มหาวิทยาลัยมหิดล
คณะแพทยศาสตร์ศิริราชพยาบาล

รายงานวิจัยฉบับสมบูรณ์

โครงการการประเมินกระบวนการสร้าง anti-CD3/28 expanded CD4+ T cell สำหรับการพัฒนาวิธีการรักษาด้วยภูมิคุ้มกันในผู้ป่วยโรคติดเชื้อเอชไอวี (Evaluation of anti-CD3/28 expanded CD4+ T cell production strategy for a development of an immune based therapy in HIV-1 infected patients)

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สัญญาเลขที่ RSA5880020

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บทคัดย่อ

รหัสโครงการ : RSA5880020

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การใช้ยาต้านไวรัสในผู้ป่วยโรคติดเชื้อเอชไอวีช่วยให้เกิดประสิทธิผลสูงสุดในการลดจำนวนไวรัส อย่างไรก็ตามการใช้ยาต้านไวรัสดังกล่าวช่วยเพียงควบคุมปริมาณไวรัสและฟื้นฟูความสามารถของภูมิคุ้มกันร่างกายเพียงบางส่วนโดยที่มีการบูรณะภูมิคุ้มกันได้อย่างเชื่องช้าและไม่สมบูรณ์ การศึกษาก่อนหน้าได้แสดงการใช้เม็ดแม่เหล็กเคลือบด้วยแอนติบอดีต่อซีดี3และซีดี28เพื่อการเพิ่มจำนวนเซลล์ในห้องปฏิบัติการซึ่งช่วยให้เซลล์สามารถควบคุมการแบ่งตัวของไวรัส รวมทั้งการถ่ายโอนเซลล์ซีดี4ที่ถูกเพิ่มจำนวนขึ้นกลับไปยังผู้ป่วยที่ได้รับยาต้านไวรัสยังช่วยให้มีการเพิ่มจำนวนทีเซลล์ชนิดซีดี4 จากผลการศึกษาเบื้องต้นดังกล่าวทำให้คณะผู้วิจัยได้พัฒนาวิธีการเพิ่มจำนวนเซลล์เพื่อการทดสอบระดับคลินิกรวมทั้งทำการจำแนกลักษณะเฉพาะและคุณสมบัติการทำงาน ในการศึกษานี้ การเพิ่มจำนวนเซลล์ปริมาณมากสามารถทำได้โดยการเลี้ยงเซลล์ในถุงสำหรับเลี้ยงเซลล์ที่ได้มาตรฐานการผลิตในระบบปิดร่วมกับการกระตุ้นโดยใช้เม็ดแม่เหล็กเคลือบด้วยแอนติบอดีต่อซีดี3และซีดี28 นอกจากนี้แล้วการเพิ่มจำนวนเซลล์โดยใช้อาหารเลี้ยงเซลล์ที่ได้มาตรฐานการผลิตร่วมกับการเสริมด้วยซีรั่มมนุษย์ยังทำให้ได้การแบ่งตัวเซลล์ในระดับที่น่าพอใจ การประเมิณโมเลกุลแบบจำเพาะบนผิวเซลล์และความสามารถในการสร้างสารไซโตไคน์แสดงให้เห็นรูปแบบของโมเลกุลบนผิวเซลล์ที่จำเพาะและลักษณะของทีเฮลเปอร์แบบที่1 โดยสรุป ผลการศึกษานี้แสดงถึงกระบวนการผลิตเซลล์ที่มีประสิทธิภาพสำหรับการนำไปใช้เพื่อการศึกษาทางคลินิกต่อไป

คำหลัก : ซีดี4 ทีลิมโฟไซต์, การเพิ่มจำนวนเซลล์, เม็ดแม่เหล็กเคลือบแอนติบอดีต่อซีดี3และซีดี28, การรักษาแบบภูมิคุ้มกันบำบัด, โรคติดเชื้อเอชไอวี

Abstract

Project Code : RSA5880020

Project Title : Evaluation of anti-CD3/28 expanded CD4+ T cell production strategy for a development of an immune based therapy in HIV-1 infected patients

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The use of antiretroviral therapy (ART) in human immunodeficiency virus (HIV) infected patients has become highly effective in lowering viral burden. However, while ART could control viremia and restore some level of immune competency, the immune reconstitution is slow and never complete. Previous studies showed that in vitro expansion of CD4+ T cells from HIV infected patients by anti-CD3/28 coated beads provided intrinsic control of viral replication and transfusion of autologous expanded CD4+ T cells showed increase in CD4+ T cells in antiretroviral treated HIV infected patients. From this preliminary finding, a clinical grade cell expansion protocol with phenotypic and functional characterization was developed. In this study, a large number of expanded CD4+ T lymphocytes can be achieved with the developed closed-culture system using Good Manufacturing Practice (GMP)-grade culture bags and anti-CD3/28 coated bead stimulation. Moreover, cell expansion using GMP-grade media with human serum supplementation rendered satisfied proliferation rates. Evaluation of specific surface molecule expressions and cytokine producing ability presented a specific surface molecule expression pattern with T helper 1-like phenotype. In conclusion, this result presented the effective cell manufacturing method for further uses in clinical trials.

Keywords : CD4+ T lymphocyte, cell expansion, anti-CD3/28 coated beads, immunotherapy, HIV infection

Executive summary

CD4 immunotherapy is potentially useful in immune reconstitution of CD4+ T cells for HIV-infected patients. Transfusion of anti-CD3/28 expanded CD4+ T cells is also proved to be safe and effective in both SIV-infected macaques and HIV-infected patients. However, there is no such standardized and practical protocol available for cell production in order to use in clinics. This study thus aimed to develop a closed-culture system for in vitro CD4+ T lymphocyte expansion by using a commercially available GMP-grade culture bag and anti-CD3/28 activation. The expansion rates and yields of expanded CD4+ T cells by using commercially available GMP-grade culture media with human serum (HS) supplementation were also determined. Moreover, a confirmation of functional-related phenotypes was assessed for ensuring the successful outcomes of treatment.

Freshly isolated CD4+ T cells by immunorosette formation from healthy donors and cryopreserved CD4+ T cells from HIV-infected patients with CD4 count over 500 cells/μL were stimulated with anti-CD3/28 coated beads. The activated cells were then expanded in conventional culture flasks and GMP-grade culture bags for three weeks. Fold expansion, cell viability, growth kinetic and phenotypic characters were observed. Results revealed that purified CD4+ T cells from healthy individuals cultured in flasks showed better expansion than those cultured in bags (797-fold and 331-fold, respectively), whereas, their cell viability, growth kinetic and expanded CD4+ T cell purity were almost similar. A large-scale production was also conducted and supported consistency of cell proliferation in the closed-culture system. Frozen CD4+ T lymphocytes from the patients were able to remain their growth function and well expanded with a good yield of 415-fold, 85% viability and 96% purity of CD4+ T cells at the end of a 3-week culture in bags.

Isolated CD4+ T cells from healthy donors were also expanded in different media supplemented with either HS or fetal bovine serum (FBS) for 3 weeks. Results showed that the expanded cells from the cultures using AIM-V and DC media with 10% HS were well expanded (~255-fold) and had similar polyfunctional patterns, however, they showed poorer proliferation rates than those from the cultures using RMPI1640 with 10% FBS (R10; ~637-fold). Moreover, cell viability of the expanded cells from R10 was also significantly higher than the others after a 3-week culture. High purity with CD3+CD4+CD8- phenotype of the expanded cells from all cultures was also observed. The expanded cells from all conditions were found predominantly producing TNF-α, IL-2 and IFN-γ and frequency of IL-2 producing cells in R10 was the highest.

As surface molecule expressions are related to cell maturation, activities and functions, this study thus explored several cell surface molecule expressions of anti-CD3/28 expanded CD4+ T cells which were divided into seven groups according to the molecules' functions and roles. The cell surface molecules included (i) chemokine receptors: CCR4, CCR5, CCR6, CCR7, CCR10, CXCR3, CXCR4 and CXCR5; (ii) adhesion molecules: CD11a, CD11b, CD11c, CD103 and $\alpha 4\beta 7$; (iii) co-stimulatory molecules: CD27, CD28, CD40, CD40L, CD134, PD-1 and ICOS; (iv) activation molecules: CD25, CD38, CD69, CD71 and HLA-DR; (v) maturation markers: CD45RO, CD45RA and

CD62L; (vi) cytokine receptors: CD126 and CD127; and (vii) other molecules: CD57, CD95, CD95L, and GITR. Changes in specific cell surface molecule expressions of the anti-CD3/28 CD4+ T cells were observed in this study. Our expanded cells also rendered a low frequency in CCR5 and twice as less CXCR4 expression than the unexpanded cells. Furthermore, our study shows that the expanded cells had a pretentious increase in $\alpha 4\beta 7$ expression suggesting that that these cells feasibly migrate to the site of depletion and improve immune response at the gut-associated lymphoid tissues.

The developed closed-culture system using culture bags and anti-CD3/28 coated beads can achieve a large number of expanded CD4+ T lymphocytes with good reproducibility, suggesting a promising protocol required for adoptive immunotherapy. Our expansion protocol using GMP-grade media, AIM-V and DC with HS supplementation rendered satisfied proliferation rates and yields without differences in polyfunctional patterns, indicating the promising method for further uses in clinical trials. Addition of FBS is also suggested for expansion improvement but under the condition that its source must be free from bovine spongiform encephalopathy. Moreover, specific surface molecule expressions of the expanded cells presented potential roles in proliferation, differentiation, homeostasis, apoptosis and organ-homing.

Introduction

Human immunodeficiency virus (HIV) infection causes a progressive decrease of CD4+ T lymphocytes and an increase of HIV viral load (or HIV RNA level), leading to higher susceptibility to opportunistic infections which can further develop to acquired immune deficiency syndrome (AIDS) [1]. HIV enters target cells through the binding of viral envelope glycoproteins to CD4 receptors along with CCR5 and CXCR4 co-receptors markedly expressed on the target CD4+ T lymphocytes [2-4]. Although highly active antiretroviral therapy (HAART) succeeds to control the HIV viral load into an undetectable level and recovers the CD4 counts in HIV-infected patients, the latent reservoir of virus still exists [5] and the immune restoration is incomplete [6-9]. A life-long treatment of HAART has also feasible consequences in cumulative drug toxicities, emergent drug-resistant viruses and unaffordable costs due to more complicated regimens. Moreover, some patients who have discordant immune responses (DIR) to HAART fail to achieve target CD4 count levels despite accomplished virological control, suggesting a higher risk in mortality [10].

An alternative approach, such as adoptive transfer of autologous activated CD4+ T lymphocytes, has been proposed to be a potential treatment for the benefit of both virological control and direct immune reconstitution. Its effectiveness and safety have been confirmed by in vivo studies in both simian deficiency virus (SIV)-infected rhesus macaques and HIV-infected patients [11-15]. To expand CD4+ T cells in vitro, anti-CD3/28 coated magnetic beads are widely used for stimulation. The anti-CD3/28 activated CD4+ T cells showed intrinsic resistance to macrophage (M)-tropic isolates of HIV-1 infection [16-18] and promoted expression of RANTES, MIP-1α and MIP-1β as well as reduced expression of CCR5 [11,13,14,16]. Furthermore, the expanded CD4+ T cells induced interferon (IFN)-γ production which is associated to type 1 T helper (Th1) cell function and increased the density of variable beta (Vβ) chain T cell receptor (TCR) repertoires [14] together with telomerase activity, resulting in a longer survival of the cells [11].

With respect to the clinical uses, a large number of CD4+ T cells expanded in vitro was required for reinfusion in HIV-infected patients [14,15]; therefore, optimization of expansion protocols is warranted. There have been established in vitro culture methods for anti-CD3/28 stimulated CD4+ T lymphocytes providing different yields [19-21] which can be related to different cell isolation methods, bead-to-cell ratios used for stimulation, and medium supplementation.

Regarding to the usage of culture media, basal culture media are generally supplemented with animal serum (e.g., fetal bovine serum (FBS)) which is essential for cell growth, metabolism and proliferation. The functions of serum in culture media are (i) to provide hormonal factors stimulating cell growth, proliferation and differentiation, (ii) to transport proteins carrying hormones, minerals, trace elements and lipids, (iii) to attach and spread factors, acting as germination points for cell attachment and (iv) to stabilize and detoxify factors needed for maintaining pH as well as to inhibit proteases and other toxic molecules. Nevertheless, the use of animal serum, particularly FBS, has been debated in some serious scientific and ethical concerns in terms of serum production and cell harvesting. Development of serum-free or animal/human protein-free media is then important for safety improvement in biological products for cell therapy and vaccination [22].

FBS can be substituted with defined chemical components in serum-free cell culture. Trickett et al. (2002) demonstrated that the expansion of functional T lymphocytes from HIV-infected patients was good when stimulated with anti-CD3/28 coated microspheres and propagated in serum-free media. However, the greater T cell proliferation was observed when supplemented with FBS in the initial period of cell expansion, whereas, human albumin (AB) serum supplementation failed to increase T cell numbers. Plasma supplementation also provided a low level of CD4+ T cells which was resulted from phenotypic switching of CD8+ T lymphocytes [23]. Carlens et al. (2000) studied expansion rates of anti-CD3 stimulated T lymphocytes in three different serum-free media (i.e., X-VIVO 15, AIM-V and Cellgro SCGM) compared to standard RPMI1640 media with 5% human serum (HS) and 10% FBS. A 3-week activation in serum-free media resulted in a small increase in expansion rates, whereas the culture with serum supplementation rendered better consistency and effectiveness in cell expansion. The additional low level of HS thus supported the T-cell expansion in all culture media types. On the other hands, supplementation with 10% FBS showed inconsistency in cell expansion when compared to that with 5% HS. Increased levels of IFN-γ secretion were detected for all media combinations when compared with serum-free culture. No IL-4 and IL-10 production was found [24].

Although the in vitro cultures with HS supplementation supported better CD4+ T cell expansion than those with serum-free media and more consistency than those with FBS supplementation, the fold expansion numbers (< 267-fold) [24] was not high when compared to our previous method [19]. While GMP-grade culture media are required for cell production, the information about the effect of serum supplementation in cell functions is limited.

More importantly, functional-associated phenotypic characters of the expanded cells are essential which are not only related to cell characterization but also maturation and activation stages as well as cell migration. Even so, there is limited information concerning specific cell surface molecule expressions of the expanded CD4+ T cells, such as chemokine receptors and maturation markers.

Objectives

1. To develop a closed-culture system for *in vitro* CD4+ T lymphocyte expansion by using a commercially available GMP-grade culture bag and anti-CD3/28 activation as well as a large-scale production aiming for adoptive immunotherapy.
2. To assess the expansion rates and yields of anti-CD3/28 expanded CD4+ T cells by using commercially available GMP-grade culture media with HS supplementation in comparison with the established protocol.
3. To investigate the expressions of surface molecule on anti-CD3/28 expanded cells including chemokine receptors, adhesion molecules, co-stimulatory molecules, activation molecules, maturation markers, cytokine receptors, and other functional-specific molecules.

Materials and Methods

1. Samples

Healthy volunteers and HIV-infected patients were recruited and signed informed consents which were approved by the Institutional Review Board of the Faculty of Medicine Siriraj Hospital at Mahidol University. Sixteen to twenty-five milliliters of whole blood were collected into sodium heparin-containing vacutainer tubes and kept at room temperature prior to phenotypic determination of whole blood and CD4+ T lymphocyte isolation. Cryopreserved CD4+ T lymphocytes were obtained from HIV-infected patients with CD4+ T cell count over 500 cells/μL and stored in a liquid nitrogen tank at -196 °C for 1.5 - 2 years.

2. Antibodies

Monoclonal antibodies (mAbs) and their conjugated fluorochromes including anti-CD3 conjugated with fluorescein isothiocyanate (FITC), anti-CD4 conjugated with phycoerythrin (PE), anti-CD8 conjugated with PE, anti-CD19 conjugated with PE, anti-CD4 conjugated with allophycocyanin (APC), anti-CD16 conjugated with APC, anti-CD56 conjugated with APC, anti-CD19 conjugated with allophycocyanin and cyanine dye (APC-Cy7), anti-CD45 conjugated with peridinin chlorophyll protein (PerCP), and anti-CD56 conjugated with phycoerythrin and cyanine dye (PE-Cy7) were purchased from Becton Dickinson Bioscience (BDB, San Jose, CA). In addition, anti-CD3 conjugated with AlexaFluor® (A700), anti-CD4 conjugated with Brilliant Violet™ 605 (BV605), anti-CD8 conjugated with PE/Dazzle™ 594, anti-CD69 conjugated with PerCP/Cy5.5, anti-IL-2 conjugated with BV510, anti-IL-4 conjugated with FITC, anti-IL-17 conjugated with PE, anti-IFN-γ conjugated with APC, anti-TNF-α conjugated with BV650 and anti-TGF-β conjugated with BV421 were obtained from BioLegend (San Diego, CA). The fluorescent-labeled mAbs used for identification of specific surface molecule expression were anti-CD4 PerCP, anti-CD3 FITC, anti-CD45RO FITC, anti-CD45RA FITC, anti-CD57 FITC, anti-CD27 FITC, anti-CCR7 PE, anti-CD62L PE, anti-CD11a PE, anti-CD11b PE, anti-CD11c PE, anti-CD126 PE, anti-CD127 PE, anti-CD95 PE, anti-CD95L PE, anti-CD154 (CD40L) PE, anti-CD40 PE, anti-CD134 (OX40) PE, anti-CD278 (ICOS) PE, anti-CD71 PE, anti-HLA-DR PE, anti-GITR PE, anti-CD28 PE, anti-CD103 PE, anti-CD38 PE, anti-CD69 PE, anti-CD25 PE, anti-CD184 (CXCR4) PE, anti-CD183 (CXCR3) PE, anti-CCR10 PE, anti-CD195 (CCR5) PE, anti-PD-1 PE, anti-CXCR5 PE, anti-CCR6 PE, anti-CCR4 PE and anti- $\alpha 4\beta 7$ PE.

3. CD4+ T lymphocyte isolation

CD4+ T lymphocytes can be directly isolated from whole blood by an immunorosettes formation method using RosetteSep® human CD4+ T cell enrichment cocktail (STEMCELL Technologies, Vancouver, BC, Canada). Briefly, CD4+ T lymphocytes were isolated from 5 mL of whole blood by adding 250 μL of RosetteSep® human CD4+ T cell enrichment cocktail. After that, the samples were thoroughly mixed and incubated at room temperature for 20 minutes. The samples were then diluted with an equal volume of phosphate buffered saline (PBS) containing 2% fetal bovine serum (FBS) and gently mixed. The diluted blood samples were carefully layered on top of LSM® lymphocyte separation medium and centrifuged at 1200g with no break at room temperature for 20 minutes. After centrifugal separation, the samples were divided into four layers including

plasma, enriched CD4+ T cells, LSM® lymphocyte separation medium and red blood cells (from top to bottom). Pasteur pipettes were used to remove the plasma layer and collect enriched CD4+ T cells from the layer interface. The collected CD4+ T cells were then washed with 10 mL of PBS containing 2% FBS and centrifuged twice at 1400 rpm at room temperature for 5 minutes. The cell pellets were collected and re-suspended with a complete medium (RPMI1640 with 10% FBS, 50 µg/mL pencillin-streptomycin and 2 mM L-glutamine). Cell number and viability of the enriched CD4+ T cells were determined by trypan blue exclusion using a hemacytometer.

4. Cell stimulation and expansion for freshly isolated CD4+ T cells

Freshly purified CD4+ T cells of $1x10^6$ cells were stimulated with anti-CD3/28 coated beads (Dynabeads® Human T-Activator CD3/CD28, Invitrogen Dynal, Oslo, Norway) at a bead-to-cell ratio of 1:1. The stimulated CD4+ T cells were then expanded in complete media (RPMI1640 with 10% fetal bovine serum (FBS)), 50 µg/mL pencillin-streptomycin and 2 mM L-glutamine). The expanded cells were incubated at 37°C and 5% CO2 humidification and reactivated on day 7. The cells were expanded for a 3-week culture period. Cell numbers and viability were observed by using trypan blue exclusion and a TC10™ automated cell counter. Phenotypic characters were analyzed by flow cytometry on days 14 and 21.

For an expansion method of flask culture, the stimulated cells of $1x10^6$ cells were placed in a 24-well plate (Costar® 24 well clear TC-treated multiple well plates, sterile, Corning Inc., Life Sciences, NY, USA) on day 0 and expanded at a concentration of 0.5x106 cells/mL before transferring to T25, T75 and T175 plastic tissue culture flasks (Corning® U-shaped canted neck cell culture flask with vent cap, Corning Inc., Life Sciences, NY, USA) on days 4, 7, and 11, respectively. Cell numbers and viability were observed on days 4, 7, 11, 14, 17 and 21 while the media were replenished with calculated amounts of fresh media on days 4, 7, 11, 14, and 17 to maintain the cell suspension concentration at $0.5x10^6$ cells/mL.

With respect to bag culture, the expansion process was similar to flask culture during the first week of cell expansion. The expanded cells were re-stimulated in T25 flasks on day 7 and replenished with calculated amounts of fresh media at a concentration of $0.5x10^6$ cells/mL before transferring to a GMP-grade culture bag (Vuelife® cell culture bags, CellGenix, Freiburg, Germany) with a size of 72c (maximum volume of 72 mL). The culture bag was clamped by half and placed on a steel grating culture stage prior to cell transfer. Fresh media were added to reach maximum volume of the bag (72 mL) on day 11. After that, the expanded cells were transferred to another culture bag with a size of 197c on day 14, filled with media up to 197 mL on day 17, and leaved for growth until day 21. Cell numbers and viability were observed on days 4, 7, 14, and 21.

Large-scale production of freshly isolated CD4+ T cells in a closed-culture system was also perform. Freshly purified CD4+ T cells of $8x10^6$ cells from healthy donors were mixed with anti-CD3/28 coated beads (Dynabeads® Human T-Activator CD3/CD28, Invitrogen Dynal, Oslo, Norway) at a bead-to-cell ratio of 1:1 in a plastic tube (Falcon® high clarity polypropylene centrifuge tube, conical bottom, sterile, Corning Inc., Life Sciences, NY, USA) before injecting into a GMP-grade culture bag (Vuelife® cell culture bags, CellGenix, Freiburg, Germany) with a size of 32c. Complete

media (RPMI1640 with 10% fetal bovine serum, 50 µg/mL pencillin-streptomycin and 2 mM L-glutamine) were added in order to achieve a concentration of $0.5x10^6$ cells/mL. The expanded cells were incubated at 37°C and 5% CO2 humidification. Only $20x10^6$ cells of anti-CD3/28 expanded CD4+ T cells were reactivated on day 7. The expanded cells were consequently transferred to larger culture bags with sizes of 72c and 196c on days 7 and 14. Fresh culture media were added to reach suggested maximum volume of individual bag size on days 4, 11 and 17. Cell numbers and viability were observed on days 7, 14 and 21 by using trypan blue exclusion and a TC10™ automated cell counter. Phenotypic characters were analyzed by flow cytometry on days 14 and 21.

For patient's cell expansion, cryopreserved CD4+ T lymphocytes from HIV-infected patients were removed from a liquid nitrogen tank at -196 °C and then thawed in a sterile water bath at 37°C. Cryopreserved cells of $1x10^6$ cells were activated and expanded following the developed bag culture protocol of freshly isolated CD4+ T cells. In addition, fresh media with IL-2 supplementation at a concentration of 100 U/mL was used on day 7 onwards. Cell numbers and viability were observed on days 4, 7, 14 and 21 by using trypan blue exclusion and a TC10™ automated cell counter. Phenotypic characters were analyzed by flow cytometry on days 14 and 21.

For comparison between culture medium and human serum (HS) supplementation, $1x10^6$ cells of purified CD4+ T cells were expanded in 4 different culture conditions including (i) RPMI1640 supplemented with 10% FBS (Gibco, Paisley, UK), (ii) RPMI1640 supplemented with 10% heat inactivated HS (Gibco, Paisley, UK), (iii) AIM-V® medium CTS™ (Therapeutic grade, Gibco, Paisley, UK) supplemented with 10% heat inactivated HS and (iv) CellGro® GMP serum-free dendritic cell medium (CellGro® DC medium, CellGenix GMBH, Freiburg, Germany) supplemented with 10% heat inactivated HS. Cell expansions were conducted by using the same protocol for flask culture described above.

For bead-to-cell ratio comparison, $1x10^6$ enriched CD4+ T cells were stimulated with anti-CD3/CD28 coated beads in the absence of exogenous interleukin (IL)-2. The bead number was calculated for 0.5:1, 1:1, and 2:1 bead-to-cell ratios to use for the expansion. Cell expansions were conducted by using the same protocol for flask culture described above.

