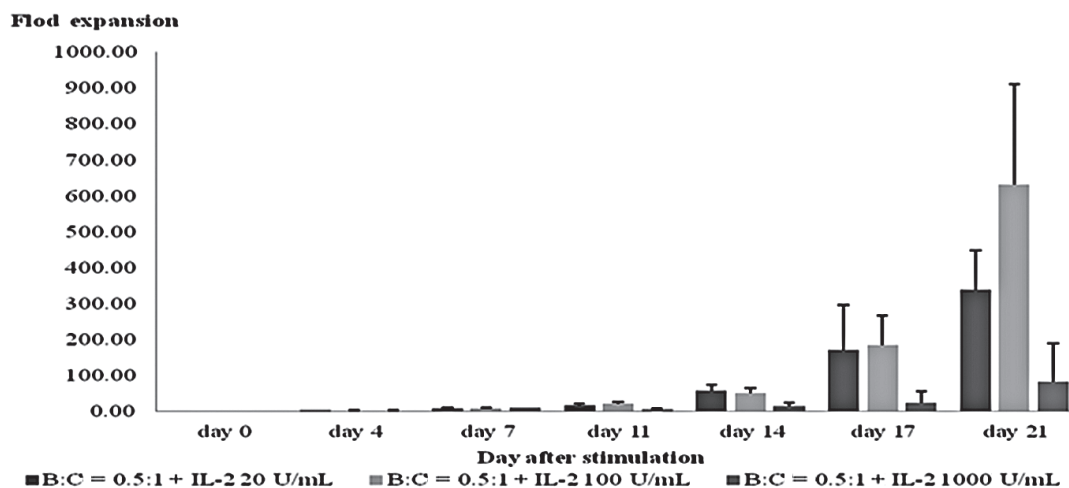


Fig. 2. The expansion rates of anti-CD3/28-expanded T lymphocytes during three weeks of culture. PBMCs were stimulated at a bead-to-cell ratio of 0.5 : 1 in the presence of three concentrations of IL-2. The expanded cells were counted on days 4, 7, 11, 14, 17 and 21. The fold expansion was calculated relative to the starting cell number and represents the mean value from three healthy individuals

Table 2. The percentages of each T lymphocyte subset in the lymphocyte population obtained from anti-CD3/28-expanded cells in three concentrations of IL-2

Cell population	Day 0	Day 14			Day 21		
		20 U/mL	100 U/mL	1000 U/mL	20 U/mL	100 U/mL	1000 U/mL
CD3+	57.7 ± 13.6	97.8 ± 0.8	98.1 ± 1.5	65.7 ± 1.4	98.6 ± 1.0	98.57 ± 1.3	66.6 ± 0.1
CD3+CD4+	30.1 ± 14.5	51.7 ± 16.6	48.7 ± 15.4	30.2 ± 28.6	12.6 ± 7.2	26.7 ± 15.5	26.2 ± 22.7
CD3+CD8+	23.7 ± 8.7	52.8 ± 14.9	53.8 ± 13.6	37.9 ± 34.2	87.2 ± 6.1	76.5 ± 15.4	42.8 ± 37.2
CD3+CD16+CD56+	4.8 ± 1.9	2.7 ± 1.0	4.4 ± 2.2	4.9 ± 5.1	2.8 ± 1.3	6.1 ± 2.3	10.9 ± 9.4
CD3- CD16+CD56+	17.6 ± 9.8	0.9 ± 0.7	1.5 ± 1.5	1.3 ± 1.9	1.2 ± 1.0	1.6 ± 1.5	0.9 ± 1.4

Results are shown as mean percentages ± standard deviation.

increase in the percentages of CD3+ T lymphocytes among the lymphocyte population on day 14 and day 21 in cells grown in the culture supplemented with 20 U/mL and 100 U/mL (more than 95%) when compared with day 0 (Table 2). The results also showed that the culture supplemented with 1000 U/mL IL-2 yielded the lowest percentages of CD3+ T lymphocytes among the lymphocyte population (66.6 ± 0.1) after three weeks of cell expansion compared with the cultures supplemented with 20 and 100 U/mL IL-2 (98.6 ± 1.0 and 98.57 ± 1.3 , respectively). An increase of approximately 50% in the percentages of CD4+ and CD8+ T lymphocytes was observed in the cultures grown in each of the different IL-2 concentrations after two weeks of cell expansion. After three weeks of cell expansion, the culture supplemented with 1000 U/mL IL-2 yielded the highest percentages of CD4+ T lymphocytes ($39.3\% \pm 1.3$) compared with the cultures supplemented with 20 and 100 U/mL IL-2 ($12.6\% \pm 7.2$ and $26.7\% \pm 15.5$, respectively), whereas the culture supplemented with 20 U/mL