With respect to IL-2 supplementation comparison, the similar activation and culture protocols were conducted by using only the 1:1 bead-to-cell ratio and cultures in the absence and in the presence of exogenous interleukin (IL)-2 at the low concentration of 20 units/mL (Prospec, Ness-Ziona, Israel). Cell numbers and viability were observed on days 4, 7, 11, 14, 17 and 21 by using trypan blue exclusion and a hematocytometer. Lymphocyte subset characters were analyzed by a flow cytometer on days 0, 14 and 21.

5. Immunofluorescent staining and flow cytometric analysis

Whole blood, purified and expanded CD4+ T cells were stained with fluorochrome-conjugated mAbs and incubated for 15 minutes before adding 1X lysing solution for red blood cell lysis. The stained cells were then washed with PBS containing 2% FBS prior to centrifugation at 1,400 rpm at 25 °C for 5 minutes. Subsequently, the stained cells were re-suspended in PBS containing 1% paraformaldehyde. The stained cells were finally acquired by a BD FACSCalibur flow

cytometer or BD FACSVerse™ flow cytometer (BDB, San Jose, CA) and the data were analyzed by using FlowJo Software (Tree Star, San Carlos, CA).

Six-parameter analysis including forward scatter (FSC), side scatter (SSC), FITC, PE, PerCP, and APC was performed using FlowJo Software (Tree Star, San Carlos, CA). The stained cells were gated using lymphogate (FSC/SSC) to determine a viable lymphocyte population. After that, lymphocyte subsets were defined using two-dimensional dot plots between CD45/SSC, CD45/CD3 and CD4/CD8 or CD19/CD16+CD56. Therefore, the lymphocyte subsets were detected into CD4+CD8-, CD4-CD8+, CD4+CD8+, CD19-CD16+CD56+ and CD19+CD16-CD56- populations. The frequencies of anti-CD3/28 expanded CD4+ T Cells that express specific surface molecules were also determined on a population of expanded cells.

6. Cell stimulation, intracellular cytokine staining (ICS) and analysis

Cryopreserved expanded CD4+ T cells from different culture conditions were used to determine different types of cytokine production. Expanded CD4+ T cells at $1x10^6$ cells/mL were stimulated with 25 ng phorbol 12-myristate 13-acetate (PMA) and 1 µg ionomycin (I) in the presence of brefeldin A (BFA) at 10 µg, whereas the expanded CD4+ T cells in the presence of BFA without stimulation were used as a control. The samples were then incubated at 37 °C and 5% CO2 for 4 h. After the incubation, PMA/I stimulated and unstimulated samples were stained with Zombie NIR™ dye (BioLegend, San Diego, CA) at 4 °C for 15 min. A washing buffer (phosphate buffered saline (PBS) with 2% FBS) were added and the samples were washed by centrifugation at 450g for 5 min. The samples were then surface stained with a combination of mAbs including anti-CD3 A700, anti-CD4 BV605, anti-CD8 PE/Dazzle™ 594 and anti-CD69 PerCP/Cy5.5 at 4 °C for 15 min and washed once. The stained samples were fixed and permeabilized in 0.5 mL of BD Cytofix/Cytoperm™ fixation and permeabilization solution (BDB, San Jose, CA) at 4 °C for 20 min. After the incubation, the samples were washed by adding 1X BD Perm/Wash™ buffer (BDB, San Jose, CA) and centrifuged at 500g for 5 min. ICS was performed by staining with a combination of mAbs including anti-IL-2 BV510, anti-IL-4 FITC, anti-IL-17 PE, anti-IFN-γ APC, anti-TNF-α BV650, anti-TGF-β BV421 at 4 °C for 30 min. After staining, the samples were washed with 1X BD Perm/Wash™ buffer (BDB, San Jose, CA) and re-suspended in PBS.

The stained cells of at least 100,000 events were acquired for each analysis by a BD Fortessa™ flow cytometer (BDB, San Jose, CA) and the data was analyzed by using FlowJo Software (Tree Star, San Carlos, CA). Cytokine producing cell subsets were determined from activated populations expressing CD69 and percentages of cytokine producing cell subsets were determined from a total cytokine producing cell population. A Boolean gating strategy was used for the analysis of polycytokine producing cell subsets in order to evaluate cytokine producing cell subsets with ability to simultaneously produce 1, 2, 3, 4, 5 or 6 cytokines.

7. Cell harvesting and quality control

Cell suspensions containing expanded CD4+ T cells from a bag culture of cryopreserved CD4+ T cells from HIV-infected patients were collected on day 21 of cell expansion. Aliquots of cell suspensions were transferred to 50-mL tubes and centrifuged at 450g for 5 min.

Culture supernatants were removed, collected in small aliquots and stored in a -80 $^{\circ}$C freezer prior to thaw for using in quality control. Cell pellets were re-suspended in 10 mL of complete media and transferred to 15-mL tubes.

To remove anti-CD3/28 coated beads, each 15-mL tube was placed in a DynaMag™-15 Magnet (Invitrogen Dynal) for 2 min and the cell suspension was transferred to another 15-mL tube. The same procedure was repeated once and aliquots of cell suspensions were pooled. The centrifugation process was repeated and media was removed. Cell pellets were re-suspended in complete media and pooled into a single tube at a volume of 20 mL. Cell numbers and viability were observed by using trypan blue exclusion and a TC10™ automated cell counter. Aliquots of the expanded CD4+ T cells at $1x10^7$ cells/mL were cryopreserved in RPMI-1640 media containing 20% FBS with 10% DMSO and stored at 180 $^{\circ}$C prior to thaw for using in quality control and intracellular cytokine staining.

For quality control, frozen expanded CD4+ T cells were thawed and re-suspended in PBS containing 5% human albumin. To detect residual bead contamination, samples containing $1x10^7$ cells were mixed with sodium hypochlorite solution and centrifuged at 450g for 5 min. Supernatants were removed and the pellets were re-suspended with PBS. Residual bead counts were determined on a hemacytometer and the total numbers of beads were calculated. For sterility testing, a 14-days United States Pharmacopoeia (USP) sterility testing on aliquots of thawed cells and culture supernatants was performed at Siriraj Hospital Microbiology Laboratory by using fluid thioglycollate medium (for the detection of anaerobic bacteria) and soybean-casein digest medium (for the detection of aerobic bacteria and fungi). Sterile results were identified as no growth of microorganism.

8. Data analysis

Fold expansion number was calculated by using the viable cell number at each indicated time point divided by the viable cell number at the beginning of cell expansion. For a large-scale production experiment, fold expansion numbers were calculated to get an approximated number if a total number of expanded cells on day 7 were expanded until day 21. Fold expansion, cell viability and frequencies of T lymphocytes and their subsets were shown as mean ± SD (standard deviation). Statistical differences of mean values of fold expansion and cell viability of expanded CD4+ T lymphocytes were analyzed by paired t-tests or 2-way ANOVA followed by Bonferroni's multiple comparisons test. Statistical significance was considered when p-value was below 0.05. For cytokine producing cell subset analysis, the percentages of specific-cytokine producing cell subsets were shown as mean ± SD. The proportions of cytokine producing cell subsets were presented as pie-charts. All data were analyzed by using Prism software (GraphPad, La Jolla, CA).

Results

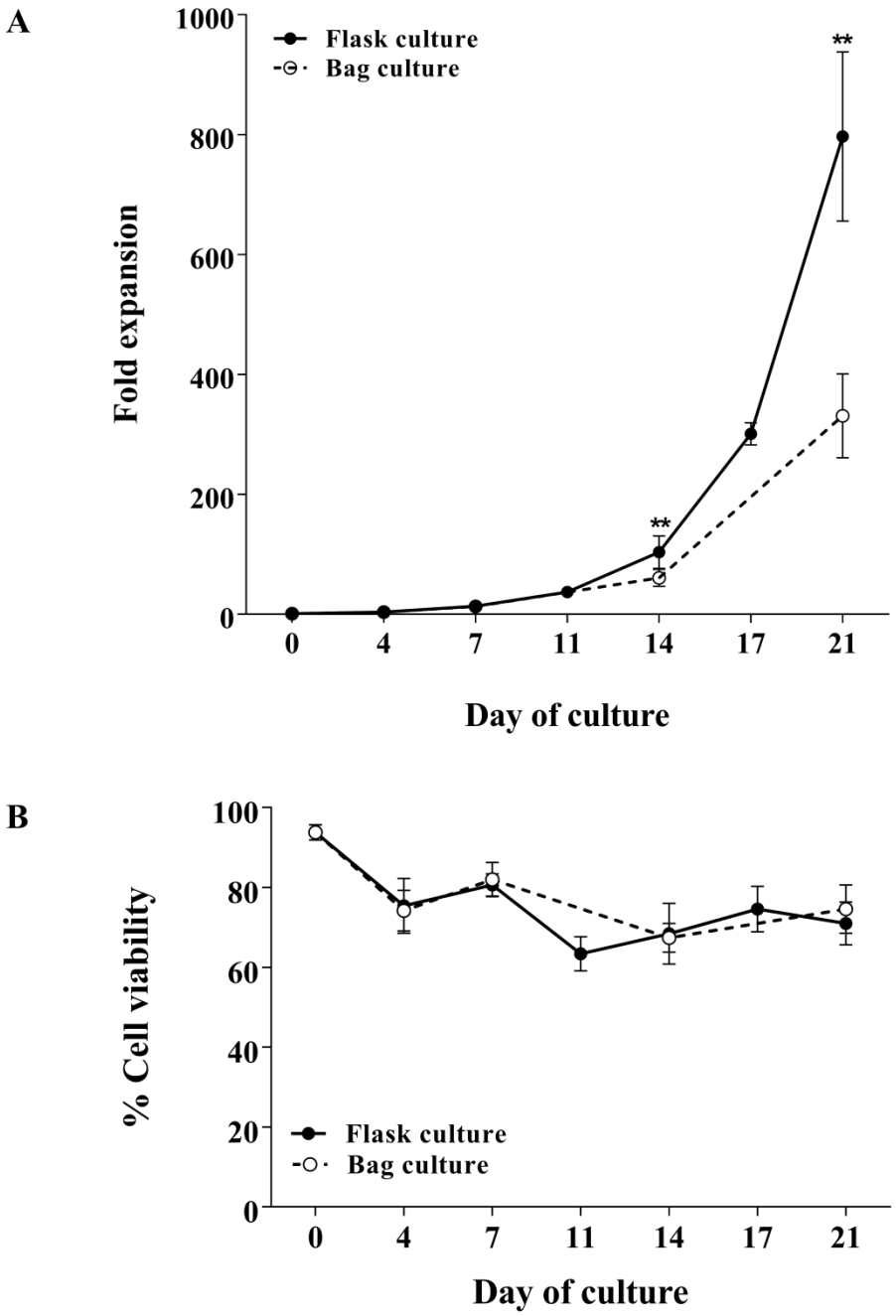
1. Expanded CD4+ T lymphocytes in small-scale plastic flasks and GMP-grade culture bags after anti-CD3/28 coated bead stimulation

To develop a closed-culture system for CD4+ T lymphocytes, GMP-grade culture bags were used for cell expansion and compared to conventional plastic flasks. Freshly isolated CD4+ T cells were obtained from five healthy volunteers for the study. Fold expansion, cell viability, growth kinetic and phenotypic characters were observed for proliferation efficiency of expanded CD4+ T cells in flasks and bags.

Fold expansion numbers of CD4+ T lymphocytes expanded in both flasks and bags were similar at the beginning and started dramatically increasing on day 14 as shown in Fig. 1A. A flask culture exhibited significantly higher fold expansion than a bag culture (103.8 ± 27.2 and 60.9 ± 14.4, respectively; p-value = 0.0075). At the end of the culture period, CD4+ T lymphocytes expanded in flasks were proliferated better than those expanded in bags (796.7 ± 141.2 –fold and 330.9 ± 70.0 –fold, respectively; p-value = 0.0078).

Cell viability of anti-CD3/28 expanded CD4+ T cells from both flask and bag cultures were similar throughout a 3-week expansion period (Fig. 1B). There were slightly decreases of viable cells from day 0 to day 21 (93.8 ± 1.9% to 71.0 ± 5.4% in flasks and 93.8 ± 1.9% to 74.6 ± 6.1% in bags) and no significant difference between the two different culture methods.

Phenotypes of whole blood, isolated CD4+ T lymphocytes, and anti-CD3/28 expanded CD4+ T lymphocytes were determined by a flow cytometer (Fig. 2). As shown in Table 1, phenotypic profiles showed that the immunorosette formation technique was effective and reproducible for CD4+ T cell isolation from whole blood with high frequency of CD3+CD4+CD8- subset (91% of lymphocytes). After a 3-week culture, anti-CD3/28 expanded CD4+ T cells (CD3+CD4+CD8-) in either flasks or bags exhibited high frequencies (> 97% of lymphocytes) on days 14 and 21. The cell purity was also confirmed with low frequencies of CD3+CD4-CD8+ and CD3+CD4+CD8+ populations (<0.3% and < 2%, respectively) throughout the expansion period.



** = p-value < 0.01

Fig. 1 Growth kinetics: (A) fold expansion, (B) cell viability of anti-CD3/28 expanded CD4+ T cells of freshly isolated CD4+ T cells from healthy volunteers expanded in flasks and bags (n = 5).

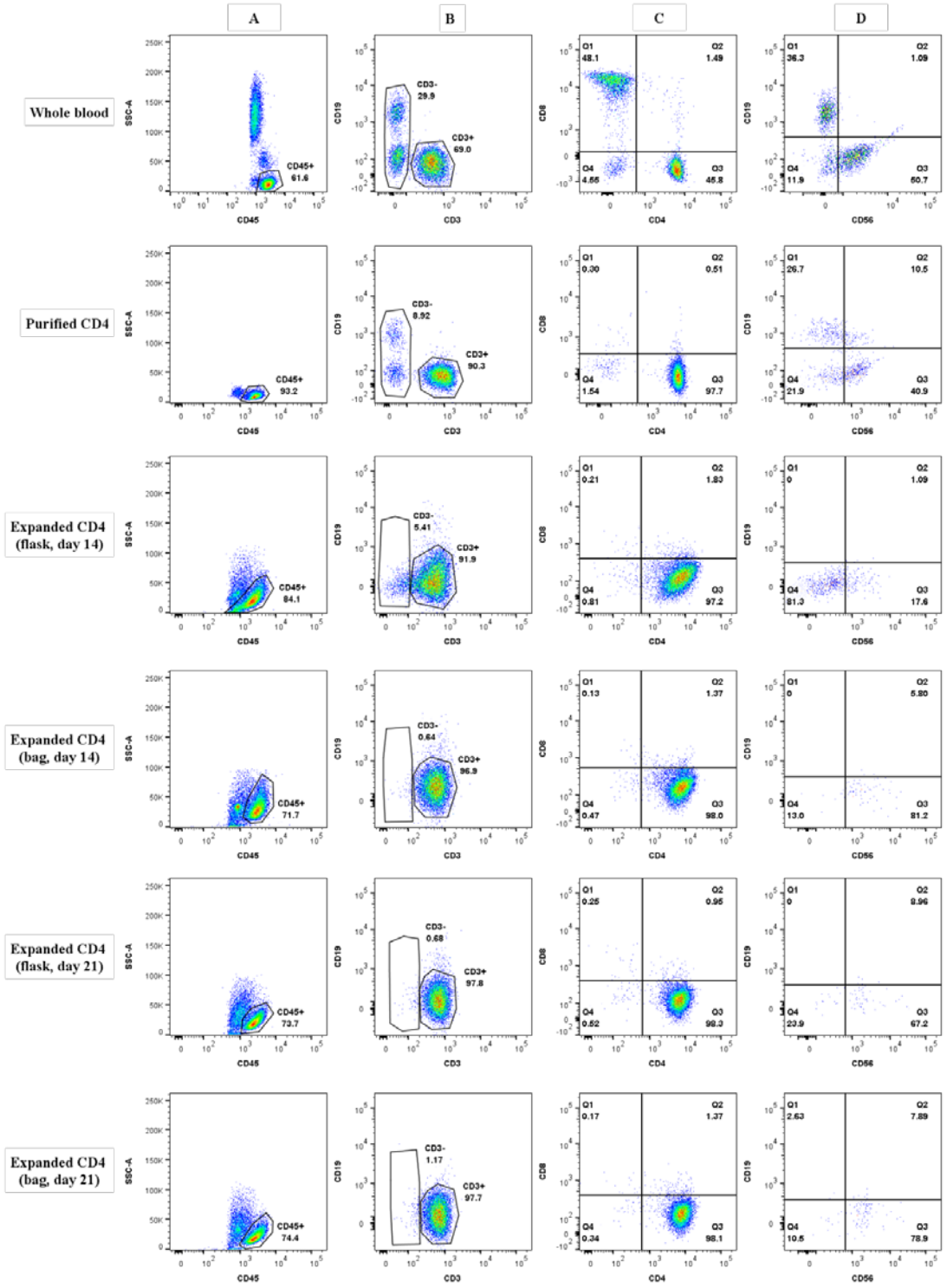


Fig. 2 Phenotype characterization of whole blood, purified CD4+ T cells, expanded CD4+ T cells on days 14 and 21 in flask and bag cultures. Gating identified (A) lymphocytes among leukocyte population, (B) CD3+ and CD3- cells among lymphocyte population, (C) CD4+ and CD8+ T cells among CD3+ cells, and (D) CD19+ B cells and CD56+ NK cells among CD3- cells.

Phenotypes	Whole blood	Isolated $CD4^+$ T cells	Anti-CD3/28 expanded $CD4^+$ T cells[a]			
	Day 0	Day 0	Day 14[b]		Day 21	
			Flasks	Bags	Flasks	Bags
% of lymphocytes						
$CD3^+CD4^-CD8^-$	4.4 ± 3.2	2.2 ± 1.3	0.8 ± 0.5	0.7 ± 0.6	0.8 ± 0.9	0.7 ± 0.4
$CD3^+CD4^+CD8^-$	32.6 ± 4.3	91.0 ± 4.8	98.2 ± 0.4	97.8 ± 1.3	98.3 ± 1.2	97.4 ± 1.5
$CD3^+CD4^-CD8^+$	36.0 ± 2.0	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	0.3 ± 0.3	0.2 ± 0.1
$CD3^+CD4^+CD8^+$	0.5 ± 0.4	2.2 ± 1.3	0.8 ± 0.5	1.4 ± 1.4	0.6 ± 0.5	1.0 ± 0.4
$CD3^-CD19^+CD56^-$	10.9 ± 3.5	1.4 ± 1.6	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^+$	13.5 ± 2.5	0.8 ± 0.8	N/A	N/A	N/A	N/A
$CD3^-CD19^+CD56^+$	0.2 ± 0.3	0.1 ± 0.0	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^-$	2.0 ± 1.4	4.2 ± 3.8	N/A	N/A	N/A	N/A
% of $CD3^+$ T cells						
$CD3^+CD4^-CD8^-$	5.9 ± 4.1	2.3 ± 1.4	N/A	N/A	N/A	N/A
$CD3^+CD4^+CD8^-$	43.9 ± 4.1	97.3 ± 1.3	N/A	N/A	N/A	N/A
$CD3^+CD4^-CD8^+$	49.5 ± 4.1	0.1 ± 0.0	N/A	N/A	N/A	N/A
$CD3^+CD4^+CD8^+$	0.7 ± 0.7	0.3 ± 0.2	N/A	N/A	N/A	N/A
% of $CD3^-$ T cells						
$CD3^-CD19^+CD56^-$	42.3 ± 7.8	24.0 ± 25.9	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^+$	51.1 ± 7.3	10.2 ± 8.5	N/A	N/A	N/A	N/A
$CD3^-CD19^+CD56^+$	0.8 ± 1.2	0.8 ± 0.4	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^-$	7.2 ± 4.9	65.0 ± 27.1	N/A	N/A	N/A	N/A

N/A = Not available; b = 4 subjects (data from one donor was ruled out due to machine error); a = gating of CD45+ lymphocytes was 100% of CD45+CD3+ T cells.

Table 1 Phenotypes of whole blood, isolated CD4+ T cells, and anti-CD3/28 expanded CD4+ T cells from healthy volunteers (mean ± SD, n = 5).

2. Large-scale expansion of CD4+ T lymphocytes in a closed culture system

As a high number of purified CD4+ T lymphocytes are demanded for cell transfusion, a large-scale production of freshly isolated CD4+ T cells from five healthy donors in a closed-culture system was developed in this study. Fold expansion, cell viability, growth kinetic and phenotypic characters were observed for large-scale proliferation.

A large-scale production of CD4+ T cells exhibited a gradual increase of fold expansion from day 0 to day 14 (1.0 ± 0.0 on day 0, 5.5 ± 1.0 on day 7, and 20.4 ± 3.4 on day 14) and a marked increase from day 14 to day 21 (109.1 ± 18.4 on day 21). Growth kinetic of this large-scale

expansion in culture bags also had a similar pattern with that of a small-scale bag culture prescribed earlier. Cell viability of large-scale anti-CD3/28 expanded CD4+ T cells was good with a slight decrease from 91.2 ± 2.6% on day 0 to 77.4 ± 6.3% on day 21.

Again, phenotypic characterization of whole blood and isolated CD4+ T cells was performed for purity confirmation of isolated CD4+ T cells before culture as presented in Table 2. Anti-CD3/28 expanded CD4+ T lymphocytes from a large-scale production showed high frequency of CD3+CD4+CD8- population (> 98%) on days 14 and 21. Low numbers of CD3+CD4-CD8+ and CD3+CD4+CD8+ populations (<0.8% and < 0.5%, respectively) were detected.

Phenotypes	**Whole blood**	**Isolated $CD4^+$ T cells**	**Anti-CD3/28 expanded $CD4^+$ T cells***	
	Day 0	**Day 0**	**Day 14**	**Day 21**
% of lymphocytes				
$CD3^+CD4^-CD8^-$	4.7 ± 1.5	1.3 ± 0.4	0.3 ± 0.1	0.8 ± 0.3
$CD3^+CD4^+CD8^-$	37.5 ± 7.2	97.5 ± 1.2	99.1 ± 0.3	98.0 ± 0.6
$CD3^+CD4^-CD8^+$	28.7 ± 6.7	0.0 ± 0.0	0.1 ± 0.1	0.8 ± 0.4
$CD3^+CD4^+CD8^+$	0.9 ± 0.3	0.1 ± 0.1	0.5 ± 0.3	0.3 ± 0.5
$CD3^-CD19^+CD56^-$	14.4 ± 5.9	0.2 ± 0.2	N/A	N/A
$CD3^-CD19^-CD56^+$	11.4 ± 3.5	0.2 ± 0.1	N/A	N/A
$CD3^-CD19^+CD56^+$	0.3 ± 0.2	0.0 ± 0.0	N/A	N/A
$CD3^-CD19^-CD56^-$	2.0 ± 1.1	0.7 ± 0.7	N/A	N/A
% of $CD3^+$ T cells				
$CD3^+CD4^-CD8^-$	6.8 ± 2.1	1.3 ± 0.4	N/A	N/A
$CD3^+CD4^+CD8^-$	52.1 ± 9.4	98.6 ± 0.5	N/A	N/A
$CD3^+CD4^-CD8^+$	39.9 ± 8.7	0.0 ± 0.0	N/A	N/A
$CD3^+CD4^+CD8^+$	1.2 ± 0.6	0.1 ± 0.1	N/A	N/A
% of $CD3^-$ T cells				
$CD3^-CD19^+CD56^-$	41.0 ± 11.1	20.6 ± 17.6	N/A	N/A
$CD3^-CD19^-CD56^+$	51.2 ± 10.8	17.0 ± 12.7	N/A	N/A
$CD3^-CD19^+CD56^+$	1.0 ± 0.5	1.3 ± 1.9	N/A	N/A
$CD3^-CD19^-CD56^-$	6.8 ± 2.0	61.1 ± 18.8	N/A	N/A

N/A = Not available; * = gating of CD45+ lymphocytes was 100% of CD45+CD3+ T cells.

Table 2 Phenotypes of whole blood, isolated CD4+ T cells, and anti-CD3/28 expanded CD4+ T cells from healthy volunteers expanded in culture bags for large-scale production (mean ± SD, n = 5).

3. Bag culture of anti-CD3/28 expanded CD4+ T cells from HIV-infected patients

Using freshly isolated CD4+ T cells from HIV-infected patients for cell expansion may possibly be inconvenient; therefore, frozen cells become alternative source and more practical for expansion. Cryopreserved CD4+ T lymphocytes from five healthy donors were able to expand in

bags with a similar fold expansion when compared to freshly isolated CD4+ T lymphocytes (data not shown). Cryopreserved CD4+ T lymphocytes from HIV-infected patients were then expanded in the developed close-culture system.

Anti-CD3/28 expanded CD4+ T cells from frozen cells showed the same trend and similar numbers of fold expansion as those from freshly isolated cells (1.0 ± 0.0 on day 0, 3.4 ± 0.4 on day 4, 13.6 ± 3.5 on day 7, 63.6 ± 16.2 on day 14 and 414.9 ± 67.6 on day 21). Cell viability of cryopreserved cells was quite high and remained stable until the end of expansion (76.7 ± 5.8% on day 0 to 85.0 ± 1.7% on day 21).

Purity of isolated CD4+ T cells from cryopreservation was ensued by phenotypic characterization (Table 3). CD3+CD4+CD8- population was detected to be dominant for anti- CD3/28 expanded CD4+ T cells throughout the expansion period (> 98%). This was confirmed by minor populations of CD3+CD4-CD8+ and CD3+CD4+CD8+ T cells (< 0.8% and <0.5%, respectively).

Phenotypes	**Cryopreserved $CD4^+$ T cells**	**Anti-CD3/28 expanded $CD4^+$ T cells***	
	Day 0	**Day 14**	**Day 21**
% of lymphocytes			
$CD3^+CD4^-CD8^-$	2.2 ± 1.1	4.7 ± 2.5	2.5 ± 2.3
$CD3^+CD4^+CD8^-$	93.6 ± 6.6	94.9 ± 2.3	95.9 ± 2.8
$CD3^+CD4^-CD8^+$	0.0 ± 0.0	0.1 ± 0.0	0.5 ± 0.5
$CD3^+CD4^+CD8^+$	0.0 ± 0.0	0.3 ± 0.2	1.1 ± 0.2
$CD3^-CD19^+CD56^-$	0.4 ± 0.5	N/A	N/A
$CD3^-CD19^-CD56^+$	0.5 ± 0.9	N/A	N/A
$CD3^-CD19^+CD56^+$	0.0 ± 0.0	N/A	N/A
$CD3^-CD19^-CD56^-$	3.1 ± 4.3	N/A	N/A

N/A = Not available; * = gating of CD45+ lymphocytes was 100% of CD45+CD3+ T cells.

Table 3 Phenotypes of cryopreserved CD4+ T cells, and anti-CD3/28 expanded CD4+ T cells from cryopreserved HIV-infected samples (CD4 counts ≥ 500 cells/μL) expanded in bags with IL-2 supplementation (mean ± SD, n = 3).

4. Determination of cytokines produced by anti-CD3/28 expanded CD4+ T cells from bag culture of cryopreserved CD4+ T cells

Although cell expansion by using anti-CD3/28 coated beads showed a homogenous population of expanded CD4+ T lymphocytes, the obtained phenotypic character were not able to provide information on a variety of expanded cells in term of function. Since CD4+ T lymphocytes can be divided into subpopulations including T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17), and regulatory T cells (Treg) based on types of cytokine produced by these cells, anti-CD3/28

expanded CD4+ T cells from a bag culture of cryopreserved CD4+ T cells from HIV-infected patients were investigated for their T helper cell subset identity.

CD69 expression was used to determine the level of activated cells after stimulation with PMA/I which is a polyclonal activator. The average percentage of CD69 expressing cells among anti-CD3/28 expanded CD4+ T cells was 95.4 ± 1.4%. Of these activated cells, the average percentage of 76.2 ± 5.0% was cytokine producing cells. Different types of cytokine producing cells including TNF-α, IL-2, IFN-γ, IL-4, IL-17 and TGF-β are shown in Fig. 3A. The major population among total cytokine producing cells was TNF-α producing cells with the average frequency of 81.4 ± 2.5%, followed by IL-2 producing cells (67.3 ± 15.1%). Determination of Th1 cells based on IFN-γ production showed the average percentage of 29.7 ± 11.9%. Low levels of Th2 and Th17 cells were detected based on the production of IL-4 and IL-17 (1.5 ± 0.9% and 1.2 ± 1.2%, respectively). On the contrary, the frequency of Treg cells based on TGF-β production was almost undetectable. When cytokine producing cell populations showing simultaneous production were determined, up to 99% of cytokine producing cells belongs to 3 major populations including single, double and triple cytokine producing cells with the average percentages of 34.3 ± 5.8%, 50.6 ± 3.9% and 14.5 ± 7.4%, respectively (Fig. 3B).

Proportions of specific-cytokine producing cell subsets determined among single, double and triple cytokine producing cells are presented in Fig. 4. For single cytokine producing cells, the majority was TNF-α producing cell with the average percentage of 51.1 ± 12.6%, followed by IL-2 and IFN-γ producing cells (32.8 ± 13.8% and 15.2 ± 4.3%, respectively). With respect of double cytokine producing cells, the highest frequency was observed for TNF-α+ IL-2+ producing cells with the average percentage of 77.7 ± 14.7%, followed by the other 2 main populations including TNF-α+ IFN-γ + (16.8 ± 14.4%) and IFN-γ + IL-2+ (4.1 ± 3.0%). For triple cytokine producing cells, TNF-α+ IL-2+ IFN-γ + producing cells showed the highest frequency with the average percentage of 89.2 ± 7.4%, followed by TNF-α+ IL-2+ IL-4+ (4.6 ± 3.7%), and TNF-α+ IL-2+ IL-17+ (2.4 ± 1.8%).

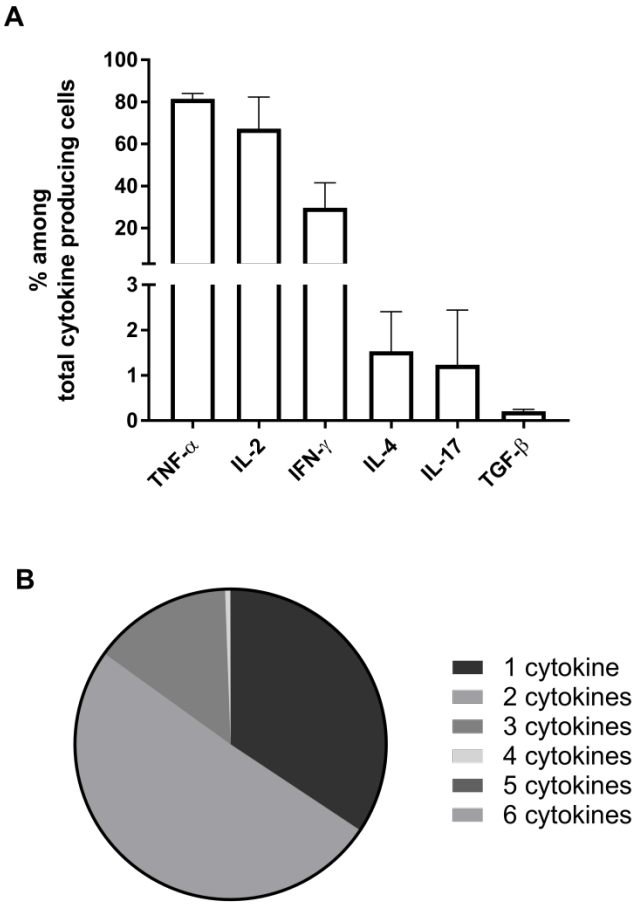


Fig. 3 Cytokine production profiles including TNF-α, IL-2, IFN-γ, IL-4, IL-17, and TGF-β of anti-CD3/28 expanded CD4+ T cells: (A) percentages (mean ± SD) of different cytokine producing cells; (B) average proportions of polyfunctional cytokine producing cells (n = 3).

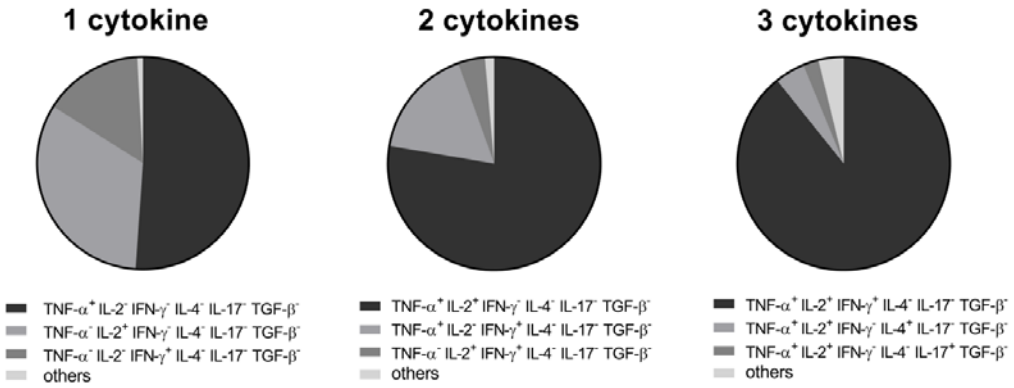


Fig. 4 Average proportions of polyfunctional cytokine producing cell subsets in single, double and triple cytokine producing cells (n = 3).

5. Residual bead detection and sterility testing

To ensure safety and sterility of the final products due to magnetic beads and microorganism contamination, a small-scale bead removal process was performed together with sterility testing on the final cell culture supernatants (day 21) and harvested expanded CD4+ T cells. Since a large volume of culture medium containing expanded CD4+ T cells was harvested, the sample was concentrated by centrifugation before bead removal. By using a small scale depletion method, the average number of $5.5x10^4$ residual beads per 10^7 expanded cell products was measured. Since the average number of $414.9x10^6$ cells was harvested on day 21, the average number of $2.3x10^6$ residual beads may be obtained in the final products. While the average number of beads before harvesting was $13.6x10^6$ beads (average cell number for reactivation on day 7), the result showed 83% depletion of beads. For sterility testing, a 14-day incubation period in specialized media was performed according to USP. The sterile results were presented as the culture supernatant colleting on the final day of cell expansion and expanded cells from cryopreservation were free from any bacterial and fungal contaminations.

6. Expanded CD4+ T lymphocytes in different culture conditions after anti-CD3/28 coated bead stimulation

To assess an optimal cell culture condition for in vitro expansion of CD4+ T lymphocytes by using anti-CD3/28 coated beads, different culture conditions based on serum supplementation and culture media usages were compared. Fold expansion, cell viability, growth kinetic and phenotypic characters from different culture conditions were observed for cell expansion efficiency.

Fold expansion numbers of CD4+ T lymphocytes expanded in all culture conditions were similar during the first 2-week period. After that, the expanded cells cultured in RPMI1640 with 10% FBS (R10) were dramatically increased on day 17 (755.7 ± 337.1-fold), whereas the other cultures maintained similar expansion rates (Fig. 5A). At the end of the culture period (day 21), slight decreases were found in every culture media. The culture in R10 showed the highest fold expansion (637.1 ± 265.3 –fold) which was approximately 2.5-fold higher than the other culture conditions with 10% HS (< 255-fold). No significant difference was observed among 10% HS supplemented media including GMP-grade media (i.e., AIM-V and DC) and standard media (i.e., RPMI1640).

Gradual declines in viable cells were observed throughout the 21-day culture period (Fig. 5B). All culture conditions exhibited a similar pattern in cell viability at over 70% during the first two weeks. Only the culture in R10 still maintained high numbers of viable cells on day 17 (75.9 ± 11.0%) before markedly decreasing on day 21 (54.8 ± 5.8%). On the other hand, the other culture media including RPMI1640, AIM-V and DC supplemented with 10% HS showed notable decreases since day 17 (47.1 ± 7.7%, 56.0 ± 6.4% and 52.6 ± 5.5%, respectively) and lowered to less than 36% on day 21.

Immunophenotypes of whole blood, isolated CD4+ T lymphocytes, and anti-CD3/28 expanded CD4+ T lymphocytes were determined by a flow cytometer (Table 4). Phenotypic profiles

revealed that purification of CD4+ T cells from whole blood by using the immunorosette formation technique provided high frequency of CD3+CD4+CD8- subset (> 95% of lymphocytes). After a 3-week culture, anti-CD3/28 expanded CD4+ T cells (CD3+CD4+CD8-) from R10 and DC with 10% HS exhibited high frequencies (> 90% of lymphocytes), whereas slightly lower frequencies were observed for RPMI1640 and AIM-V with 10% HS. The cell purity was also confirmed with low frequencies of CD3+CD4-CD8+ and CD3+CD4+CD8+ populations at the end of the expansion period.

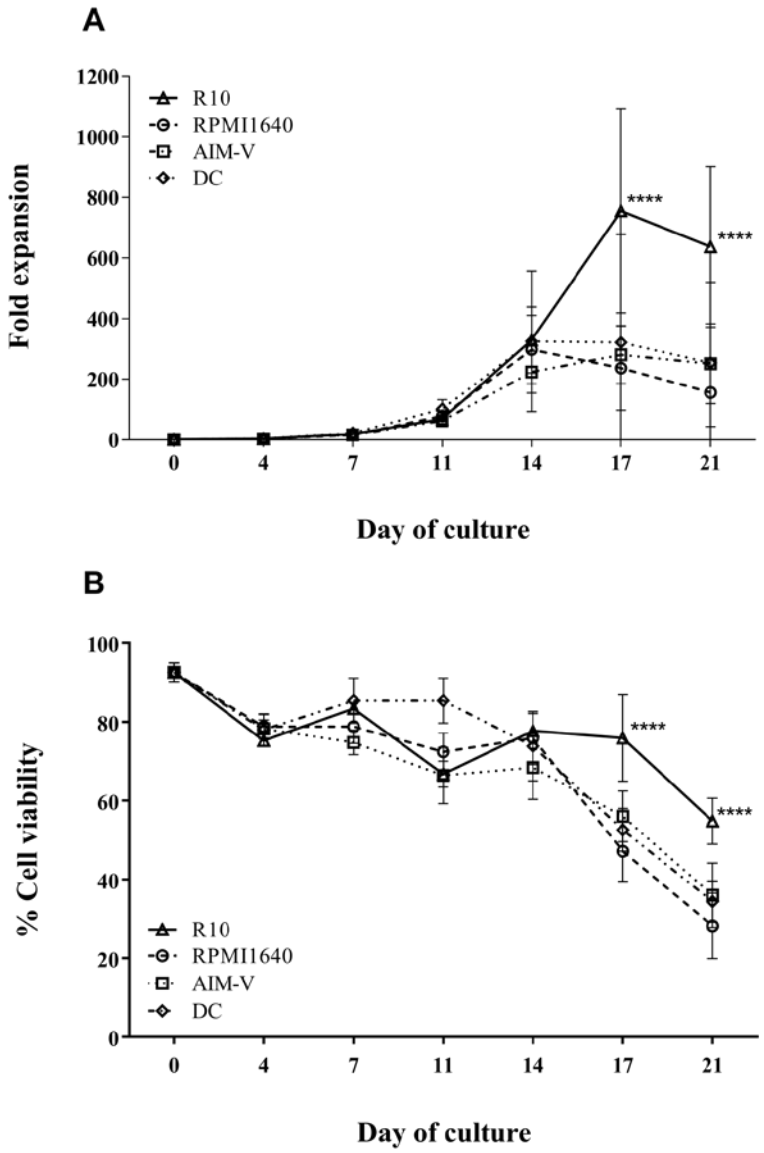


Fig. 5 Growth kinetics of anti-CD3/28 expanded CD4+ T cells from different culture conditions. (A) Fold expansion and (B) cell viability of the expanded cells were observed from different cultures in R10 (RPMI1640 with 10% FBS) and media containing 10% human serum including RPMI1640, AIM-V, and DC. All data are presented as mean ± SD (n = 5, ****p-value < 0.0001).

Phenotypes	Whole blood	Isolated $CD4^+$ T cells	Anti-CD3/28 expanded $CD4^+$ T cells			
			10% FBS	10% HS		
			RPMI1640*	RPMI1640	AIM-V	DC
% of lymphocytes						
$CD3^+CD4^-CD8^-$	6.1 ± 1.9	1.6 ± 0.6	5.7 ± 7.9	7.4 ± 9.3	10.0 ± 6.4	3.5 ± 2.1
$CD3^+CD4^+CD8^-$	34.7 ± 6.2	96.0 ± 2.6	91.9 ± 8.1	89.0 ± 11.3	84.8 ± 7.5	94.1 ± 3.9
$CD3^+CD4^-CD8^+$	30.3 ± 7.2	0.0 ± 0.0	1.0 ± 0.8	2.5 ± 2.2	3.6 ± 1.8	1.2 ± 1.2
$CD3^+CD4^+CD8^+$	0.5 ± 0.3	0.1 ± 0.1	1.4 ± 1.0	0.9 ± 0.5	1.5 ± 1.3	1.0 ± 0.9
$CD3^-CD19^+CD56^-$	11.2 ± 5.1	0.5 ± 0.8	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^+$	15.8 ± 7.4	0.6 ± 0.7	N/A	N/A	N/A	N/A
$CD3^-CD19^+CD56^+$	0.3 ± 0.3	0.0 ± 0.1	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^-$	1.1 ± 0.2	1.0 ± 1.2	N/A	N/A	N/A	N/A

*RPMI1640 with 10% FBS is R10

Table 4 Lymphocyte subset analyses of whole blood, isolated CD4+ T cells, and anti-CD3/28 expanded CD4+ T cells (mean ± SD, n = 5).

7. Determination of cytokines produced by anti-CD3/28 expanded CD4+ T cells from different culture conditions

Although a homogenous population of expanded CD4+ T lymphocytes was observed in all culture conditions with different degree of expansion ability, it remains unclear whether there are any functional changes induced by different culture conditions. Anti-CD3/28 expanded CD4+ T cells from various culture conditions were then investigated in this study for their functional variances based on types of cytokines produced by these cells.

To determine the levels of activated cells among anti-CD3/28 expanded CD4+ T cells after stimulation with PMA/I, the CD69 expression levels were assessed. There was no significant difference in frequencies of CD69 expressing cells among different culture conditions (Fig. 6A). These CD69 expressing cell populations were also consequently determined for cytokine producing cells in order to ensure the presence of cytokine production in the activated cells. While total cytokine producing cells (i.e., a combination of all types of cytokine producing cells) from all culture conditions were not significantly different, the expanded cells from R10 exhibited higher percentages of total cytokine producing cells than the others (Fig. 6B).

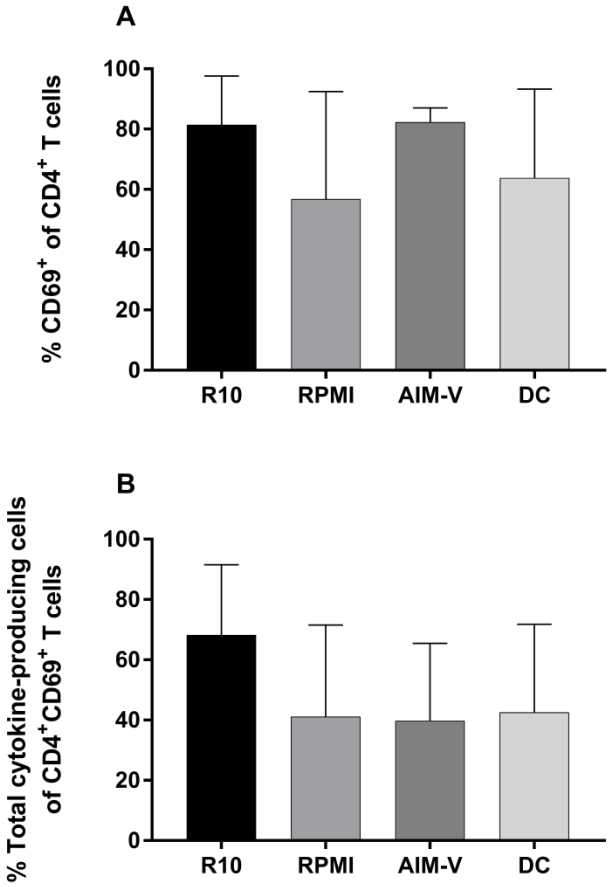


Fig. 6 Activation and cytokine production of anti-CD3/28 expanded CD4+ T cells from different culture conditions. (A) Percentages of CD69 expressing cells and (B) percentages of cytokine producing cells were observed from different cultures in R10 (RPMI1640 with 10% FBS) and media containing 10% human serum including RPMI1640, AIM-V, and DC. All data are presented as mean ± SD (n = 3).

When different cytokine-producing cell populations including TNF-α, IL-2, IFN-γ, IL-4, IL-17 and TGF-β were determined, the major population among total cytokine-producing cells was TNF-α producing cells (Fig. 7A). No difference in all types of cytokine-producing cells was observed among different culture conditions, except IL-2 producing cells with a significant high percentage (58.0 ± 18.6 % of CD4+CD69+ T cells) from the R10 culture. Ability in simultaneous production of cytokines was also determined. Results demonstrated that up to 99% of cytokine-producing cells from all culture conditions belong to 3 main populations including single, double and triple cytokine-producing cells in which a single cytokine-producing cell population showed the highest percentages of over 50% of total cytokine-producing cells (Fig. 7B).

The 3 main populations of single, double and triple cytokine-producing cells were then evaluated for specific-cytokine producing cell subsets. IL-2 producing cells were predominantly found in the single cytokine-producing cells from all culture conditions. For the double cytokine-producing cells, TNF-α+ IL-2+ producing cells showed the highest frequency for all culture conditions, except

the DC culture presenting the highest proportion of IFN-γ+ IL-2+ producing cells instead. The triple cytokine-producing cells in all media supplemented with 10% HS exhibited TNF-α+ IL-2+ IFN-γ+ producing cells as a majority, whereas TNF-α+ IL-2+ IL-4+ producing cells were found predominant in the R10 culture (data not shown).

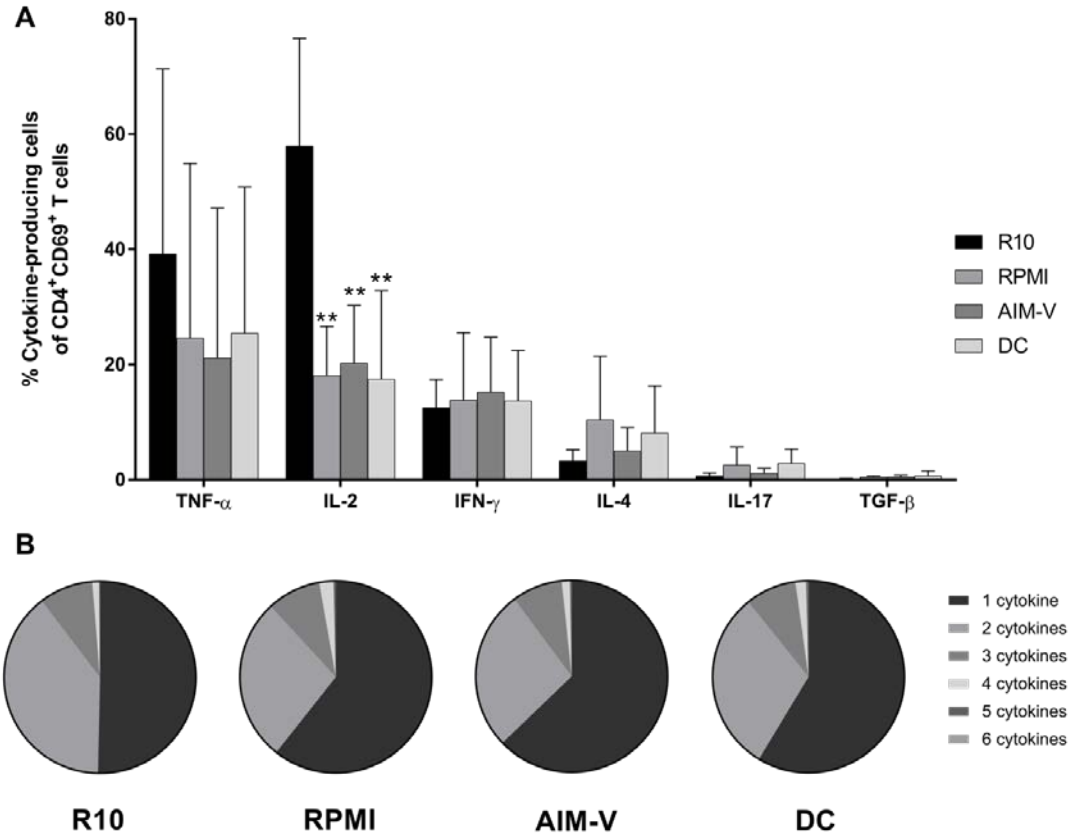


Fig. 7 Cytokine production profiles including TNF-α, IL-2, IFN-γ, IL-4, IL-17, and TGF-β of anti-CD3/28 expanded CD4+ T cells. (A) Percentages (mean ± SD) of different cytokine producing cells and (B) average proportions of polyfunctional cytokine producing cells were observed from different cultures in R10 (RPMI1640 with 10% FBS) and media containing 10% human serum including RPMI1640, AIM-V, and DC. (n = 3, ****p-value < 0.0001).