IL-2 yielded the highest percentages of CD8+ T lymphocytes ($87.2\% \pm 6.1$) compared with the cultures supplemented with 100 and 1000 U/mL IL-2 ($76.5\% \pm 15.4$ and $64.2\% \pm 5.1$, respectively). The culture supplemented with 1000 U/mL IL-2 continually increased and yielded the highest percentages of NKT T cells from day 14 to day 21 ($7.4\% \pm 4.0$ and $16.3\% \pm 0.4$, respectively). No increases in B lymphocytes and NK cells were found in any of the three cultures after bead stimulation for three weeks of cell expansion.

Discussion

Although several studies have shown that many parameters have an effect on the proliferation of PBMCs, the optimum conditions for cell expansion have not previously been reported. A previous study showed that unfractionated cells in culture are more useful than purified populations of T cells for cell proliferation due to the action

of autocrine and paracrine, such as in unfractionated cells, monocytes and macrophages, secrete IL-1 and IL-12 which stimulate T cell proliferation [6]. Studies have also demonstrated that the activation of cells during cell expansion might lead to a reduction of T lymphocyte functions, including proliferation and cytokine production, and to an alteration of the phenotypic characterization of the cells [11–12].

However, there are several intrinsic and extrinsic factors that influence cell activation, proliferation and differentiation, such as TRC strengthening and the cytokine environment. Previous studies have shown that the expansion rate positively correlates with the number of beads per lymphocytes in the range of 0.1–5 beads, but that bead-to-cell ratios over 5 : 1 do not promote an increase in the proliferation rate [6]. At the same time, it has been found that there is no difference in the proliferation rate of stimulated CD4⁺ T lymphocytes between cultures grown in 4 : 1, 2 : 1 and 1 : 1 bead-to-cell ratios in *rhesus macaques* [5]. The present study investigated the effects of the strength of T cell stimulation and the IL-2 concentration on the expansion rate and phenotypic characterization of cells stimulated by anti-CD3/CD28-coated beads. In contrast to previous studies, the results showed that PBMC stimulation with anti-CD3/CD28-coated beads at a 0.5 : 1 bead-to-cell ratio in the presence of 100 U/mL of exogenous IL-2 was the optimum condition for expanding T lymphocytes *ex vivo*. Under these conditions the expansion rate increased by more than 1000-fold within three weeks, with a high cell viability. The results suggest that a number of bead-to-cell ratios should be considered while devising a protocol for optimizing cell expansion. The results of the present study are in agreement with a previous study that demonstrated that cell stimulation with a low bead-to-cell ratio showed an increase in the proliferation and survival of antigen-specific T cells, a reduction in the possibility of cultured cells suffering from bead withdrawal near the end of the culture and also a reduction in material costs [4].

While cell expansion using PBMCs provided 10,000-fold expansion, the proliferation of CD8⁺ T lymphocytes was higher than the proliferation of CD4⁺ T lymphocytes [6]. Recently it was reported that by varying either antigen dose, co-stimulatory signals, or the affinity of antigenic peptide/MHC complexes for the TCR, low-strength stimulation is sufficient for Th2 differentiation, whereas high-strength stimulation is required for Th1 differentiation [13]. These studies suggested that varying the strength of the T cell stimulation provided by anti-CD3/CD28-coated beads could lead to a different phenotypic characterization of the expand-

ed cells. In the present study, reducing the bead-to-cell ratio to 0.1 : 1 led to NK and NKT cell expansion, whereas the strength of the signal from bead stimulation at a 0.5 : 1 ratio led to an expansion of CD8⁺ T lymphocytes. Increasing the strength of the signal provided to T lymphocytes by increasing the number of beads to a 1 : 1 bead-to-cell ratio supported CD4⁺ T lymphocyte expansion. Moreover, almost all of the expanded cells at the 0.5 : 1 bead-to-cell ratio were CD8⁺ T lymphocytes in all of the different IL-2 concentrations that were tested. These data indicate that the strength of T cell stimulation plays an important role in the dramatic change in the phenotype of the expanded cells.