8. Bead-to-cell ratio comparison for anti-CD3/28 CD4+ T cell expansion

To achieve satisfied yields of the expanded cells, it is important to determine the optimum bead-to-cell ratio used for stimulation. In this study, three healthy volunteers were recruited for blood collection. Isolated CD4+ T cells were activated with anti-CD3/28 coated magnetic beads at different bead-to-cell ratios (i.e., 0.5:1, 1:1 and 2:1) and cultured in the absence of IL-2 for 21 days. Fold expansion, cell viability, growth kinetic and phenotypic characters were observed for proliferation efficiency of the expanded CD4+ T cells on days 0, 4, 7, 11, 14, 17 and 21.

There was no difference in fold expansion among 3 different bead-to-cell ratios during the first 14 days of culture, however, the fold expansion number of CD4+ T cells expanded with the 1:1 bead-to-cell ratio on day 17 showed remarkably higher than the others (Fig. 8A). On day 21 of culture, it was obvious that stimulation with the 1:1 bead-to-cell ratio provided the highest yield of the anti-CD3/28 expanded CD4+ T cells followed by the 2:1 and 0.5 bead-to-cell ratios (1,044 ± 259 –, 629 ± 457 –, and 301 ± 167 – fold, respectively). Cell viabilities of the expanded cells from the 3 different ratios were comparable with over 90% throughout the 3-week culture period (Fig. 8B). There were only slightly decreases in viable cells at the end of the culture for the 1:1 and 2:1 ratios (88 ± 7% and 83 ± 15%, respectively).

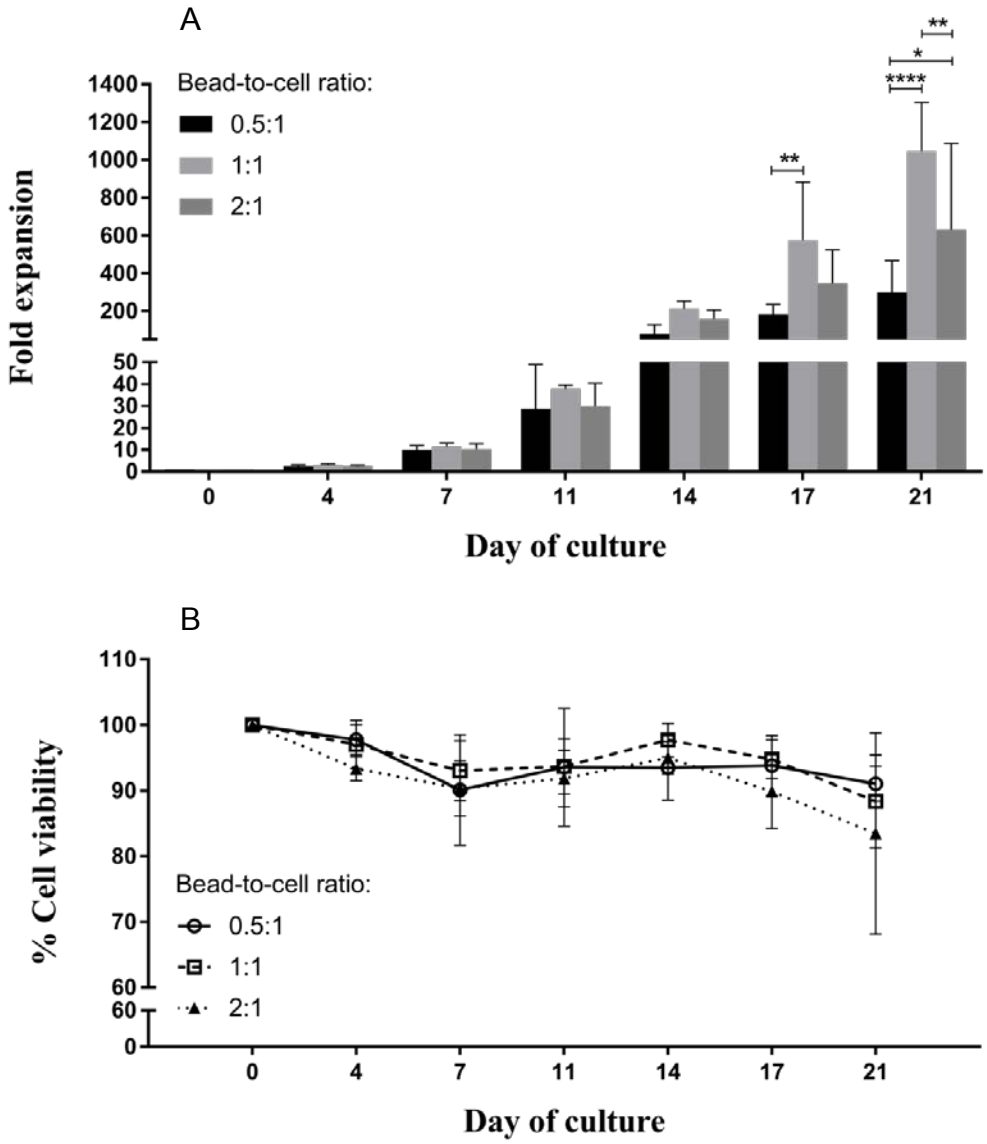


Fig. 8 Growth kinetics of anti-CD3/28 expanded CD4+ T cells when using different bead-to-cell ratios for stimulation. Fold expansion (a) and cell viability (b) of the expanded cells at different bead-to-cell ratios (i.e., 0.5:1, 1:1, and 2:1) were observed over the 21-day culture. All data are presented as mean ± SD (n = 3, *p-value < 0.05, **p-value < 0.01 and ****p-value < 0.0001).

Lymphocyte subset characters of the anti-CD3/28 stimulated CD4+ T cells were analyzed by a flow cytometer (Table 5). It was clearly demonstrated that the major population of the expanded cells was CD3+ T cells (> 99% of lymphocytes) with the dominant subset of CD3+CD4+CD8- for all bead-to-cell ratio groups over the culture period (> 98% of lymphocytes). The purity of the expanded cells was also confirmed with low frequencies of CD3+CD4-CD8+, CD3+CD4+CD8+, CD3-CD19+, CD3-CD16+CD56+, CD3+CD16+CD56+, and CD3+CD19+ (< 3.5% of expanded cells).

Cell population	**Day of culture**	**Bead-to-cell ratio**		
		0.5:1	**1:1**	**2:1**
$CD3^+$	0	99.3 ± 0.4	99.3 ± 0.4	98.8 ± 1.2
	14	99.8 ± 0.2	99.9 ± 0.1	99.9 ± 0.1
	21	99.6 ± 0.5	99.9 ± 0.1	99.9 ± 0.2
$CD3^+CD4^+CD8^-$	0	97.8 ± 1.3	98.1 ± 1.4	97.5 ± 1.7
	14	99.1 ± 0.6	99.8 ± 0.1	99.7 ± 0.1
	21	98.7 ± 1.5	99.5 ± 0.3	99.3 ± 0.4
$CD3^+CD4^-CD8^+$	0	0.0	0.0	0.0
	14	0.0 ± 0.1	0.0	0.0
	21	0.0	0.0	0.0
$CD3^+CD4^+CD8^+$	0	0.0	0.0	0.0
	14	0.0	0.0	0.0
	21	0.0 ± 0.1	0.0 ± 0.1	0.0
Others[a]	0	0.9 ± 0.5[b]	0.7 ± 0.4[b]	1.1 ± 0.9[b]
	14	1.7 ± 0.4[b]	3.5 ± 1.5[b]	2.4 ± 0.3[b]
	21	1.3 ± 0.7[b]	1.3 ± 0.4[b]	1.2 ± 0.4[b]

[a]Other cell populations include $CD3^-CD19^+$, $CD3^-CD16^+CD56^+$, $CD3^+CD16^+CD56^+$, and $CD3^+CD19^+$.
[b]A sum of average percentages of frequencies of the other cell populations.

Table 5 Characterization of anti-CD3/28 stimulated CD4+ T lymphocytes at different bead-to-cell ratios over the 21-day culture. Frequencies of all cell populations are represented as percentages of lymphocytes (mean ± SD, n = 3).

9. Effects of IL-2 supplementation on cell expansion

IL-2 supplementation has been generally used to promote cell proliferation in addition to the autocrine/paracrine IL-2 production by the activated T cells. High concentrations of IL-2 (100 and 300 IU/mL) have been reported to predominantly affect CD8+ T cell development. This study, therefore, used a low concentration of IL-2 at 20 IU/mL to support cell expansion and compared this expansion effect of IL-2 to the autocrine/paracrine IL-2 production (i.e., cell culture in the absence of

IL-2). Fold expansion, cell viability, growth kinetic and lymphocyte subset characters were observed for proliferation efficiency of the expanded CD4+ T cells on days 0, 4, 7, 11, 14, 17 and 21.

Data showed that fold expansion numbers between the culture without and with IL-2 supplementation were similar throughout the 21-day culture period (Fig. 9A). Only the expanded cells cultured in the absence of IL-2 supplementation on day 17 proliferated significantly higher than those in the presence of IL-2 supplement (582 ± 166 – and 455 ± 125 – fold, respectively). At the end of the culture, there was no significant difference in proliferation between the two culture groups. With respect to cell viability, the cultures without or with IL-2 supplementation maintained great numbers of viable cells with over 90% throughout the 3-week culture period (Fig. 9B).

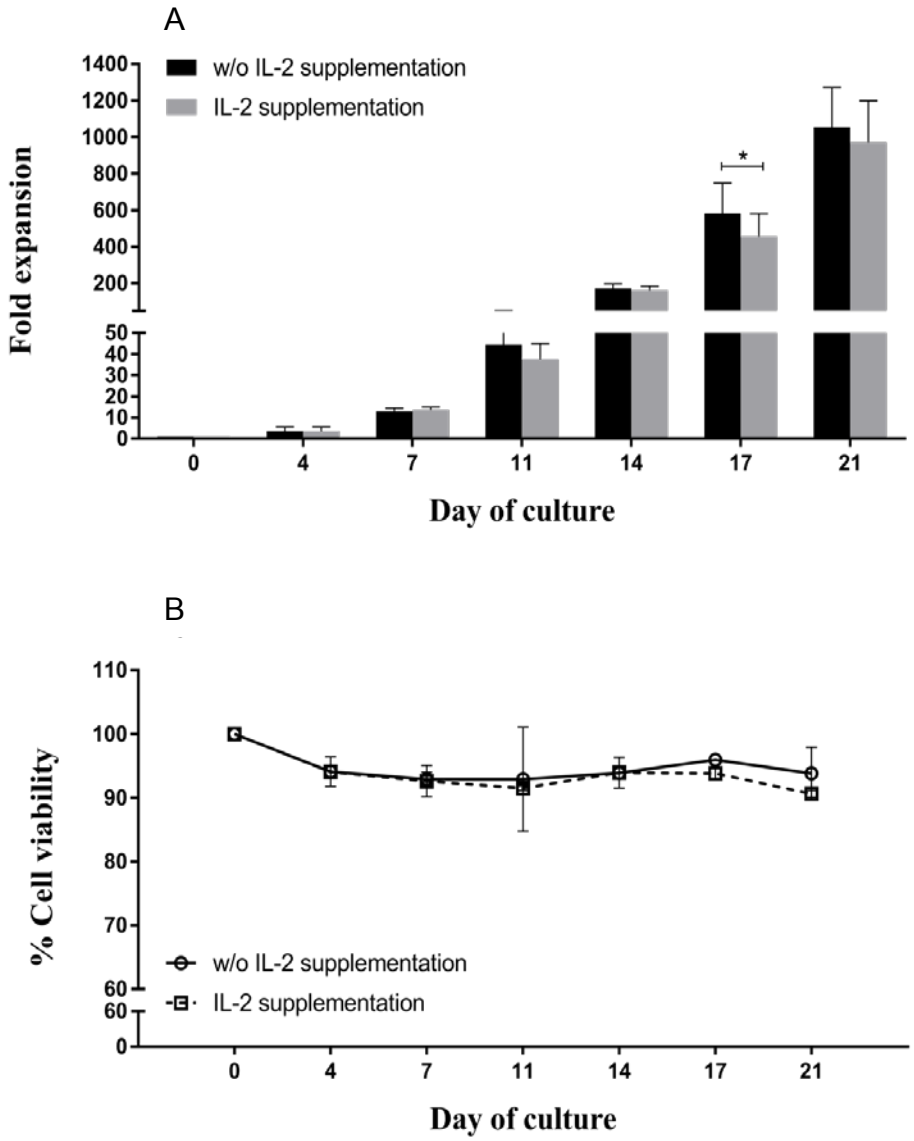


Fig. 9 Growth kinetics of anti-CD3/28 expanded CD4+ T cells when cultured without and with IL-2 supplementation. Fold expansion (a) and cell viability (b) of the expanded cells when cultured without (w/o) and with IL-2 supplementation at the concentration of 20 units/mL were observed over 21 days. All data are presented as mean ± SD (n = 3 and *p-value < 0.05).

Predominant phenotypes of the expanded cells from both culture groups were CD3+ T cells (> 97% of expanded cells) with the major CD3+CD4+CD8- subset (> 94% of expanded cells) as presented in Table 6. The minor cell populations including CD3+CD4-CD8+, CD3+CD4+CD8+, CD3-CD19+, CD3-CD16+CD56+, CD3+CD16+CD56+ and CD3+CD19+ were also found with very low frequencies (< 2% of expanded cells), suggesting a specific expansion of purified CD4+ T cells.

Cell population	**Day of culture**	**Supplementation**	
		w/o IL-2	**IL-2**
$CD3^+$	0	99.5 ± 0.2	99.2 ± 0.7
	14	96.7 ± 5.7	99.8 ± 0.1
	21	99.9 ± 0.1	99.9 ± 0.0
$CD3^+CD4^+CD8^-$	0	98.3 ± 0.6	97.9 ± 1.7
	14	94.4 ± 5.2	99.6 ± 0.1
	21	99.2 ± 0.1	99.0 ± 0.9
$CD3^+CD4^-CD8^+$	0	0.0	0.0
	14	0.0	0.0
	21	0.1 ± 0.1	0.0
$CD3^+CD4^+CD8^+$	0	0.0	0.0
	14	1.4 ± 2.4	0.1 ± 0.1
	21	0.0	0.0
Others[a]	0	0.7 ± 0.4[b]	0.8 ± 0.6[b]
	14	2.0 ± 1.0[b]	1.9 ± 0.6[b]
	21	1.3 ± 0.4[b]	0.6 ± 0.4[b]

[a]Other cell populations include $CD3^-CD19^+$, $CD3^-CD16^+CD56^+$, $CD3^+CD16^+CD56^+$, and $CD3^+CD19^+$.
[b]A sum of average percentages of frequencies of the other cell populations.

Table 6 Characterization of anti-CD3/28 stimulated CD4+ T lymphocytes when cultured without (w/o) and with IL-2 supplementation over 21 days. Frequencies of all cell populations are represented as percentages of lymphocytes (mean ± SD, n = 3).

10. Specific cell surface molecule expressions of anti-CD3/28 CD4+ T cells

As surface molecule expressions are related to cell maturation, activities and functions, this study thus explored several cell surface molecule expressions of anti-CD3/28 expanded CD4+ T cells which were divided into seven groups according to the molecules' functions and roles. The cell surface molecules included (i) chemokine receptors: CCR4, CCR5, CCR6, CCR7, CCR10, CXCR3, CXCR4 and CXCR5; (ii) adhesion molecules: CD11a, CD11b, CD11c, CD103 and $\alpha4\beta7$; (iii) co-stimulatory molecules: CD27, CD28, CD40, CD40L, CD134, PD-1 and ICOS; (iv) activation molecules: CD25, CD38, CD69, CD71 and HLA-DR; (v) maturation markers: CD45RO, CD45RA and CD62L; (vi) cytokine receptors: CD126 and CD127; and (vii) other molecules: CD57, CD95, CD95L,

and GITR. Frequencies of the expanded cells expressing these surface molecules in the cultures without and with IL-2 supplementation on day 21 were observed and compared to those of whole blood on day 0 as a baseline control.

For chemokine receptors, the expanded cells from cultures whether IL-2 supplementation or not exhibited significant lower expressions of CCR6, CCR7 and CXCR4 and dramatic higher expression of CXCR3 when compared to the unexpanded cells (i.e., whole blood) as seen in Fig. 10A. Other molecules, CCR4, CCR5, CCR10, CXCR5, remained similar after the expansion. The expanded cells also had marked increases in expressions of adhesion molecules, CD11b, CD11c and $\alpha 4\beta 7$ when compared to the unexpanded cells (Fig. 10B). There was also no change in expressions of CD11a and CD103 between the expanded and unexpanded cells.

With respect to co-stimulatory molecules, only frequencies of CD40L of the expanded cells from both expansion groups were significantly increased when compared to the unexpanded cells (Fig. 10C). All other molecules including CD27, CD28, CD40, CD134, PD-1 and ICOS remained unchanged after the expansion. Furthermore, expressions of all activation molecules, except CD69, on the expanded cells were significantly upregulated when compared to those of the unexpanded cells (Fig. 10D). The expanded cells also showed significant lower frequencies of CD45RO and higher frequencies of CD45RA, while their CD62L expression was similar to the unexpanded cells (Fig. 10E).

The expanded cells also exhibited notable downregulation of cytokine receptor, CD126, and upregulation in CD127 when compared to the unexpanded cells (Fig. 10F). Expressions of other molecules including CD57, CD95 and GITR were dramatically raised after the expansion, whereas there were slight increases in CD95L (Fig. 10G). Moreover, no significant difference in numbers of any chemokine receptors, adhesion molecules, co-stimulatory molecules, activation molecules, maturation markers, cytokine receptors and other molecules was found between the two culture groups with and without IL-2 supplementation (Figs. 10A-10G).

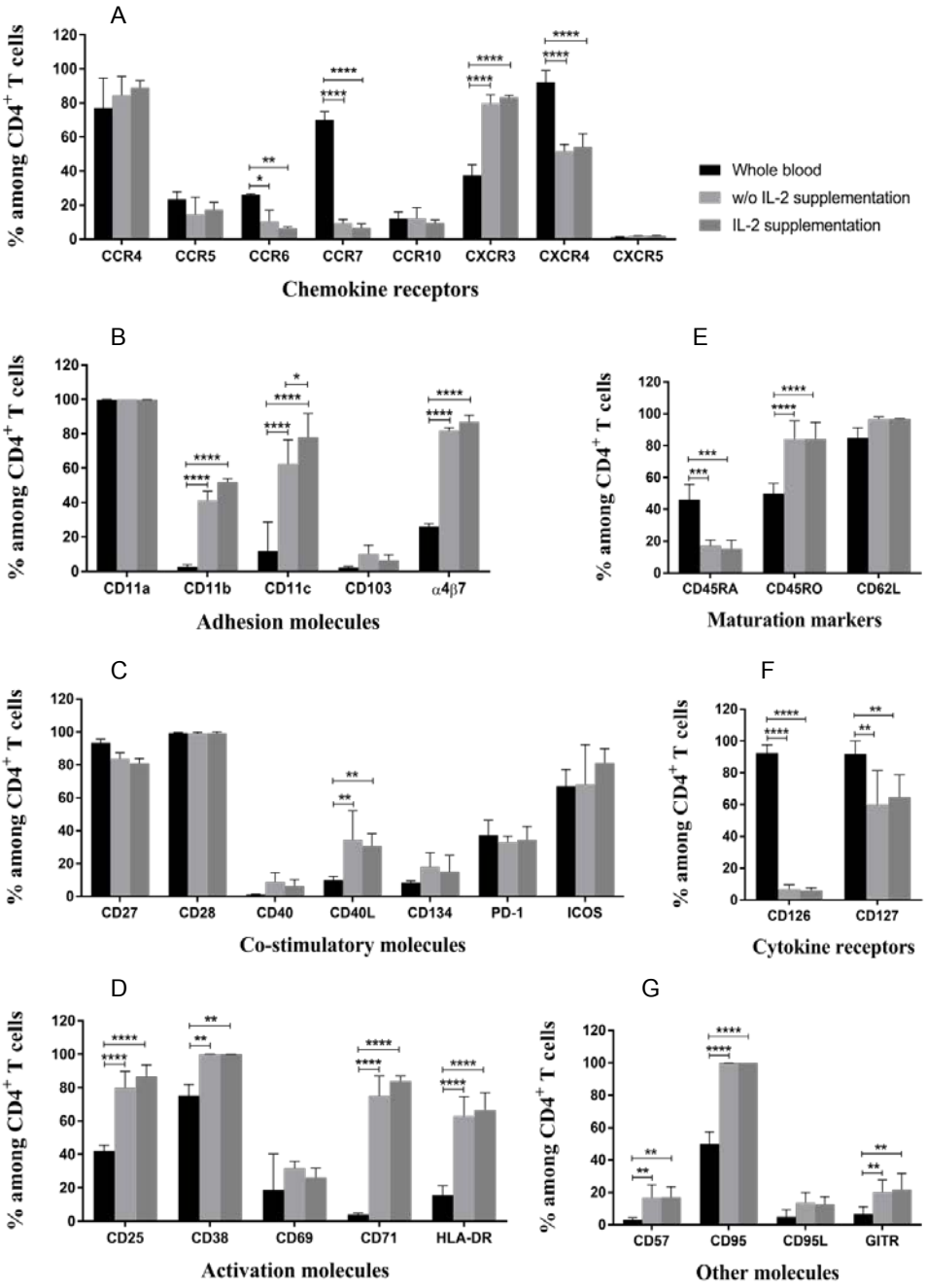


Fig. 10 Specific cell surface molecule expressions of anti-CD3/28 expanded CD4+ T cells. Expressions of a) chemokine receptors, b) adhesion molecules, c) co-stimulatory molecules, d) activation molecules, e) maturation markers, f) cytokine receptors and g) other molecules of the expanded cells when cultured without (w/o) and with IL-2 supplementation were observed on day 21 of the culture and compared to the expressions of whole blood observed on day 0. All data are presented as mean ± SD (n = 3, *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001 and ****p-value < 0.0001).

Discussions and conclusions

With respect to implementation of in vitro CD4+ T lymphocyte expansion for adoptive immunotherapy in human, there is a clinical study conducting adoptive transfer of up to 3x1010 autologous anti-CD3/28 activated CD4+T cells in HIV-infected patients and the transfusion was scheduled approximately $5x10^9$ cells at 8-week intervals [14, 15], indicating that large-scale expanded CD4+ T lymphocytes is required. A large-scale production of CD4+ T cells from HIV-1-infected donors using anti-CD3/28 coated beads, IL-2 supplementation, and gas-permeable bags was established and achieved 37-fold expansion after 14 days [21] which is not yet satisfied for CD4+ T cell numbers demanded for transfusion.

GMP-grade culture bags are in a concern to reduce contamination risks from microorganisms during a cell production aiming for treatment in clinics. GMP-grade Teflon cell culture (TCC) bags were used to compare a growth ability of normal human CD8+ T cells with conventional tissue culture flasks and plates [25]. Expanded CD8+ T cells in TCC bags yielded considerably higher than those in flasks and plates after 10 days (73-fold, 41-fold and 48-fold, respectively). Nevertheless, our study shows that anti-CD3/28 expanded CD4+ T cells from healthy donors in flasks proliferated better than those in commercially available GMP-grade culture bags (797-fold and 331-fold, respectively) after 21 days. Even so, fold expansion of CD4+ T cells from bag culture is still considerably high enough for cell production. High cell viability (75%) and high purity (97%) of expanded CD4+ T cells also support the use of bag for cell expansion.

Previous studies showed that expansion of both human and rhesus macaque CD4+ T cells with anti-CD3/28 coated beads provided a large number of homogenous CD4+ T cell population with Th1-like phenotype [16, 26]. Moreover, TNF-α production of anti-CD3/28 expanded CD4+ T cells played an important role in regulating synthesis of various β-chemokines [27] which support cellular resistance to R5-tropic HIV strain. Our results show that TNF-α producing cells were a major population presenting in the expanded cells which can play a protective role in resistance to infection. The presence of high frequency of IL-2 producing cells can also provide sufficient amount of IL-2 that supports in vitro expansion without exogenous IL-2 supplementation. Interestingly, although only one-third of anti-CD3/28 expanded CD4+ T cells showed Th1-like phenotype, other CD4+ T cell types including Th2, Th17 and Treg were almost diminished suggesting that anti-CD3/28 expansion promotes Th1 dominant population of the expanded cells. More importantly, simultaneous detection of cytokine production at a single cell level by polychromatic flow cytometry demonstrates that the majority of expanded cells simultaneously produced 2-3 cytokines (approximately 65%), suggesting a polyfunctional role of these cells.

To optimize the protocol for clinical adoptive T-cell therapy, other serum supplements besides FBS were considered in this study. HS was chosen for CD4+ T cell expansion development as it is commercially available and can be obtained as human AB serum. The culture media including RPMI1640, AIM-V and DC were then added with 10% HS for expansion comparison with RPMI1640 supplemented with 10% FBS. Culture media with 10% HS supplementation supported CD4+ T cell expansion similar to normal culture medium (i.e., culture medium with 10% FBS

supplementation or R10 in our study) in the first 2 weeks of culture. The growth rates of expanded cells from all culture media were 224-330 -fold and cell viability was approximately 70% after two weeks of cell activation and expansion. Fold expansions of all media, except R10, maintained at the same levels on day 17 before gradual declines on day 21. Unlike the others, fold expansion of R10 was considerably augmented on day 17 and then decreased on day 21. However, the expanded cells from the R10 culture on day 21 still proliferated ~2–fold higher than those on day 14. CD4+ T cell expansion in R10 provided the highest growth rate and the most consistency when compared to that in the others. Of all cultures using specialized media, RPMI1640 supplemented with 10% HS rendered the lowest value of fold expansion after a 3-week culture. It is then assumed that FBS supplementation render the higher expansion rates, and yields as well as better consistency than HS supplementation. Our findings are opposed to the previous study demonstrating that 10% FBS provided inconsistency in cell expansion when compared to HS supplementation [24]. Therefore, FBS supplementation can be a choice of interest for clinical uses to achieve the best expansion as long as its source is from an area free of bovine spongiform encephalopathy (BSE), such as Australia or New Zealand [28].

Furthermore, AIM-V and DC with 10% HS supplementation were able to provide suitable CD4+ T cell expansion with average 250-fold which is similar to the results achieved from AIM-V and DC with 5% HS supplementation from the previous study [24]. It is suggested that 5% HS supplementation is sufficient to support CD4+ T cell expansion and higher concentration of HS does not improve the proliferation rates and yields. Both AIM-V and DC are also good candidates as GMP-grade media used for cell expansion following requirement of clinical trials.

Besides FBS and HS supplementation, a xeno-free serum replacement (SR) was introduced as a novel platform strategy for clinical-grade cell manufacturing [29]. In this study, T-cell expansion was conducted by using a serum-free medium AIM-V supplemented with 5% SR and anti-CD3/28 coated bead stimulation. The expansion kinetic of T cells using SR was comparable and polyfunctional profiles were similar to that using FBS and HS [29]. SR supplementation, therefore, has become an alternative choice for FBS and HS substitution and for clinical-grade cell production since a potential risk of cross-species contamination can be avoided. Addition of exogenous cytokines, particularly IL-2, is worth considerable for improvement in cell expansion. Previous studies showed that growth rates of the expanded T cells cultured in media with HS and IL-2 supplementation were increased when compared to those without IL-2 supplementation [24, 29]. In this case, addition of HS and IL-2 together can synergistically promote the cell expansion.

Changes in specific cell surface molecule expressions of the anti-CD3/28 CD4+ T cells were also observed in this study. Several chemokine receptors have been identified as coreceptors for the HIV entry, such as CCR5, CXCR4, CCR4, CCR6 and CCR10. The in vitro expanded CD4+ T cells with anti-CD3/28 activation were proved to be resistant to HIV-1 infection via the reduction in frequencies and densities of CCR5 molecules [18,30]. Our expanded cells also rendered a low frequency in CCR5 (< 15% of CD4+ T cells) which presumably maintain at this low level because the recovery of CCR5 expression was low when activation with anti-CD3/28 coated beads compared

to stimulation with anti-CD3/28 immobilized on the surface of a tissue culture plate [30]. Our anti-CD3/28 activation protocol also rendered the expanded CD4+ T cells with twice as less CXCR4 expression than the unexpanded cells. Although a high number of the expanded cells still expressed CXCR4 (52% of CD4+ T cells), the chance of viral entry when switching coreceptor usage from CCR5 to CXCR4 [31] will feasibly diminished when compared to the unexpanded cells.

CCR4, CCR6 and CCR10 were also reported to be other HIV-1 coreceptors of primary HIV-1 isolates [32-34]. Our expanded cells showed that CCR4 was highly expressed throughout the expansion period (84% of CD4+ T cells), whereas a significant downregulation in CCR6 by 2.5 – fold and low frequency of CCR10 (12% of CD4+ T cells) were found. Although CCR4 expression was high, soluble viral protein gp120 had greater affinity to CCR5 than CCR4 [34]. CCR4+ T cells are also able to be defined as Th2 cells, while Th1 cells are classified by CXCR3+ [35]. In our study, the frequency of CXCR3+ T cells was 2-fold higher than that of the unexpanded cells (~83% of CD4+ T cells) which was almost equal to CCR4+ T cells. However, we cannot specify that our CXCR3+ T cells are absolutely purified Th1 cells as they need to be further characterized with expressions of CCR4-, CCR5+, and CXCR6+ [35]. These evidences clearly demonstrate that our expanded cells are suitable for reinfusion due to their highly feasible ability for HIV-1 resistance in vivo.

As the gut compartment is a major portal for HIV entry, the considerable depletion in mucosal CD4+ T cells was observed during acute infection [36]. Our study shows that the expanded cells had a pretentious increase in $\alpha 4\beta 7$ expression to 82% of CD4+ T cells. In contrary, the level of another gut mucosal specific adhesion molecule, CD103 [37], was low at the end of the culture (10% of CD4+ T cells) even though it was 5 – fold higher expressed in the expanded cells. It is then worth proposing that these cells feasibly migrate to the site of depletion and improve immune response at the gut-associated lymphoid tissues.

In conclusion, results from this study address that our developed closed-culture system for CD4+ T lymphocyte expansion using anti-CD3/28 coated beads and GMP-grade gas-permeable culture bags is practical, effective, and reproducible. Using our protocol, a large number of anti-CD3/28 expanded CD4+ T lymphocytes is achieved together with high viability and high purity within reasonable time (21 days). This method is also successful to expand both freshly isolated CD4+ T cells from healthy volunteers and cryopreserved CD4+ T cells from HIV-infected patients. It is then suggested that our refined procedure is useful for cell production used for adoptive transfer immunotherapy, although, there are still many aspects require further improved and investigated, such as effects of different culture media, impact of exogenous supplementation, and cell manufacturing processes under GMP standard. This study also addresses the expansion and polyfunctional profiles of expanded CD4+ T cells when cultured in commercially available GMP-grade media with human serum supplementation. We found that AIM-V and DC are good candidates for clinical-grade cell manufacturing and HS supplementation can well support cell expansion. Although animal-derived substances, mainly FBS, is not recommended to use in cell products due to their risk in cross-species contamination, FBS supplementation is still able to render the highest fold expansion with high viability and consistency when compared to HS. There is also no significant

difference in polyfunctional profiles between FBS and HS. To use FBS for cell production, its origin must be free of BSE. Therefore, our expansion protocol using either AIM-V or DC supplemented with HS provides satisfied yields of the expanded cells of which polyfunctions are similar, suggesting the suitable protocol for further uses in clinical studies.

Moreover, this study demonstrated that the 1:1 bead-to-cell ratio of anti-CD3/28 coated magnetic beads for CD4+ T cell expansion was the most optimum bead quantity to achieve the satisfied yield of the expanded cells. The autocrine cytokines, mainly IL-2, produced by the expanded cells themselves are also adequate for a 3-week proliferation without additional IL-2 supplementation. After the expansion, phenotypic profiles of the expanded cells were changed. The expanded cells likely become more resistant to HIV-1 via downregulation of dominant coreceptors for HIV entry, CCR5 and CXCR4, as well as migrate to the site of depletion and improve immune response at the gut-associated lymphoid tissues due to higher expressions of gut-homing molecules, $\alpha_4\beta_7$ integrin. Furthermore, other specific surface molecule expressions related to activation, proliferation, differentiation, homeostasis and apoptosis revealed certain functions of the expanded cells. It is thus worth suggesting that these expanded cells following our optimized protocol are suitable for CD4+ T cell immunotherapy used in HIV-infected patients, even though further investigation on CD4+ T cells from HIV-infected patients and a large-scale production are required.

Suggestion for further study

A study to develop cell based immunotherapy using autologous transferred of CD3/28 expanded CD4+ T cells with an aim at increasing CD4+ T cell level can be a promising therapeutic tool for immunological discordant patients to support a conventional antiretroviral drug treatment. Moreover, a successful effort to reduce antiretroviral drug cost in HIV infected patients with HAART is possible since CD4 transfusion will supplement with HAART interruption in successive HIV viral control patients by providing long-term anti-HIV viral control using patient's immune response. More importantly, when HAART interruption is needed to reduce drug toxicity and prevent a development of drug resistant HIV viral strain, HAART supplemented with CD4 transfusion before treatment interruption will ensure a successful control of HIV viral replication during treatment cessation.

With this developed protocol, clinical grade expansion procedure could be applied to regulatory T cell which can be used for the treatment of autoimmune disease. Furthermore, Tumor specific T cell from infiltrated T lymphocytes in a tissue from solid tumor can be isolated and expanded for being used in a treatment of certain type of cancer. The cell expansion protocol can also be used for the expansion of Chimeric Antigen Receptor (CAR) T cells to obtain a large number of CAR T cells for infusion in patients with specific cancer type.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ

   1.1 Thitilertdecha P, Suwannachod P, Poungpairoj P, Tantithavorn V, Khowawisetsut L, Ammaranond P, Onlamoon N. A closed-culture system using a GMP-grade culture bag and anti-CD3/28 coated bead stimulation for CD4+ T cell expansion from healthy and HIV-infected donors. J Immunol Methods. 2018 Jun 9. pii: S0022-1759(17)30458-1. doi: 10.1016/j.jim.2018.06.004. [Epub ahead of print]

   1.2 Thitilertdecha P, Tantithavorn V, Poungpairoj P, Ammaranond P, Loharungsikul S, Onlamoon N. Comparison of serum supplementation and culture medium for CD4+ T cell expansion by using anti-CD3/28 coated beads. (Submitted to Biological procedures online)

   1.3 Thitilertdecha P, Poungpairoj P, Tantithavorn V, Ammaranond P, Onlamoon N. Differential expression of functional-associated cell surface molecules on anti-CD3/28 expanded CD4+ T cells. (Submitted to Human Immunology)

2. การนำผลงานวิจัยไปใช้ประโยชน์
   - เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป) : ไม่มี
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   - เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง) : ไม่มี
   - เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่) : มีการฝึกนักวิจัยรุ่นใหม่ให้ร่วมดำเนินงานในโครงการวิจัย

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Appendix

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Research paper

A closed-culture system using a GMP-grade culture bag and anti-CD3/28 coated bead stimulation for CD4$^+$ T cell expansion from healthy and HIV-infected donors

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ABSTRACT

CD4 immunotherapy is potentially useful in immune reconstitution of CD4$^+$ T cells for HIV-infected patients. Transfusion of anti-CD3/28 expanded CD4$^+$ T cells is also proved to be safe and effective in both SIV-infected macaques and HIV-infected patients. However, there is no such standardized and practical protocol available for cell production in order to use in clinics. This study thus aimed to develop a closed-culture system for *in vitro* CD4$^+$ T lymphocyte expansion by using a commercially available GMP-grade culture bag and anti-CD3/28 activation. Freshly isolated CD4$^+$ T cells by immunorosette formation from healthy donors and cryopreserved CD4$^+$ T cells from HIV-infected patients with CD4 count over 500 cells/μL were stimulated with anti-CD3/28 coated beads. The activated cells were then expanded in conventional culture flasks and GMP-grade culture bags for three weeks. Fold expansion, cell viability, growth kinetic and phenotypic characters were observed. Results revealed that purified CD4$^+$ T cells from healthy individuals cultured in flasks showed better expansion than those cultured in bags (797-fold and 331-fold, respectively), whereas, their cell viability, growth kinetic and expanded CD4$^+$ T cell purity were almost similar. A large-scale production was also conducted and supported consistency of cell proliferation in the closed-culture system. Frozen CD4$^+$ T lymphocytes from the patients were able to remain their growth function and well expanded with a good yield of 415-fold, 85% viability and 96% purity of CD4$^+$ T cells at the end of a 3-week culture in bags. This developed closed-culture system using culture bags and anti-CD3/28 coated beads, therefore, can achieve a large number of expanded CD4$^+$ T lymphocytes with good reproducibility, suggesting a promising protocol required for adoptive immunotherapy.

1. Introduction

Highly active antiretroviral therapy (HAART) is greatly effective and safe for human immunodeficiency virus (HIV)-infected patients by lowering the HIV viral load into an undetectable level. However, this therapy does not eradicate latent reservoirs of virus (Finzi et al., 1997) and not completely restore immune system (Carcelain et al., 2001; Lange and Lederman, 2003; Valdez et al., 2002; Valdez et al., 2003). Adoptive transfer of autologous activated CD4$^+$ T cells then becomes an alternative approach for HIV treatment due to its tentative benefit for immune reconstitution. This approach is proved to be effective and safe by several *in vivo* studies in both simian immune deficiency virus (SIV)-infected rhesus macaques and HIV-infected patients (Onlamoon et al., 2007; Villinger et al., 2002; Onlamoon et al., 2006; Bernstein et al., 2004; Levine et al., 2002).

CD4$^+$ T cell expansion method was established with results showing that expanded CD4$^+$ T cells were intrinsic resistant to macrophage-tropic isolates of HIV-1 infection when using anti-CD3/28 coated magnetic beads for cell stimulation (Levine et al., 1996; Carroll et al., 1997; Riley et al., 1997). Anti-CD3/28 activated CD4$^+$ T cells also had

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lower expression of CCR5 as well as greater expression of RANTES, MIP-1α, and MIP-1β (Onlamoon et al., 2007; Onlamoon et al., 2006; Bernstein et al., 2004; Levine et al., 1996). Furthermore, cytokine secretion of these expanded $CD4^+$ T cells was associated with T helper cell type 1 function along with increases of telomerase activity and diversity of TCR Vβ repertoires (Onlamoon et al., 2007; Bernstein et al., 2004).

Although several *in vitro* culture methods are available for anti-CD3/28 activated $CD4^+$ T lymphocytes (Levine et al., 1997; Onlamoon et al., 2013; Garland et al., 1999; Levine et al., 1998), none of them is yet standardized and practical to be used in clinics. There are many concerns in cell production for adoptive immunotherapy, such as contamination risk, production scale, and source of starting cells. To reduce contamination risk of microorganisms, a closed-culture system is introduced by expanding the cells in gas-permeable culture bags instead of conventional flasks. No current protocols have yet reached satisfaction in cell yields for transfusion. According to the production scale for clinical uses, previous studies demonstrated that a large number of up to 3×10^{10} expanded $CD4^+$ T cells were demanded of reinfusion into HIV-infected patients (Bernstein et al., 2004; Levine et al., 2002), suggesting that a large number of purified $CD4^+$ T cells are required for *in vitro* expansion. Most HIV-infected patients, however, have low $CD4^+$ T cell counts and then becoming a limitation for sufficient cell number.

Our study then purposed to develop a closed-culture system for *in vitro* $CD4^+$ T lymphocyte expansion by using a commercially available GMP-grade culture bag and anti-CD3/28 activation as well as a large-scale production aiming for adoptive immunotherapy. This developed procedure was also utilized for purified $CD4^+$ T cells from HIV-infected patients.

2. Materials and methods

2.1. Samples

Five healthy volunteers were recruited and signed informed consents which were approved by the Institutional Review Board of the Faculty of Medicine Siriraj Hospital at Mahidol University. Sixteen to twenty-five milliliters of whole blood were collected into sodium heparin-containing vacutainer tubes and kept at room temperature prior to phenotypic determination of whole blood and $CD4^+$ T lymphocyte isolation.

Cryopreserved $CD4^+$ T lymphocytes were obtained from HIV-infected patients with $CD4^+$ T cell count over 500 cells/μL and stored in a liquid nitrogen tank at −196 °C for 1.5–2 years.

2.2. Antibodies

Monoclonal antibodies (mAbs) and their conjugated fluorochromes including anti-CD3 conjugated with fluorescein isothiocyanate (FITC), anti-CD4 conjugated with allophycocyanin (APC), anti-CD8 conjugated with phycoerythrin (PE), anti-CD19 conjugated with allophycocyanin and cyanine dye (APC-Cy7), anti-CD45 conjugated with peridinin chlorophyll protein (PerCP), and anti-CD56 conjugated with phycoerythrin and cyanine dye (PE-Cy7) were purchased from Becton Dickinson Bioscience (BDB, San Jose, CA). In addition, anti-CD3 conjugated with AlexaFluor® (A700), anti-CD4 conjugated with Brilliant Violet™ 605 (BV605), anti-CD8 conjugated with PE/Dazzle™ 594, anti-CD69 conjugated with PerCP/Cy5.5, anti-IL-2 conjugated with BV510, anti-IL-4 conjugated with FITC, anti-IL-17 conjugated with PE, anti-IFN-γ conjugated with APC, anti-TNF-α conjugated with BV650 and anti-TGF-β conjugated with BV421 were obtained from BioLegend (San Diego, CA).

2.3. $CD4^+$ T lymphocyte isolation

$CD4^+$ T lymphocytes were directly isolated from fresh whole blood through immunorosette formation by using RosetteSep® Human $CD4^+$ T cell enrichment cocktail (STEMCELL Technologies, Vancouver, BC, Canada) according to the manufacturer's instruction. Purified $CD4^+$ T lymphocytes were isolated by a standard Ficoll-Hypaque gradient centrifugation (Histopaque, Sigma-Aldrich,Co., St.Louis, MO, USA) and were ready for phenotypic characterization and cell expansion.

2.4. Cell stimulation and expansion for freshly isolated $CD4^+$ T cells

Freshly purified $CD4^+$ T cells of 1×10^6 cells were stimulated with anti-CD3/28 coated beads (Dynabeads® Human T-Activator CD3/CD28, Invitrogen Dynal, Oslo, Norway) at a bead-to-cell ratio of 1:1. The stimulated $CD4^+$ T cells were then expanded in complete media (RPMI1640 with 10% fetal bovine serum (FBS)), 50 μg/mL pencillin-streptomycin and 2 mM L-glutamine. The expanded cells were incubated at 37 °C and 5% CO_2 humidification and reactivated on day 7. The cells were expanded for a 3-week culture period. Cell numbers and viability were observed by using trypan blue exclusion and a TC10™ automated cell counter. Phenotypic characters were analyzed by flow cytometry on days 14 and 21.

For an expansion method of flask culture, the stimulated cells of 1×10^6 cells were placed in a 24-well plate (Costar® 24 well clear TC-treated multiple well plates, sterile, Corning Inc., Life Sciences, NY, USA) on day 0 and expanded at a concentration of 0.5×10^6 cells/mL before transferring to T25, T75 and T175 plastic tissue culture flasks (Corning® U-shaped canted neck cell culture flask with vent cap, Corning Inc., Life Sciences, NY, USA) on days 4, 7, and 11, respectively. Cell numbers and viability were observed on days 4, 7, 11, 14, 17 and 21 while the media were replenished with calculated amounts of fresh media on days 4, 7, 11, 14, and 17 to maintain the cell suspension concentration at 0.5×10^6 cells/mL.

With respect to bag culture, the expansion process was similar to flask culture during the first week of cell expansion. The expanded cells were re-stimulated in T25 flasks on day 7 and replenished with calculated amounts of fresh media at a concentration of 0.5×10^6 cells/mL before transferring to a GMP-grade culture bag (Vuelife® cell culture bags, CellGenix, Freiburg, Germany) with a size of 72c (maximum volume of 72 mL). The culture bag was clamped by half and placed on a steel grating culture stage prior to cell transfer. Fresh media were added to reach maximum volume of the bag (72 mL) on day 11. After that, the expanded cells were transferred to another culture bag with a size of 197c on day 14, filled with media up to 197 mL on day 17, and leaved for growth until day 21. Cell numbers and viability were observed on days 4, 7, 14, and 21.

2.5. Large-scale production of freshly isolated $CD4^+$ T cells in a closed-culture system

Freshly purified $CD4^+$ T cells of 8×10^6 cells from healthy donors were mixed with anti-CD3/28 coated beads (Dynabeads® Human T-Activator CD3/CD28, Invitrogen Dynal, Oslo, Norway) at a bead-to-cell ratio of 1:1 in a plastic tube (Falcon® high clarity polypropylene centrifuge tube, conical bottom, sterile, Corning Inc., Life Sciences, NY, USA) before injecting into a GMP-grade culture bag (Vuelife® cell culture bags, CellGenix, Freiburg, Germany) with a size of 32c. Complete media (RPMI1640 with 10% fetal bovine serum, 50 μg/mL pencillin-streptomycin and 2 mM L-glutamine) were added in order to achieve a concentration of 0.5×10^6 cells/mL. The expanded cells were incubated at 37 °C and 5% CO_2 humidification. Only 20×10^6 cells of anti-CD3/28 expanded $CD4^+$ T cells were reactivated on day 7. The expanded cells were consequently transferred to larger culture bags with sizes of 72c and 196c on days 7 and 14. Fresh culture media were added to reach suggested maximum volume of individual bag size on

days 4, 11 and 17. Cell numbers and viability were observed on days 7, 14 and 21 by using trypan blue exclusion and a TC10™ automated cell counter. Phenotypic characters were analyzed by flow cytometry on days 14 and 21.

2.6. Cell stimulation and expansion for cryopreserved CD4$^+$ T cells

Cryopreserved CD4$^+$ T lymphocytes from HIV-infected patients were removed from a liquid nitrogen tank at −196 °C and then thawed in a sterile water bath at 37 °C. Cryopreserved cells of 1×10^6 cells were activated and expanded following the developed bag culture protocol of freshly isolated CD4$^+$ T cells. In addition, fresh media with IL-2 supplementation at a concentration of 100 U/mL was used on day 7 onwards. Cell numbers and viability were observed on days 4, 7, 14 and 21 by using trypan blue exclusion and a TC10™ automated cell counter. Phenotypic characters were analyzed by flow cytometry on days 14 and 21.

2.7. Immunofluorescence staining and analysis

Samples of whole blood, purified and expanded CD4$^+$ T cells were stained with fluorochrome-conjugated mAbs for 15 min before adding FACS lysing solution (BDB, San Jose, CA) in order to lyse and fix the cells. The stained cells of at least 100,000 events were acquired for each analysis by a BD FACSVerse™ flow cytometer (BDB, San Jose, CA) and the data were analyzed by using FlowJo Software (Tree Star, San Carlos, CA). The percentages of lymphocyte subsets were determined by the expression of CD3, CD4, CD8, CD19 and CD56.

2.8. Cell stimulation, intracellular cytokine staining (ICS) and analysis

Expanded CD4$^+$ T cells from a bag culture of cryopreserved CD4$^+$ T cells from HIV-infected patients were used to determined different types of cytokine production. Expanded CD4$^+$ T cells at 1×10^6 cells/mL were stimulated with 25 ng phorbol 12-myristate 13-acetate (PMA) and 1 μg ionomycin (I) in the presence of brefeldin A (BFA) at 10 μg, whereas expanded CD4$^+$ T cells in the presence of BFA without stimulation were used as a control. The samples were then incubated at 37 °C/5% CO_2 for 4 h. After incubation, PMA/I stimulated and unstimulated samples were stained with Zombie NIR™ dye (BioLegend, San Diego, CA) at 4 °C for 15 min. A washing buffer (phosphate buffered saline (PBS) with 2% FBS) were added and the samples were washed by centrifugation at 450 *g* for 5 min. The samples were then surface stained with a combination of mAbs including anti-CD3 A700, anti-CD4 BV605, anti-CD8 PE/Dazzle™ 594 and anti-CD69 PerCP/Cy5.5 at 4 °C for 15 min and washed once. The stained samples were fixed and permeabilized in 0.5 mL of BD Cytofix/Cytoperm™ Fixation and Permeabilization Solution (BDB, San Jose, CA) at 4 °C for 20 min. After incubation, the samples were washed by adding 1× BD Perm/Wash™ Buffer (BDB, San Jose, CA) and centrifuged at 500 *g* for 5 min. ICS were performed by staining with a combination of mAbs including anti-IL-2 BV510, anti-IL-4 FITC, anti-IL-17 PE, anti-IFN-γ APC, anti-TNF-α BV650, anti-TGF-β BV421 at 4 °C for 30 min. After staining, the samples were washed with 1× BD Perm/Wash™ Buffer and resuspended in PBS. The stained cells of at least 100,000 events were acquired for each analysis by a BD Fortessa™ flow cytometer (BDB, San Jose, CA) and the data were analyzed by using FlowJo Software. The cytokine producing cell subsets were determined from activated populations expressing CD69 and the percentages of cytokine producing cell subsets were determined from a total cytokine producing cell population. A Boolean gating strategy was used for the analysis of polycytokine producing cell subsets in order to evaluate cytokine producing cell subsets with ability to simultaneously produce 1, 2, 3, 4, 5 or 6 cytokines.