Other factors that have an effect on the cell growth of anti-CD3/CD28-activated T lymphocytes, such as IL-2 and serum supplementation, have been reported. Some studies have shown that IL-2 supplementation was not essential for stimulated CD4⁺ T lymphocytes with anti-CD3/CD28-coated beads, since autocrine IL-2 production was sufficient to support their proliferation [6, 14]. Moreover, cell cultures at low IL-2 concentrations induce fewer cells within the population to enter cell division and the cells take a longer time to complete subsequent divisions, with larger fractions of them dying off before the next division begins [14]. In contrast, a study by Cornish et al. reported that IL-2 induces the differentiation of effector CD8⁺ T lymphocytes, and IL-2 stimulation induces the protein synthesis that is required for cell-cycle progression and an increase in cell size [15]. Levine et al. reported that exogenous IL-2 helps extend the time for cell proliferation and growth to 83 days because its population doubling times (PDs) are slower than in a culture lacking exogenous IL-2 [7].

In the present study there were no differences in the mean percentages of CD4⁺ T lymphocytes and CD8⁺ T lymphocytes from cells grown in the three different IL-2 concentrations on day 14. It is possible that autocrine IL-2 production from activated CD4⁺ T lymphocytes in the cell culture supernatant might induce the activation and proliferation of CD8⁺ T lymphocytes after two weeks of cell expansion.

The present study also investigated whether extended time periods of expansion promoted an increase in the expansion rates and cell viability. The results showed that the expansion rates of stimulated cells at a bead-to-cell ratio of 0.5 : 1 in the presence of 100 U/mL IL-2 continually increased, but their viability continually decreased after three weeks of cell expansion (data not shown). These data indicate that the rate of cell proliferation slowed down, and the cells in the suspension culture subsequently reached a sta-

tionary phase and a decline phase. There are multiple possible influences that might reduce cell viability, including nutrient limitation, toxic waste accumulation or a critical population density being reached. Repeated stimulation induces expression of the death receptor Fas (CD95) and its ligand FasL (CD95L), and also repression of the anti-apoptotic protein (FLIP), leading to activation-induced cell death [9]. Moreover, the highest dose of IL-2 used in the present study (1000 U/mL) led to an increase in cell death among the expanded cells from day 7 to day 21, which was observed continually (data not shown). Although IL-2 induces anti-apoptotic Bcl family proteins for promoting T cell survival, a high IL-2 concentration enhances the transcription of CD95L and inhibits the transcription and expression of the cellular FLIP/FLICE-inhibitory protein [16]. The cells were susceptible to AICD following CD95 engagement. However, the expression of these markers on the surface of expanded cells needs further investigation, and three weeks was the appropriate time period for cell expansion.

The ultimate goal of PBMC expansion is to explore a potential supplementary treatment by autologous transfer of expanded PBMCs to HIV in-

fecting patients. In this study, PBMCs exhibited more than 1000-fold expansion in three weeks with a high cell viability and a high purity. This protocol might be useful for the expansion of PBMCs from HIV-infected patients because the cellular dysfunction associated with several markers (including CD57, CTLA-4 and PD-1) which leads to impairment of proliferation, cytokine production or cell exhaustion, occurs during the chronic phase of HIV infection [17]. Because the number of PBMCs that can be obtained from HIV patients is low when compared to healthy donors [18], the fact that the expansion procedure presented in this study reduces the number of isolated cells required to generate large-scale expansion of PBMCs is particularly noteworthy when considering the treatment of HIV-infected patients.

In conclusion, the present study describes a method for the optimal expansion of T lymphocytes through adjustments of the IL-2 concentration and the strength of the signal provided by anti-CD3/CD28-coated beads to T cells *ex vivo*. These findings will improve the generation and manipulation of human T cells *ex vivo* for future clinical applications.

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