2.9. Cell harvesting and quality control

Cell suspensions containing expanded CD4$^+$ T cells from a bag culture of cryopreserved CD4$^+$ T cells from HIV-infected patients were collected on day 21 of cell expansion. Aliquots of cell suspensions were transferred to 50-mL tubes and centrifuged at 450 *g* for 5 min. Culture supernatants were removed, collected in small aliquots and stored in a − 80 °C freezer prior to thaw for using in quality control. Cell pellets were re-suspended in 10 mL of complete media and transferred to 15-mL tubes.

To remove anti-CD3/28 coated beads, each 15-mL tube was placed in a DynaMag™-15 Magnet (Invitrogen Dynal) for 2 min and the cell suspension was transferred to another 15-mL tube. The same procedure was repeated once and aliquots of cell suspensions were pooled. The centrifugation process was repeated and media was removed. Cell pellets were re-suspended in complete media and pooled into a single tube at a volume of 20 mL. Cell numbers and viability were observed by using trypan blue exclusion and a TC10™ automated cell counter. Aliquots of the expanded CD4$^+$ T cells at 1×10^7 cells/mL were cryopreserved in RPMI-1640 media containing 20% FBS with 10% DMSO and stored at 180 °C prior to thaw for using in quality control and intracellular cytokine staining.

For quality control, frozen expanded CD4$^+$ T cells were thawed and re-suspended in PBS containing 5% human albumin. To detect residual bead contamination, samples containing 1×10^7 cells were mixed with sodium hypochlorite solution and centrifuged at 450 *g* for 5 min. Supernatants were removed and the pellets were re-suspended with PBS. Residual bead counts were determined on a hemacytometer and the total numbers of beads were calculated. For sterility testing, a 14-days United States Pharmacopoeia (USP) sterility testing on aliquots of thawed cells and culture supernatants was performed at Siriraj Hospital Microbiology Laboratory by using fluid thioglycollate medium (for the detection of anaerobic bacteria) and soybean-casein digest medium (for the detection of aerobic bacteria and fungi). Sterile results were identified as no growth of microorganism.

2.10. Data analysis

Fold expansion number was calculated by using the viable cell number at each indicated time point divided by the viable cell number at the beginning of cell expansion. For a large-scale production experiment, fold expansion numbers were calculated to get an approximated number if a total number of expanded cells on day 7 was expanded until day 21. Fold expansion, cell viability and frequencies of T lymphocytes and their subsets were shown as mean ± SD (standard deviation). Statistical differences of mean values of fold expansion and cell viability of expanded CD4$^+$ T lymphocytes were analyzed by paired *t*-tests. Statistical significance was considered when *p*-value was below 0.05. For cytokine producing cell subset analysis, the percentages of specific-cytokine producing cell subsets were shown as mean ± SD. The proportions of cytokine producing cell subsets were presented as pie-charts. All data were analyzed by using Prism software (GraphPad, La Jolla, CA).

3. Results

3.1. Expanded CD4$^+$ T lymphocytes in small-scale plastic flasks and GMP-grade culture bags after anti-CD3/28 coated bead stimulation

To develop a closed-culture system for CD4$^+$ T lymphocytes, GMP-grade culture bags were used for cell expansion and compared to conventional plastic flasks. Freshly isolated CD4$^+$ T cells were obtained from five healthy volunteers for the study. Fold expansion, cell viability, growth kinetic and phenotypic characters were observed for proliferation efficiency of expanded CD4$^+$ T cells in flasks and bags.

Fold expansion numbers of CD4$^+$ T lymphocytes expanded in both

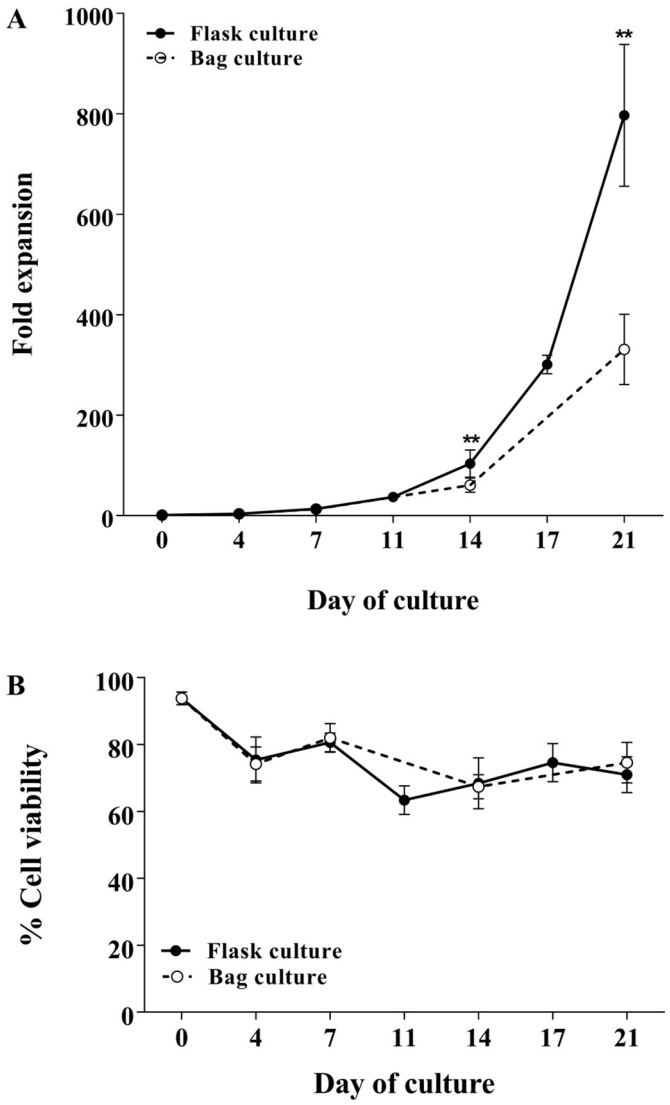

Fig. 1. Growth kinetics: (A) fold expansion, (B) cell viability of anti-CD3/28 expanded $CD4^+$ T cells of freshly isolated $CD4^+$ T cells from healthy volunteers expanded in flasks and bags ($n = 5$, ** p-value < 0.01).

flasks and bags were similar at the beginning and started dramatically increasing on day 14 as shown in Fig. 1A. A flask culture exhibited significantly higher fold expansion than a bag culture (103.8 ± 27.2 and 60.9 ± 14.4, respectively, p-value = 0.0075). At the end of the culture period, $CD4^+$ T lymphocytes expanded in flasks were proliferated better than those expanded in bags (796.7 ± 141.2 –fold and 330.9 ± 70.0 –fold, respectively, p-value = 0.0078).

Cell viability of anti-CD3/28 expanded $CD4^+$ T cells from both flask and bag cultures were similar throughout a 3-week expansion period (Fig. 1B). There were slightly decreases of viable cells from day 0 to day 21 (93.8 ± 1.9% to 71.0 ± 5.4% in flasks and 93.8 ± 1.9% to 74.6 ± 6.1% in bags) and no significant difference between the two different culture methods.

Phenotypes of whole blood, isolated $CD4^+$ T lymphocytes, and anti-CD3/28 expanded $CD4^+$ T lymphocytes were determined by a flow cytometer (Fig. 2). As shown in Table 1, phenotypic profiles showed that the immunorosette formation technique was effective and reproducible for $CD4^+$ T cell isolation from whole blood with high frequency of $CD3^+CD4^+CD8^-$ subset (91% of lymphocytes). After a 3-week culture, anti-CD3/28 expanded $CD4^+$ T cells ($CD3^+CD4^+CD8^-$) in either flasks or bags exhibited high frequencies (> 97% of lymphocytes) on days 14 and 21. The cell purity was also confirmed with low frequencies of $CD3^+CD4^-CD8^+$ and $CD3^+CD4^+CD8^+$ populations (< 0.3% and < 2%, respectively) throughout the expansion period.

3.2. Large-scale expansion of $CD4^+$ T lymphocytes in a closed-culture system

As a high number of purified $CD4^+$ T lymphocytes are demanded for cell transfusion, a large-scale production of freshly isolated $CD4^+$ T cells from five healthy donors in a closed-culture system was developed in this study. Fold expansion, cell viability, growth kinetic and phenotypic characters were observed for large-scale proliferation.

A large-scale production of $CD4^+$ T cells exhibited a gradual increase of fold expansion from day 0 to day 14 (1.0 ± 0.0 on day 0, 5.5 ± 1.0 on day 7, and 20.4 ± 3.4 on day 14) and a marked increase from day 14 to day 21 (109.1 ± 18.4 on day 21). Growth kinetic of this large-scale expansion in culture bags also had a similar pattern with that of a small-scale bag culture prescribed earlier. Cell viability of large-scale anti-CD3/28 expanded $CD4^+$ T cells was good with a slight decrease from 91.2 ± 2.6% on day 0 to 77.4 ± 6.3% on day 21.

Again, phenotypic characterization of whole blood and isolated $CD4^+$ T cells was performed for purity confirmation of isolated $CD4^+$ T cells before culture as presented in Table 2. Anti-CD3/28 expanded $CD4^+$ T lymphocytes from a large-scale production showed high frequency of $CD3^+CD4^+CD8^-$ population (> 98%) on days 14 and 21. Low numbers of $CD3^+CD4^-CD8^+$ and $CD3^+CD4^+CD8^+$ populations (< 0.8% and < 0.5%, respectively) were detected.

3.3. Bag culture of anti-CD3/28 expanded $CD4^+$ T cells from HIV-infected patients

Using freshly isolated $CD4^+$ T cells from HIV-infected patients for cell expansion may possibly be inconvenient; therefore, frozen cells become alternative source and more practical for expansion. Cryopreserved $CD4^+$ T lymphocytes from five healthy donors were able to expand in bags with a similar fold expansion when compared to freshly isolated $CD4^+$ T lymphocytes (data not shown). Cryopreserved $CD4^+$ T lymphocytes from HIV-infected patients were then expanded in the developed close-culture system.

Anti-CD3/28 expanded $CD4^+$ T cells from frozen cells showed the same trend and similar numbers of fold expansion as those from freshly isolated cells (1.0 ± 0.0 on day 0, 3.4 ± 0.4 on day 4, 13.6 ± 3.5 on day 7, 63.6 ± 16.2 on day 14 and 414.9 ± 67.6 on day 21). Cell viability of cryopreserved cells was quite high and remained stable until the end of expansion (76.7 ± 5.8% on day 0 to 85.0 ± 1.7% on day 21).

Purity of isolated $CD4^+$ T cells from cryopreservation was ensued by phenotypic characterization (Table 3). $CD3^+CD4^+CD8^-$ population was detected to be dominant for anti-CD3/28 expanded $CD4^+$ T cells throughout the expansion period (> 98%). This was confirmed by minor populations of $CD3^+CD4^-CD8^+$ and $CD3^+CD4^+CD8^+$ T cells (< 0.8% and < 0.5%, respectively).

3.4. Determination of cytokines produced by anti-CD3/28 expanded $CD4^+$ T cells

Although cell expansion by using anti-CD3/28 coated beads showed a homogenous population of expanded $CD4^+$ T lymphocytes, the obtained phenotypic character were not able to provide information on a variety of expanded cells in term of function. Since $CD4^+$ T lymphocytes can be divided into subpopulations including T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17), and regulatory T cells (Treg) based on types of cytokine produced by these cells, anti-CD3/28 expanded $CD4^+$ T cells from a bag culture of cryopreserved $CD4^+$ T cells from HIV-infected patients were investigated for their T helper cell subset identity.

CD69 expression was used to determine the level of activated cells

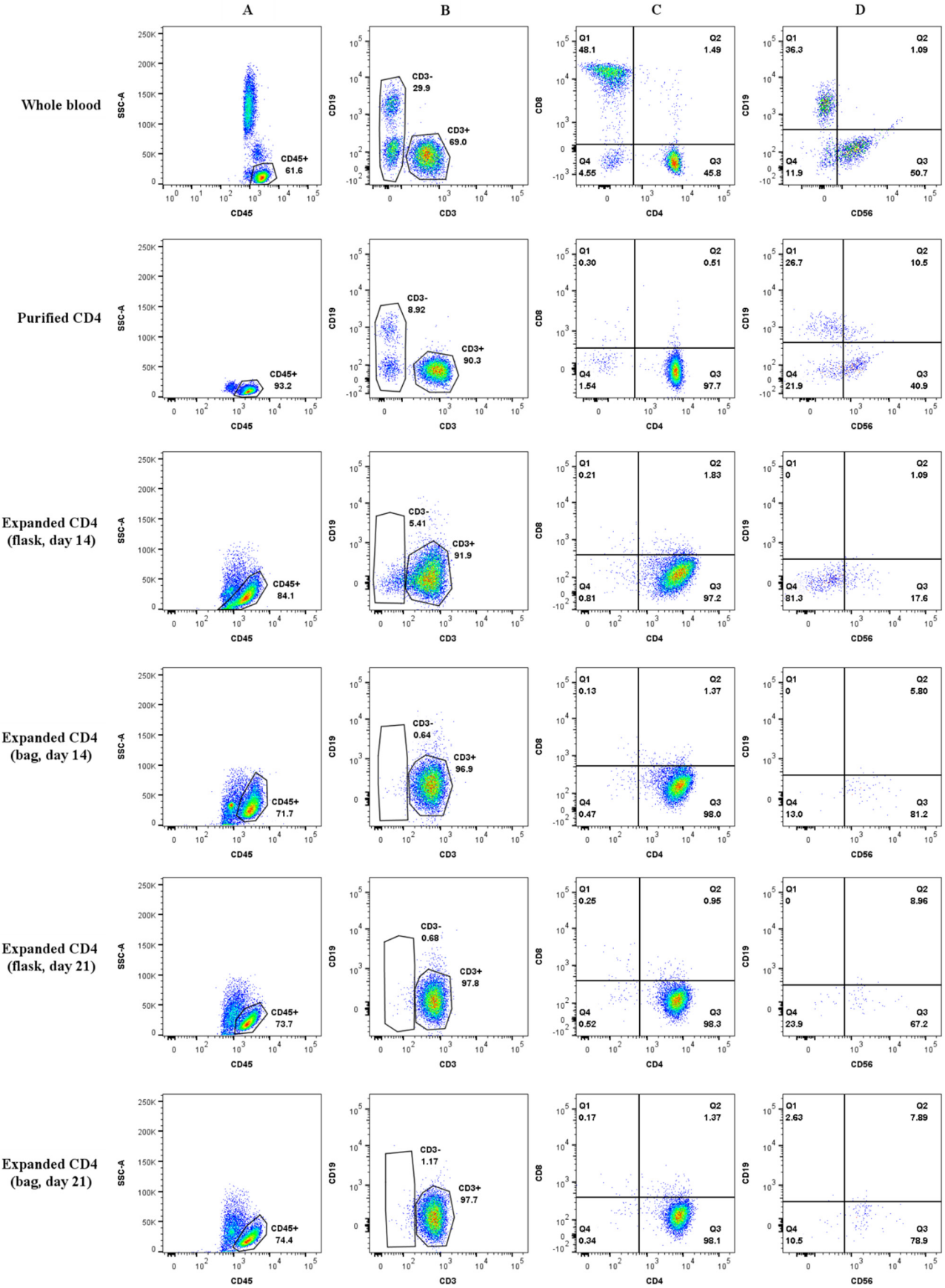

Fig. 2. Phenotype characterization of whole blood, purified $CD4^+$ T cells, expanded $CD4^+$ T cells on days 14 and 21 in flask and bag cultures. Gating identified (A) lymphocytes among leukocyte population, (B) $CD3^+$ and $CD3^-$ cells among lymphocyte population, (C) $CD4^+$ and $CD8^+$ T cells among $CD3^+$ cells, and (D) $CD19^+$ B cells and $CD56^+$ NK cells among $CD3^-$ cells.

Table 1
Phenotypes of whole blood, isolated $CD4^+$ T cells, and anti-CD3/28 expanded $CD4^+$ T cells from healthy volunteers (mean ± SD, $n = 5$).

Phenotypes	Whole blood	Isolated $CD4^+$ T cells	Anti-CD3/28 expanded $CD4^+$ T cells[a]			
	Day 0	Day 0	Day 14[b]		Day 21	
			Flasks	Bags	Flasks	Bags
% of lymphocytes						
$CD3^+CD4^-CD8^-$	4.4 ± 3.2	2.2 ± 1.3	0.8 ± 0.5	0.7 ± 0.6	0.8 ± 0.9	0.7 ± 0.4
$CD3^+CD4^+CD8^-$	32.6 ± 4.3	91.0 ± 4.8	98.2 ± 0.4	97.8 ± 1.3	98.3 ± 1.2	97.4 ± 1.5
$CD3^+CD4^-CD8^+$	36.0 ± 2.0	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	0.3 ± 0.3	0.2 ± 0.1
$CD3^+CD4^+CD8^+$	0.5 ± 0.4	2.2 ± 1.3	0.8 ± 0.5	1.4 ± 1.4	0.6 ± 0.5	1.0 ± 0.4
$CD3^-CD19^+CD56^-$	10.9 ± 3.5	1.4 ± 1.6	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^+$	13.5 ± 2.5	0.8 ± 0.8	N/A	N/A	N/A	N/A
$CD3^-CD19^+CD56^+$	0.2 ± 0.3	0.1 ± 0.0	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^-$	2.0 ± 1.4	4.2 ± 3.8	N/A	N/A	N/A	N/A
% of $CD3^+$ T cells						
$CD3^+CD4^-CD8^-$	5.9 ± 4.1	2.3 ± 1.4	N/A	N/A	N/A	N/A
$CD3^+CD4^+CD8^-$	43.9 ± 4.1	97.3 ± 1.3	N/A	N/A	N/A	N/A
$CD3^+CD4^-CD8^+$	49.5 ± 4.1	0.1 ± 0.0	N/A	N/A	N/A	N/A
$CD3^+CD4^+CD8^+$	0.7 ± 0.7	0.3 ± 0.2	N/A	N/A	N/A	N/A
% of $CD3^-$ T cells						
$CD3^-CD19^+CD56^-$	42.3 ± 7.8	24.0 ± 25.9	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^+$	51.1 ± 7.3	10.2 ± 8.5	N/A	N/A	N/A	N/A
$CD3^-CD19^+CD56^+$	0.8 ± 1.2	0.8 ± 0.4	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^-$	7.2 ± 4.9	65.0 ± 27.1	N/A	N/A	N/A	N/A

N/A = Not available; [b] = 4 subjects (data from one donor was ruled out due to machine error); [a] = gating of $CD45^+$ lymphocytes was 100% of $CD45^+CD3^+$ T cells.

Table 2
Phenotypes of whole blood, isolated $CD4^+$ T cells, and anti-CD3/28 expanded $CD4^+$ T cells from healthy volunteers expanded in culture bags for large-scale production (mean ± SD, $n = 5$).

Phenotypes	Whole blood	Isolated $CD4^+$ T cells	Anti-CD3/28 expanded $CD4^+$ T cells*	
	Day 0	Day 0	Day 14	Day 21
% of lymphocytes				
$CD3^+CD4^-CD8^-$	4.7 ± 1.5	1.3 ± 0.4	0.3 ± 0.1	0.8 ± 0.3
$CD3^+CD4^+CD8^-$	37.5 ± 7.2	97.5 ± 1.2	99.1 ± 0.3	98.0 ± 0.6
$CD3^+CD4^-CD8^+$	28.7 ± 6.7	0.0 ± 0.0	0.1 ± 0.1	0.8 ± 0.4
$CD3^+CD4^+CD8^+$	0.9 ± 0.3	0.1 ± 0.1	0.5 ± 0.3	0.3 ± 0.5
$CD3^-CD19^+CD56^-$	14.4 ± 5.9	0.2 ± 0.2	N/A	N/A
$CD3^-CD19^-CD56^+$	11.4 ± 3.5	0.2 ± 0.1	N/A	N/A
$CD3^-CD19^+CD56^+$	0.3 ± 0.2	0.0 ± 0.0	N/A	N/A
$CD3^-CD19^-CD56^-$	2.0 ± 1.1	0.7 ± 0.7	N/A	N/A
% of $CD3^+$ T cells				
$CD3^+CD4^-CD8^-$	6.8 ± 2.1	1.3 ± 0.4	N/A	N/A
$CD3^+CD4^+CD8^-$	52.1 ± 9.4	98.6 ± 0.5	N/A	N/A
$CD3^+CD4^-CD8^+$	39.9 ± 8.7	0.0 ± 0.0	N/A	N/A
$CD3^+CD4^+CD8^+$	1.2 ± 0.6	0.1 ± 0.1	N/A	N/A
% of $CD3^-$ T cells				
$CD3^-CD19^+CD56^-$	41.0 ± 11.1	20.6 ± 17.6	N/A	N/A
$CD3^-CD19^-CD56^+$	51.2 ± 10.8	17.0 ± 12.7	N/A	N/A
$CD3^-CD19^+CD56^+$	1.0 ± 0.5	1.3 ± 1.9	N/A	N/A
$CD3^-CD19^-CD56^-$	6.8 ± 2.0	61.1 ± 18.8	N/A	N/A

N/A = Not available; * = gating of $CD45^+$ lymphocytes was 100% of $CD45^+CD3^+$ T cells.

Table 3
Phenotypes of cryopreserved $CD4^+$ T cells, and anti-CD3/28 expanded $CD4^+$ T cells from cryopreserved HIV-infected samples (CD4 counts ≥ 500 cells/μL) expanded in bags with IL-2 supplementation (mean ± SD, $n = 3$).

Phenotypes	Cryopreserved $CD4^+$ T cells	Anti-CD3/28 expanded $CD4^+$ T cells*	
	Day 0	Day 14	Day 21
% of lymphocytes			
$CD3^+CD4^-CD8^-$	2.2 ± 1.1	4.7 ± 2.5	2.5 ± 2.3
$CD3^+CD4^+CD8^-$	93.6 ± 6.6	94.9 ± 2.3	95.9 ± 2.8
$CD3^+CD4^-CD8^+$	0.0 ± 0.0	0.1 ± 0.0	0.5 ± 0.5
$CD3^+CD4^+CD8^+$	0.0 ± 0.0	0.3 ± 0.2	1.1 ± 0.2
$CD3^-CD19^+CD56^-$	0.4 ± 0.5	N/A	N/A
$CD3^-CD19^-CD56^+$	0.5 ± 0.9	N/A	N/A
$CD3^-CD19^+CD56^+$	0.0 ± 0.0	N/A	N/A
$CD3^-CD19^-CD56^-$	3.1 ± 4.3	N/A	N/A

N/A = Not available; * = gating of $CD45^+$ lymphocytes was 100% of $CD45^+CD3^+$ T cells.

after stimulation with PMA/I which is a polyclonal activator. The average percentage of CD69 expressing cells among anti-CD3/28 expanded $CD4^+$ T cells was 95.4 ± 1.4%. Of these activated cells, the average percentage of 76.2 ± 5.0% was cytokine producing cells. Different types of cytokine producing cells including TNF-α, IL-2, IFN-γ, IL-4, IL-17 and TGF-β are shown in Fig. 3A. The major population among total cytokine producing cells was TNF-α producing cells with the average frequency of 81.4 ± 2.5%, followed by IL-2 producing cells (67.3 ± 15.1%). Determination of Th1 cells based on IFN-γ production showed the average percentage of 29.7 ± 11.9%. Low levels of Th2 and Th17 cells were detected based on the production of IL-4 and IL-17 (1.5 ± 0.9% and 1.2 ± 1.2%, respectively). On the contrary, the frequency of Treg cells based on TGF-β production was almost undetectable. When cytokine producing cell populations showing simultaneous production were determined, up to 99% of cytokine producing cells belongs to 3 major populations including single, double and triple cytokine producing cells with the average percentages of 34.3 ± 5.8%, 50.6 ± 3.9% and 14.5 ± 7.4%, respectively (Fig. 3B).

Proportions of specific-cytokine producing cell subsets determined among single, double and triple cytokine producing cells are presented in Fig. 4. For single cytokine producing cells, the majority was TNF-α producing cell with the average percentage of 51.1 ± 12.6%, followed by IL-2 and IFN-γ producing cells (32.8 ± 13.8% and 15.2 ± 4.3%, respectively). With respect of double cytokine producing cells, the highest frequency was observed for $TNF\text{-}\alpha^+$ $IL\text{-}2^+$ producing cells with the average percentage of 77.7 ± 14.7%, followed by the other 2 main populations including $TNF\text{-}\alpha^+$ $IFN\text{-}\gamma^+$ (16.8 ± 14.4%) and $IFN\text{-}\gamma^+$ $IL\text{-}2^+$ (4.1 ± 3.0%). For triple cytokine producing cells, $TNF\text{-}\alpha^+$ $IL\text{-}2^+$ $IFN\text{-}\gamma^+$ producing cells showed the highest frequency with the average percentage of 89.2 ± 7.4%, followed by $TNF\text{-}\alpha^+$ $IL\text{-}2^+$ $IL\text{-}4^+$ (4.6 ± 3.7%), and $TNF\text{-}\alpha^+$ $IL\text{-}2^+$ $IL\text{-}17^+$ (2.4 ± 1.8%).

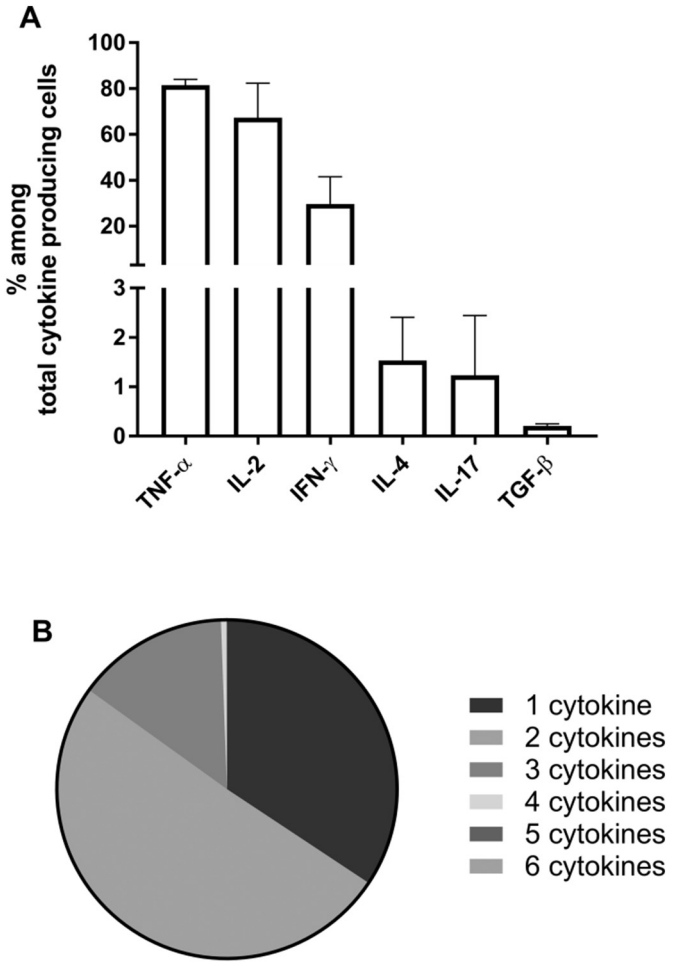

Fig. 3. Cytokine production profiles including TNF-α, IL-2, IFN-γ, IL-4, IL-17, and TGF-β of anti-CD3/28 expanded $CD4^+$ T cells: (A) percentages (mean ± SD) of different cytokine producing cells; (B) average proportions of polyfunctional cytokine producing cells ($n = 3$).

3.5. Residual bead detection and sterility testing

To ensure safety and sterility of the final products due to magnetic beads and microorganism contamination, a small-scale bead removal process was performed together with sterility testing on the final cell culture supernatants (day 21) and harvested expanded $CD4^+$ T cells. Since a large volume of culture medium containing expanded $CD4^+$ T cells was harvested, the sample was concentrated by centrifugation before bead removal. By using a small scale depletion method, the average number of 5.5×10^4 residual beads per 10^7 expanded cell products was measured. Since the average number of 414.9×10^6 cells was harvested on day 21, the average number of 2.3×10^6 residual beads may be obtained in the final products. While the average number of beads before harvesting was 13.6×10^6 beads (average cell number for reactivation on day 7), the result showed 83% depletion of beads. For sterility testing, a 14-day incubation period in specialized media was performed according to USP. The sterile results were presented as the culture supernatant colleting on the final day of cell expansion and expanded cells from cryopreservation were free from any bacterial and fungal contaminations.

4. Discussion

This report introduces a promising closed-culture system for anti-CD3/28 expanded $CD4^+$ T lymphocytes by using GMP-grade culture bags. Immunorosette formation was chosen for $CD4^+$ T cell purification in our study as this method showed the best expansion (1000-fold) with high viability (90%) and high purity of $CD4^+$ T cells (> 95%) after a 3-week culture with anti-CD3/28 activation in flasks when compared to other methods (*i.e.*, negative selection and CD8 depletion) (Onlamoon et al., 2013). Our phenotypic profiles for all freshly isolated $CD4^+$ T cells confirm effectiveness and reproducibility of the immunorosette formation technique with high purity of $CD3^+CD4^+CD8^-$ populations (> 91% of lymphocytes).

Levine et al. (1997) conducted a long-term *in vitro* proliferation of polyclonal adult $CD4^+$ T cells using immobilized anti-CD3/28 stimulation in flasks, showing an exponential growth of $CD4^+$ T cells for over 60 days (Levine et al., 1997). The culture condition with IL-2 supplement provided significant higher expansion than that without IL-2 supplementation (10^9- to 10^{11}-fold and 10^5-fold, respectively). However, Onlamoon et al. (2013) presented that autocrine cytokines were sufficient to support proliferation of anti-CD3/28 expanded $CD4^+$ T cells in absence of IL-2 throughout a 3-week culture (1000-fold expansion) (Onlamoon et al., 2013). Our enriched $CD4^+$ T cells expanded in flasks thus underpin good proliferation of approximately 797-fold after 21 days without IL-2 supplementation.

GMP-grade culture bags are in a concern to reduce contamination risks from microorganisms during a cell production aiming for treatment in clinics. GMP-grade Teflon cell culture (TCC) bags were used to compare a growth ability of normal human $CD8^+$ T cells with conventional tissue culture flasks and plates (Garland et al., 1999). Expanded $CD8^+$ T cells in TCC bags yielded considerably higher than those in flasks and plates after 10 days (73-fold, 41-fold and 48-fold, respectively). Nevertheless, our study shows that anti-CD3/28 expanded $CD4^+$ T cells from healthy donors in flasks proliferated better than those in commercially available GMP-grade culture bags (797-fold and 331-fold, respectively) after 21 days. Even so, fold expansion of

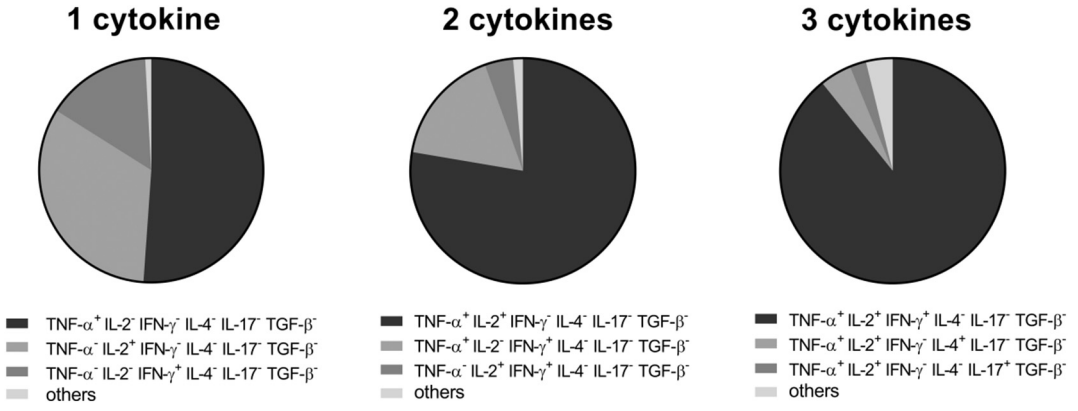

Fig. 4. Average proportions of polyfunctional cytokine producing cell subsets in single, double and triple cytokine producing cells ($n = 3$).

$CD4^+$ T cells from bag culture is still considerably high enough for cell production. High cell viability (75%) and high purity (97%) of expanded $CD4^+$ T cells also support the use of bag for cell expansion.

With respect to implementation of *in vitro* $CD4^+$ T lymphocyte expansion for adoptive immunotherapy in human, there is a clinical study conducting adoptive transfer of up to 3×10^{10} autologous anti-CD3/28 activated $CD4^+$ T cells in HIV-infected patients and the transfusion was scheduled approximately 5×10^9 cells at 8-week intervals (Bernstein et al., 2004; Levine et al., 2002), indicating that large-scale expanded $CD4^+$ T lymphocytes is required. A large-scale production of $CD4^+$ T cells from HIV-1-infected donors using anti-CD3/28 coated beads, IL-2 supplementation, and gas-permeable bags was established and achieved 37-fold expansion after 14 days (Levine et al., 1998) which is not yet satisfied for $CD4^+$ T cell numbers demanded for transfusion. Therefore, our study provides an improved expansion method under the closed-culture system for large-scale anti-CD3/28 expanded $CD4^+$ T cells from healthy volunteers with 109-fold increase after 21 days.

For expansion of anti-CD3/28 activated $CD4^+$ T cells from HIV-infected patients in our developed closed-culture system, $CD4^+$ T cells from patients with CD4 count > 500 cells/μL were selected for the study. Anti-CD3/28 expanded $CD4^+$ T cells from HIV-infected patients with CD4 count > 500 cells/μL were found to have better proliferation ability than patients with CD4 counts 200–500 and < 200 cells/μL (Onlamoon et al., 2015). They also reached 1000-fold expansion when supplemented with IL-2 which was quite similar level as seen in healthy volunteers (Onlamoon et al., 2013). Nevertheless, freshly isolated $CD4^+$ T cells from the patients may be not usually accessible and cryopreserved $CD4^+$ T cells become substitute. Frozen $CD4^+$ T cells from healthy donors were successfully expanded (data not shown) before we carried out the expansion of those from the patients. Our results show that anti-CD3/28 activated $CD4^+$ T cells from cryopreserved cells of HIV-infected patients still remained their growth ability even after a long storage (1.5–2 years). The proliferation was great with 415-fold when IL-2 in presence for growth enhancement, although it was only half of expanded cells from flask culture system of freshly isolated $CD4^+$ T cells from HIV-infected patients reported previously (Onlamoon et al., 2013). This achievement may be resulted from ability of IL-2 for restoration of $CD4^+$ T cell proliferation by either prevention or reversion of T cell unresponsiveness. Although using IL-2 supplementation was considered to tentatively promote apoptosis of expanded cells which rapidly disappeared after transfusion (Tan et al., 1999), our protocol still provides high cell viability (85%) at the beginning and indeed requires further investigation for a relation between anti-CD3/28 stimulation with IL-2 supplementation and induction of apoptotic signaling molecules (*e.g.*, Bcl-2, Bcl-xL and capase-3).

Previous studies showed that expansion of both human and rhesus macaque $CD4^+$ T cells with anti-CD3/28 coated beads provided a large number of homogenous $CD4^+$ T cell population with Th1-like phenotype (Levine et al., 1996; Brice et al., 1998). Moreover, TNF-α production of anti-CD3/28 expanded $CD4^+$ T cells played an important role in regulating synthesis of various β-chemokines (Brice et al., 2000) which support cellular resistance to R5-tropic HIV strain. Our results show that TNF-α producing cells were a major population presenting in the expanded cells which can play a protective role in resistance to infection. The presence of high frequency of IL-2 producing cells can also provide sufficient amount of IL-2 that supports *in vitro* expansion without exogenous IL-2 supplementation. Interestingly, although only one-third of anti-CD3/28 expanded $CD4^+$ T cells showed Th1-like phenotype, other $CD4^+$ T cell types including Th2, Th17 and Treg were almost diminished suggesting that anti-CD3/28 expansion promotes Th1 dominant population of the expanded cells. More importantly, simultaneous detection of cytokine production at a single cell level by polychromatic flow cytometry demonstrates that the majority of expanded cells simultaneously produced 2–3 cytokines (approximately 65%), suggesting a polyfunctional role of these cells.

With respect of safety concern, before releasing the expanded cell product for cell transfusion, anti-CD3/28 coated beads have to be removed prior to preparing the final product due to the potential toxicity hazard associated with the administration of microspheres. Interestingly, the previous study showed the absence of an adverse toxicity in a rat model receiving an intravenous administration of sheep anti-mouse immunoglobulin G coated paramagnetic-polystyrene beads at a high level of bead exposure (White et al., 1995). However, a removal of beads from cell therapy products has become a general practice for the manufacturing of *in vitro* expanded T cell products (Hollyman et al., 2009; Tumaini et al., 2013). In these studies, a large-scale depletion of anti-CD3/28 coated beads was achieved by using the Dynal ClinExVIVO magnetic particle concentrator magnet which consists of a large primary magnet allowing the major removal of beads and a smaller secondary magnet that traps residual beads. The effectiveness of bead depletion was assessed in a final product for the presence of residual beads by lysing 10^7 cells of the final product in chloride bleach. The lysed sample was centrifuged and supernatant was removed. The pellet was then re-suspended in a buffer and placed on a hemacytometer for residual bead counting and calculation of residual bead quantity. While approximately 80% bead depletion was achieved in this study, a higher depletion level may be obtained by using a large-scale depletion method.

More importantly, sterility testing must be performed on cell therapy products in order to ensure that the products are free of bacterial and fungal contamination. Sterility testing can be conducted in a hospital microbiology laboratory where Gram staining and a 14-day culture in culture medium detecting aerobic and anaerobic microorganisms are tested. In this study, all cell culture supernatants and expanded cells of the final products had no contamination of bacteria and fungi when testing with standard USP methods. Alternatively, automated culture methods such as Bact/ALERT (bioMérieux) and Bactec (Becton Dickinson) have already been validated and used for sterility testing of cell therapy products (Khuu et al., 2006; Hocquet et al., 2014). The routine evaluation for sterility testing of cell therapy products also showed the effectiveness in decreasing rate of contamination of cell harvest, suggesting the significances of routine sterility testing (Golay et al., 2018).

5. Conclusions

Results from this study address that our developed closed-culture system for $CD4^+$ T lymphocyte expansion using anti-CD3/28 coated beads and GMP-grade gas-permeable culture bags is practical, effective, and reproducible. Using our protocol, a large number of anti-CD3/28 expanded $CD4^+$ T lymphocytes is achieved together with high viability and high purity within reasonable time (21 days). This method is also successful to expand both freshly isolated $CD4^+$ T cells from healthy volunteers and cryopreserved $CD4^+$ T cells from HIV-infected patients. It is then suggested that our refined procedure is useful for cell production used for adoptive transfer immunotherapy, although, there are still many aspects require further improved and investigated, such as effects of different culture media, impact of exogenous supplementation, and cell manufacturing processes under GMP standard.

Conflicts of interest

None.

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Biological Procedures Online

Comparison of serum supplementation and culture medium for CD4+ T cell expansion by using anti-CD3/28 coated beads

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Abstract:	Background: Although several in vitro expansion methods for CD4+ T cells have been established for adoptive immunotherapy, the information about effects of commercially available GMP-grade media with serum supplementation in cell expansion and functions is limited. Results: Isolated CD4+ T cells from healthy donors were stimulated with anti-CD3/28 coated beads and expanded in different media supplemented with either human serum (HS) or fetal bovine serum (FBS) for 3 weeks. Fold expansion, cell viability, growth kinetics and phenotypic characters were periodically observed throughout the culture period. Cytokine production of IL-2, IL-4, IL-17, IFN-γ, TNF-α and TGF-β of the expanded cells was detected by intracellular cytokine staining. Results showed that the expanded cells from the cultures using AIM-V and DC media with 10% HS were well expanded (~255-fold) and had similar polyfunctional patterns, however, they showed poorer proliferation rates than those from the cultures using RMPI1640 with 10% FBS (R10; ~637-fold). Moreover, cell viability of the expanded cells from R10 was also significantly higher than the others after a 3-week culture. High purity with CD3+CD4+CD8- phenotype of the expanded cells from all cultures was also observed. The expanded cells from all conditions were found predominantly producing TNF-α, IL-2 and IFN-γ and frequency of IL-2 producing cells in R10 was the highest. Conclusions: Our expansion protocol using GMP-grade media, AIM-V and DC with HS supplementation rendered satisfied proliferation rates and yields without differences in polyfunctional patterns, indicating the promising method for further uses in clinical trials. Addition of FBS is also suggested for expansion improvement but under the condition that its source must be free from bovine spongiform encephalopathy.
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Comparison of serum supplementation and culture medium for CD4$^+$ T cell expansion by using anti-CD3/28 coated beads

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ABSTRACT

Background: Although several *in vitro* expansion methods for $CD4^+$ T cells have been established for adoptive immunotherapy, the information about effects of commercially available GMP-grade media with serum supplementation in cell expansion and functions is limited.

Results: Isolated $CD4^+$ T cells from healthy donors were stimulated with anti-CD3/28 coated beads and expanded in different media supplemented with either human serum (HS) or fetal bovine serum (FBS) for 3 weeks. Fold expansion, cell viability, growth kinetics and phenotypic characters were periodically observed throughout the culture period. Cytokine production of IL-2, IL-4, IL-17, IFN-γ, TNF-α and TGF-β of the expanded cells was detected by intracellular cytokine staining. Results showed that the expanded cells from the cultures using AIM-V and DC media with 10% HS were well expanded (~255-fold) and had similar polyfunctional patterns, however, they showed poorer proliferation rates than those from the cultures using RMPI1640 with 10% FBS (R10; ~637-fold). Moreover, cell viability of the expanded cells from R10 was also significantly higher than the others after a 3-week culture. High purity with $CD3^+CD4^+CD8^-$ phenotype of the expanded cells from all cultures was also observed. The expanded cells from all conditions were found predominantly producing TNF-α, IL-2 and IFN-γ and frequency of IL-2 producing cells in R10 was the highest.

Conclusions: Our expansion protocol using GMP-grade media, AIM-V and DC with HS supplementation rendered satisfied proliferation rates and yields without differences in polyfunctional patterns, indicating the promising method for further uses in clinical trials. Addition of FBS is also suggested for expansion improvement but under the condition that its source must be free from bovine spongiform encephalopathy.

Keywords: $CD4^+$ T lymphocytes; anti-CD3/28 coated beads; *in vitro* cell expansion; culture medium; serum supplementation.

BACKGROUND

Anti-CD3/28 co-stimulation is an effective method to induce *in vitro* expansion of $CD4^+$ T cells. A major advantage of this method is that anti-CD3/28 expanded $CD4^+$ T cells became resistant to the infection of M-tropic isolates of human immunodeficiency virus (HIV)-1 [1]. Some experiments in non-human primates showed that expanded autologous $CD4^+$ T cells from pre- and post- simian immunodeficiency virus (SIV) infection promoted the immune responses for viral suppression and plasma viremia control by using in combination with a transient initial antiretroviral treatment in SIV-infected models [2,3]. An adoptive transfer of the expanded autologous $CD4^+$ T cells also exhibited an increased level of $CD4^+$ T cells in antiretroviral-treated HIV-infected patients [4]. Therefore, the optimized protocol of a large-scale $CD4^+$ T cell expansion is warranted for clinical uses. This expanded $CD4^+$ T cell reinfusion can be beneficial for improvement in immune restoration and become an alternative treatment for HIV-infected patients with immunological discordance.

Several studies demonstrated many parameters affecting T cell proliferation, such as cell isolation, disease progression, and culture media. Onlamoon *et al.* (2013) determined the effect of cell isolation on cell expansion rates and cell phenotypes. Of three different $CD4^+$ T cell isolation methods including immunorosette formation, negative selection and CD8 depletion, enriched $CD4^+$ T lymphocytes from normal whole blood by immunorosette formation exhibited the highest yield of over 1,000-fold expansion with good viability (90%) and high purity of $CD4^+$ T lymphocytes (> 95%) within three weeks of culture. $CD4^+$ T lymphocyte enrichment through immunorosette formation thus provided the optimal isolation method for a large-scale expansion of anti-CD3/28 expanded $CD4^+$ T lymphocytes [5].

Following the same protocol, the expansion of $CD4^+$ T cells from the HIV-infected patients was conducted and found different growth kinetics among the patients with CD4 counts < 200, 200-500, and > 500 cells/μL [6]. Isolated $CD4^+$ T cells from the patients still retained proliferation ability and their yields depended on disease progression. Even so, their fold expansion numbers were still lower than those of healthy volunteers. IL-2 supplementation at the concentration of 100 U/mL was also found to significantly help increasing fold expansion of $CD4^+$ T cells from HIV-infected patients with both low and high CD4 counts (< 200 and > 500 cells/μL, respectively). However, $CD4^+$ T cells isolated from the patients with more progressive disease (CD4 count < 200 cell/μL) showed lower rates in cell survival and

proliferative responses to anti-CD3/CD28 co-stimulation with IL-2 supplementation when compared to those from HIV-infected patients with high CD4 counts [6].

For media supplementation, basal culture media are generally supplemented with animal serum (e.g., fetal bovine serum (FBS)) which is essential for cell growth, metabolism and proliferation. The functions of serum in culture media are (i) to provide hormonal factors stimulating cell growth, proliferation and differentiation, (ii) to transport proteins carrying hormones, minerals, trace elements and lipids, (iii) to attach and spread factors, acting as germination points for cell attachment and (iv) to stabilize and detoxify factors needed for maintaining pH as well as to inhibit proteases and other toxic molecules. Nevertheless, the use of animal serum, particularly FBS, has been debated in some serious scientific and ethical concerns in terms of serum production and cell harvesting. Development of serum-free or animal/human protein-free media is then important for safety improvement in biological products for cell therapy and vaccination [7].

FBS can be substituted with defined chemical components in serum-free cell culture. Trickett *et al.* (2002) demonstrated that the expansion of functional T lymphocytes from HIV-infected patients was good when stimulated with anti-CD3/28 coated microspheres and propagated in serum-free media. However, the greater T cell proliferation was observed when supplemented with FBS in the initial period of cell expansion, whereas, human albumin (AB) serum supplementation failed to increase T cell numbers. Plasma supplementation also provided a low level of $CD4^+$ T cells which was resulted from phenotypic switching of $CD8^+$ T lymphocytes [8]. Carlens *et al.* (2000) studied expansion rates of anti-CD3 stimulated T lymphocytes in three different serum-free media (i.e., X-VIVO 15, AIM-V and Cellgro SCGM) compared to standard RPMI1640 media with 5% human serum (HS) and 10% FBS. A 3-week activation in serum-free media resulted in a small increase in expansion rates, whereas the culture with serum supplementation rendered better consistency and effectiveness in cell expansion. The additional low level of HS thus supported the T-cell expansion in all culture media types. On the other hands, supplementation with 10% FBS showed inconsistency in cell expansion when compared to that with 5% HS. Increased levels of IFN-γ secretion were detected for all media combinations when compared with serum-free culture. No IL-4 and IL-10 production was found [9].

Although the *in vitro* cultures with HS supplementation supported better $CD4^+$ T cell expansion than those with serum-free media and more consistency than those with FBS

supplementation, the fold expansion numbers (< 267-fold) [9] was not high when compared to our previous method [5]. While GMP-grade culture media are required for cell production, the information about the effect of serum supplementation in cell functions is limited. Our study, therefore, aimed to assess the expansion rates and yields of expanded $CD4^+$ T cells by using commercially available GMP-grade culture media with HS supplementation in comparison with the established protocol. Their cytokine production was also investigated to observe the potential diversity of cell functions.

RESULTS

Expanded $CD4^+$ T lymphocytes in different culture conditions after anti-CD3/28 coated bead stimulation

To assess an optimal cell culture condition for *in vitro* expansion of $CD4^+$ T lymphocytes by using anti-CD3/28 coated beads, different culture conditions based on serum supplementation and culture media usages were compared. Fold expansion, cell viability, growth kinetic and phenotypic characters from different culture conditions were observed for cell expansion efficiency.

Fold expansion numbers of $CD4^+$ T lymphocytes expanded in all culture conditions were similar during the first 2-week period. After that, the expanded cells cultured in RPMI1640 with 10% FBS (R10) were dramatically increased on day 17 (755.7 ± 337.1-fold), whereas the other cultures maintained similar expansion rates (Fig. 1A). At the end of the culture period (day 21), slight decreases were found in every culture media. The culture in R10 showed the highest fold expansion (637.1 ± 265.3 –fold) which was approximately 2.5-fold higher than the other culture conditions with 10% HS (< 255-fold). No significant difference was observed among 10% HS supplemented media including GMP-grade media (i.e., AIM-V and DC) and standard media (i.e., RPMI1640).

Gradual declines in viable cells were observed throughout the 21-day culture period (Fig. 1B). All culture conditions exhibited a similar pattern in cell viability at over 70% during the first two weeks. Only the culture in R10 still maintained high numbers of viable cells on day 17 (75.9 ± 11.0%) before markedly decreasing on day 21 (54.8 ± 5.8%). On the other hand, the other culture media including RPMI1640, AIM-V and DC supplemented with 10% HS showed notable decreases since day 17 (47.1 ± 7.7%, 56.0 ± 6.4% and 52.6 ± 5.5%, respectively) and lowered to less than 36% on day 21.

Immunophenotypes of whole blood, isolated $CD4^+$ T lymphocytes, and anti-CD3/28 expanded $CD4^+$ T lymphocytes were determined by a flow cytometer (Table 1). Phenotypic profiles revealed that purification of $CD4^+$ T cells from whole blood by using the immunorosette formation technique provided high frequency of $CD3^+CD4^+CD8^-$ subset (> 95% of lymphocytes). After a 3-week culture, anti-CD3/28 expanded $CD4^+$ T cells ($CD3^+CD4^+CD8^-$) from R10 and DC with 10% HS exhibited high frequencies (> 90% of lymphocytes), whereas slightly lower frequencies were observed for RPMI1640 and AIM-V with 10% HS. The cell purity was also confirmed with low frequencies of $CD3^+CD4^-CD8^+$ and $CD3^+CD4^+CD8^+$ populations at the end of the expansion period.

Table 1 Lymphocyte subset analyses of whole blood, isolated $CD4^+$ T cells, and anti-CD3/28 expanded $CD4^+$ T cells (mean ± SD, n = 5).

Phenotypes	**Whole blood**	**Isolated $CD4^+$ T cells**	**Anti-CD3/28 expanded $CD4^+$ T cells**			
			10% FBS	**10% HS**		
			RPMI1640*	**RPMI1640**	**AIM-V**	**DC**
% of lymphocytes						
$CD3^+CD4^-CD8^-$	6.1 ± 1.9	1.6 ± 0.6	5.7 ± 7.9	7.4 ± 9.3	10.0 ± 6.4	3.5 ± 2.1
$CD3^+CD4^+CD8^-$	34.7 ± 6.2	96.0 ± 2.6	91.9 ± 8.1	89.0 ± 11.3	84.8 ± 7.5	94.1 ± 3.9
$CD3^+CD4^-CD8^+$	30.3 ± 7.2	0.0 ± 0.0	1.0 ± 0.8	2.5 ± 2.2	3.6 ± 1.8	1.2 ± 1.2
$CD3^+CD4^+CD8^+$	0.5 ± 0.3	0.1 ± 0.1	1.4 ± 1.0	0.9 ± 0.5	1.5 ± 1.3	1.0 ± 0.9
$CD3^-CD19^+CD56^-$	11.2 ± 5.1	0.5 ± 0.8	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^+$	15.8 ± 7.4	0.6 ± 0.7	N/A	N/A	N/A	N/A
$CD3^-CD19^+CD56^+$	0.3 ± 0.3	0.0 ± 0.1	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^-$	1.1 ± 0.2	1.0 ± 1.2	N/A	N/A	N/A	N/A

N/A = Not available as gated population of $CD45^+$ lymphocytes was 100% of $CD45^+CD3^+$ T cells.
*RPMI1640 with 10% FBS is R10

Determination of cytokines produced by anti-CD3/28 expanded $CD4^+$ T cells

Although a homogenous population of expanded $CD4^+$ T lymphocytes was observed in all culture conditions with different degree of expansion ability, it remains unclear whether there are any functional changes induced by different culture conditions. Anti-CD3/28 expanded $CD4^+$ T cells from various culture conditions were then investigated in this study for their functional variances based on types of cytokines produced by these cells.

To determine the levels of activated cells among anti-CD3/28 expanded $CD4^+$ T cells after stimulation with PMA/I, the CD69 expression levels were assessed. There was no significant difference in frequencies of CD69 expressing cells among different culture conditions (Fig. 2A). These CD69 expressing cell populations were also consequently determined for cytokine producing cells in order to ensure the presence of cytokine production in the activated cells. While total cytokine producing cells (i.e., a combination of all types of cytokine producing cells) from all culture conditions were not significantly different, the expanded cells from R10 exhibited higher percentages of total cytokine producing cells than the others (Fig. 2B).

When different cytokine-producing cell populations including TNF-α, IL-2, IFN-γ, IL-4, IL-17 and TGF-β were determined, the major population among total cytokine-producing cells was TNF-α producing cells (Fig. 3A). No difference in all types of cytokine-producing cells was observed among different culture conditions, except IL-2 producing cells with a significant high percentage (58.0 ± 18.6 % of $CD4^+CD69^+$ T cells) from the R10 culture. Ability in simultaneous production of cytokines was also determined. Results demonstrated that up to 99% of cytokine-producing cells from all culture conditions belong to 3 main populations including single, double and triple cytokine-producing cells in which a single cytokine-producing cell population showed the highest percentages of over 50% of total cytokine-producing cells (Fig. 3B).

The 3 main populations of single, double and triple cytokine-producing cells were then evaluated for specific-cytokine producing cell subsets. IL-2 producing cells were predominantly found in the single cytokine-producing cells from all culture conditions. For the double cytokine-producing cells, TNF-α^+ IL-2^+ producing cells showed the highest frequency for all culture conditions, except the DC culture presenting the highest proportion of IFN-γ^+ IL-2^+ producing cells instead. The triple cytokine-producing cells in all media supplemented with 10% HS exhibited TNF-α^+ IL-2^+ IFN-γ^+ producing cells as a majority, whereas TNF-α^+ IL-2^+ IL-4^+ producing cells were found predominant in the R10 culture (data not shown).

DISCUSSION

Over the last decade, manufacturing processes for T-cell therapy have been extensively refined to improve the quality of effector cells and to increase the speed of production [4,10]. Adoptive transfer of anti-CD3/28 expanded $CD4^+$ T lymphocytes was already achieved in HIV-infected patients and SIV-infected nonhuman primates for effectiveness in their immune responses [2-4,11], suggesting a promising treatment strategy for HIV-infected patients. According to our previous studies, the isolation and *ex vivo* expansion protocols of $CD4^+$ T cells from healthy and HIV-infected individuals by using anti-CD3/28 coated bead stimulation were optimized [5,6]. Even so, the platform of clinical cell transfusion for patient safety is still demanded for further investigation and was thus proposed in this study.

To optimize the protocol for clinical adoptive T-cell therapy, other serum supplements besides FBS were considered in this study. HS was chosen for $CD4^+$ T cell expansion development as it is commercially available and can be obtained as human AB serum. The culture media including RPMI1640, AIM-V and DC were then added with 10% HS for expansion comparison with RPMI1640 supplemented with 10% FBS. Culture media with 10% HS supplementation supported $CD4^+$ T cell expansion similar to normal culture medium (i.e., culture medium with 10% FBS supplementation or R10 in our study) in the first 2 weeks of culture. The growth rates of expanded cells from all culture media were 224-330 -fold and cell viability was approximately 70% after two weeks of cell activation and expansion. Fold expansions of all media, except R10, maintained at the same levels on day 17 before gradual declines on day 21. Unlike the others, fold expansion of R10 was considerably augmented on day 17 and then decreased on day 21. However, the expanded cells from the R10 culture on day 21 still proliferated ~2–fold higher than those on day 14. $CD4^+$ T cell expansion in R10 provided the highest growth rate and the most consistency when compared to that in the others. Of all cultures using specialized media, RPMI1640 supplemented with 10% HS rendered the lowest value of fold expansion after a 3-week culture. It is then assumed that FBS supplementation render the higher expansion rates, and yields as well as better consistency than HS supplementation. Our findings are opposed to the previous study demonstrating that 10% FBS provided inconsistency in cell expansion when compared to HS supplementation [9]. Therefore, FBS supplementation can be a choice of interest for clinical uses to achieve the best expansion as long as its source is from an area free of bovine spongiform encephalopathy (BSE), such as Australia or New Zealand [12].

Furthermore, AIM-V and DC with 10% HS supplementation were able to provide suitable $CD4^+$ T cell expansion with average 250-fold which is similar to the results achieved from AIM-V and DC with 5% HS supplementation from the previous study [9]. It is suggested that 5% HS supplementation is sufficient to support $CD4^+$ T cell expansion and higher concentration of HS does not improve the proliferation rates and yields. Both AIM-V and DC are also good candidates as GMP-grade media used for cell expansion following requirement of clinical trials.

Besides FBS and HS supplementation, a xeno-free serum replacement (SR) was introduced as a novel platform strategy for clinical-grade cell manufacturing [13]. In this study, T-cell expansion was conducted by using a serum-free medium AIM-V supplemented with 5% SR and anti-CD3/28 coated bead stimulation. The expansion kinetic of T cells using SR was comparable and polyfunctional profiles were similar to that using FBS and HS [13]. SR supplementation, therefore, has become an alternative choice for FBS and HS substitution and for clinical-grade cell production since a potential risk of cross-species contamination can be avoided. Addition of exogenous cytokines, particularly IL-2, is worth considerable for improvement in cell expansion. Previous studies showed that growth rates of the expanded T cells cultured in media with HS and IL-2 supplementation were increased when compared to those without IL-2 supplementation [9,13]. In this case, addition of HS and IL-2 together can synergistically promote the cell expansion.

Regarding to functions of the expanded $CD4^+$ T cells, previous studies revealed that $CD4^+$ T cell population with type 1 T helper cell (Th1)-like phenotype was expanded when *in vitro* expansion of both human and rhesus macaque $CD4^+$ T cells was performed by using anti-CD3/28 coated beads [14,15]. We found that anti-CD3/28 expanded $CD4^+$ T cells produced 3 major cytokines including TNF-α, IL-2 and IFN-γ. Interestingly, a frequency of IL-2 producing cells in the culture condition using FBS was the highest which was correlated to its superior fold expansion when compared to the other conditions. This can presume that a higher level of IL-2 released from these cells under FBS supplementation may self-support such high *in vitro* expansion. A simultaneous detection of cytokine production at a single cell level also confirmed the presence of IL-2 producing cells in the largest proportion when compared to other cytokines, highlighting its significant role in promoting cell expansion.

CONCLUSIONS

This study addresses the expansion and polyfunctional profiles of expanded CD4+ T cells when cultured in commercially available GMP-grade media with human serum supplementation. We found that AIM-V and DC are good candidates for clinical-grade cell manufacturing and HS supplementation can well support cell expansion. Although animal-derived substances, mainly FBS, is not recommended to use in cell products due to their risk in cross-species contamination, FBS supplementation is still able to render the highest fold expansion with high viability and consistency when compared to HS. There is also no significant difference in polyfunctional profiles between FBS and HS. To use FBS for cell production, its origin must be free of BSE. Therefore, our expansion protocol using either AIM-V or DC supplemented with HS provides satisfied yields of the expanded cells of which polyfunctions are similar, suggesting the suitable protocol for further uses in clinical studies.

MATERIALS AND METHODS

Samples

Five healthy volunteers were recruited and signed informed consents which were approved by the Institutional Review Board of the Faculty of Medicine Siriraj Hospital at Mahidol University. Sixteen to twenty-five milliliters of whole blood were collected into sodium heparin-containing vacutainer tubes and kept at room temperature prior to phenotypic determination of whole blood and $CD4^+$ T lymphocyte isolation.

Antibodies

Monoclonal antibodies (mAbs) and their conjugated fluorochromes including anti-CD3 conjugated with fluorescein isothiocyanate (FITC), anti-CD4 conjugated with allophycocyanin (APC), anti-CD8 conjugated with phycoerythrin (PE), anti-CD19 conjugated with allophycocyanin and cyanine dye (APC-Cy7), anti-CD45 conjugated with peridinin chlorophyll protein (PerCP), and anti-CD56 conjugated with phycoerythrin and cyanine dye (PE-Cy7) were purchased from Becton Dickinson Bioscience (BDB, San Jose, CA). In addition, anti-CD3 conjugated with AlexaFluor® (A700), anti-CD4 conjugated with Brilliant Violet™ 605 (BV605), anti-CD8 conjugated with PE/Dazzle™ 594, anti-CD69 conjugated with PerCP/Cy5.5, anti-IL-2 conjugated with BV510, anti-IL-4 conjugated with

FITC, anti-IL-17 conjugated with PE, anti-IFN-γ conjugated with APC, anti-TNF-α conjugated with BV650 and anti-TGF-β conjugated with BV421 were obtained from BioLegend (San Diego, CA).

$CD4^+$ T lymphocyte isolation

$CD4^+$ T lymphocytes were freshly isolated from whole blood through immunorosette formation by using RosetteSep® Human $CD4^+$ T cell enrichment cocktail (STEMCELL Technologies, Vancouver, BC, Canada) following the manufacturer's instruction. Purified $CD4^+$ T lymphocytes were isolated by a standard Ficoll-Hypaque gradient centrifugation (Histopaque, Sigma-Aldrich,Co., St.Louis, MO, USA) prior to phenotypic characterization and cell expansion.

Cell stimulation and expansion of purified $CD4^+$ T cells

Freshly purified $CD4^+$ T cells ($1x10^6$ cells) were stimulated with anti-CD3/28 coated beads (Dynabeads® Human T-Activator CD3/CD28, Invitrogen Dynal, Oslo, Norway) at a bead-to-cell ratio of 1:1 in a 24-well plate (Costar® 24 well clear TC-treated multiple well plates, sterile, Corning Inc., Life Sciences, NY). The stimulated $CD4^+$ T cells were then expanded in complete media composing RPMI1640 supplemented with 10% heat inactivated FBS, 50 µg/mL pencillin-streptomycin, and 2 mM L-glutamine (Gibco, Paisley, UK) at a concentration of $0.5x10^6$ cells/mL. The expanded cells were incubated at 37 °C and 5% CO_2 humidification before reactivation on day 7 at a similar bead-to-cell ratio. Cell numbers and viability were observed by using trypan blue exclusion and a TC10™ automated cell counter on days 4, 7, 11, 14, 17 and the calculated amounts of fresh media were replenished to maintain the cell suspension concentration at $0.5x10^6$ cells/mL. The cell suspensions were also transferred to T25, T75 and T175 plastic tissue culture flasks (Corning® U-shaped canted neck cell culture flask with vent cap, Corning Inc., Life Sciences, NY) on days 4, 7, 11 and maintained in T175 until the end of a 3-week culture period. Final cell numbers and viability were determined on day 21. The beads were removed and the expanded cells were re-suspended in a cryopreservation media and stored in a liquid nitrogen tank at -196 °C for further analyses.

For comparison between culture medium and serum supplementation, $1x10^6$ cells of purified $CD4^+$ T cells were expanded in 3 different culture conditions including (i) RPMI1640 supplemented with 10% heat inactivated HS (Gibco, Paisley, UK), (ii) AIM-V® medium

CTS™ (Therapeutic grade, Gibco, Paisley, UK) supplemented with 10% heat inactivated HS and (iii) CellGro® GMP serum-free dendritic cell medium (CellGro® DC medium, CellGenix GMBH, Freiburg, Germany) supplemented with 10% heat inactivated HS. Cell expansions were conducted by using the same protocol described above.

Immunofluorescence staining and analysis

For phenotypic characterization, samples of whole blood, purified and expanded $CD4^+$ T cells were stained with fluorochrome-conjugated mAbs for 15 min before adding FACS lysing solution (BDB, San Jose, CA) for cell lysis and fixation. The stained cells of at least 100,000 events were acquired for each analysis by a BD FACSVerse™ flow cytometer (BDB, San Jose, CA) and the data was analyzed by using FlowJo Software (Tree Star, San Carlos, CA). Percentages of lymphocyte subsets were determined by expressions of CD3, CD4, CD8, CD19 and CD56.

Intracellular cytokine staining (ICS) and analysis

Cryopreserved expanded $CD4^+$ T cells from different culture conditions were used to determine different types of cytokine production. Expanded $CD4^+$ T cells at $1x10^6$ cells/mL were stimulated with 25 ng phorbol 12-myristate 13-acetate (PMA) and 1 µg ionomycin (I) in the presence of brefeldin A (BFA) at 10 µg, whereas the expanded $CD4^+$ T cells in the presence of BFA without stimulation were used as a control. The samples were then incubated at 37 °C and 5% CO_2 for 4 h. After the incubation, PMA/I stimulated and unstimulated samples were stained with Zombie NIR™ dye (BioLegend, San Diego, CA) at 4 °C for 15 min. A washing buffer (phosphate buffered saline (PBS) with 2% FBS) were added and the samples were washed by centrifugation at 450g for 5 min. The samples were then surface stained with a combination of mAbs including anti-CD3 A700, anti-CD4 BV605, anti-CD8 PE/Dazzle™ 594 and anti-CD69 PerCP/Cy5.5 at 4 °C for 15 min and washed once. The stained samples were fixed and permeabilized in 0.5 mL of BD Cytofix/Cytoperm™ fixation and permeabilization solution (BDB, San Jose, CA) at 4 °C for 20 min. After the incubation, the samples were washed by adding 1X BD Perm/Wash™ buffer (BDB, San Jose, CA) and centrifuged at 500g for 5 min. ICS was performed by staining with a combination of mAbs including anti-IL-2 BV510, anti-IL-4 FITC, anti-IL-17 PE, anti-IFN-γ APC, anti-TNF-α BV650, anti-TGF-β BV421 at 4 °C for 30 min. After staining, the samples were washed with 1X BD Perm/Wash™ buffer (BDB, San Jose, CA) and re-suspended in PBS.

The stained cells of at least 100,000 events were acquired for each analysis by a BD Fortessa™ flow cytometer (BDB, San Jose, CA) and the data was analyzed by using FlowJo Software (Tree Star, San Carlos, CA). Cytokine producing cell subsets were determined from activated populations expressing CD69 and percentages of cytokine producing cell subsets were determined from a total cytokine producing cell population. A Boolean gating strategy was used for the analysis of polycytokine producing cell subsets in order to evaluate cytokine producing cell subsets with ability to simultaneously produce 1, 2, 3, 4, 5 or 6 cytokines.

Data analysis

Fold expansion numbers were calculated by using the viable cell numbers at each indicated time point divided by viable cell numbers at the beginning of cell expansion. All statistical analyses were performed using GraphPad Prism® version 7.02 (GraphPad Software Inc., CA, USA). Datasets were expressed as mean ± standard deviation (S.D.) and compared for statistical significance at p-value ≤ 0.05 with 2-way ANOVA followed by Bonferroni's multiple comparisons test. Proportions of cytokine producing cell subsets were presented as pie-charts.

LIST OF ABBREVIATIONS

A700	AlexaFluor®
AB	Albumin
APC	Allophycocyanin
APC-Cy7	Allophycocyanin and cyanine dye
BFA	Brefeldin A
BSE	Bovine spongiform encephalopathy
BV605	Brilliant Violet™ 605
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
HIV	Human immunodeficiency virus
HS	Human serum
I	Ionomycin
IFN	Interferon

IL	Interleukin
mAbs	Monoclonal antibodies
OKT3	Muromonab-CD3
PBS	Phosphate buffered saline
PE	Phycoerythrin
PE-Cy7	Phycoerythrin and cyanine dye
PerCP	Peridinin chlorophyll protein
PMA	Phorbol 12-myristate 13-acetate
RPMI	Roswell Park Memorial Institute
SIV	Simian immunodeficiency virus
SR	Serum replacement
TGF	Transforming growth factor
Th1	Type 1 T helper T cells
TNF	Tumor necrosis factor

DECLARATIONS

Ethical approval and consent to participate:

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable standards. The study was approved by the Institutional Review Board of the Faculty of Medicine Siriraj Hospital at Mahidol University. Informed consent was obtained from all individual participants recruited in the study.

Consent for publication:

Written informed consent was obtained from the patients and participants for publication of their individual details and accompanying images in this manuscript. The consent form is held by the authors and is available for review by the Editor-in-Chief.

Availability of data and material:

All data generated or analyzed during this study are included in this published article.

Competing interests:

The authors declare that they have no conflict of interest.

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Authors' contributions:

PT: performed experiments, data analysis and manuscript writing; VT, PP and PA: performed experiments and data collection; NO: research idea formation, research monitoring, and manuscript editing. All authors read and approved the final manuscript.

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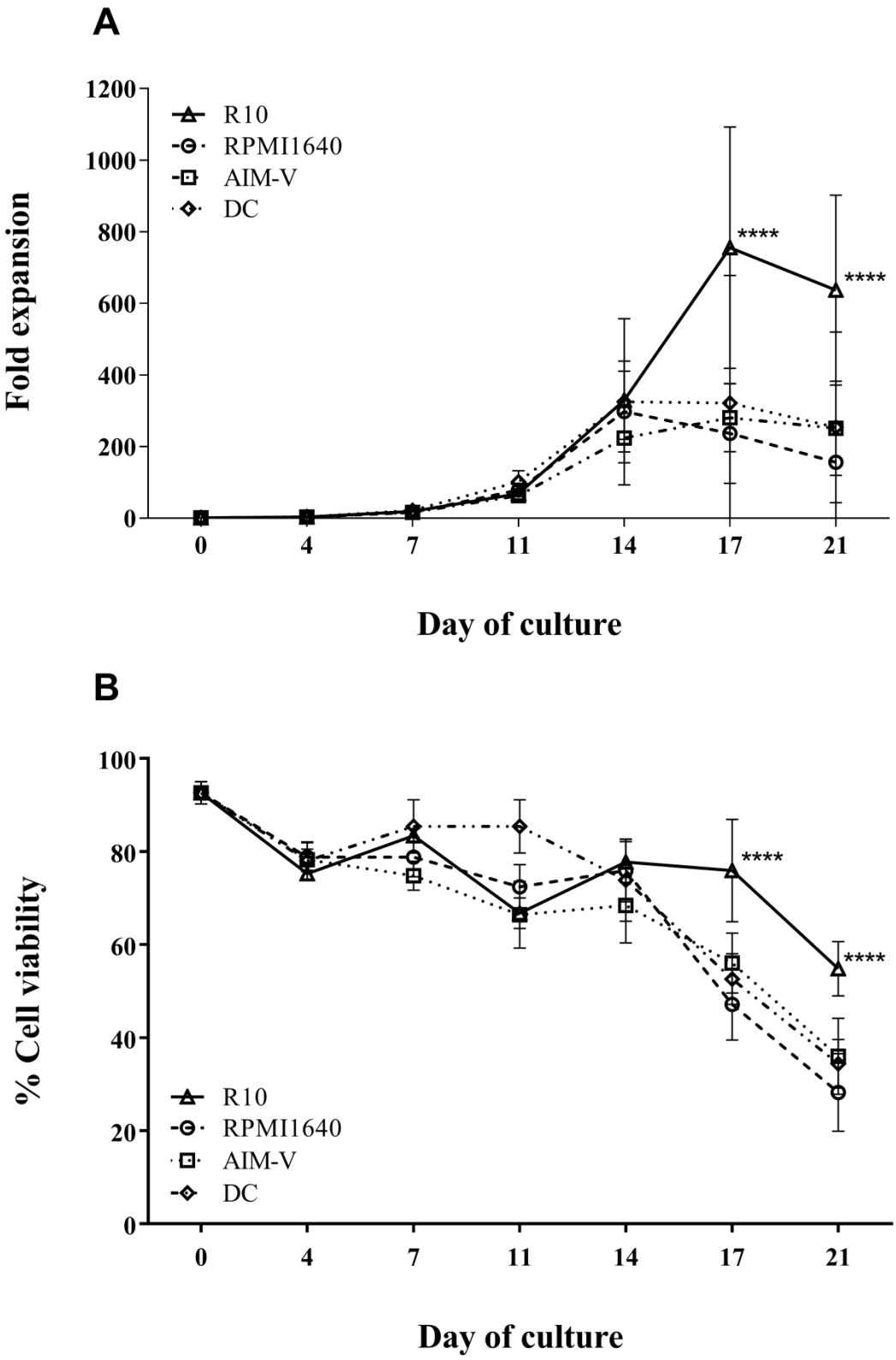

Fig. 1 Growth kinetics of anti-CD3/28 expanded $CD4^+$ T cells from different culture conditions. (A) Fold expansion and (B) cell viability of the expanded cells were observed from different cultures in R10 (RPMI1640 with 10% FBS) and media containing 10% human serum including RPMI1640, AIM-V, and DC. All data are presented as mean ± SD (n = 5, ****p-value < 0.0001).

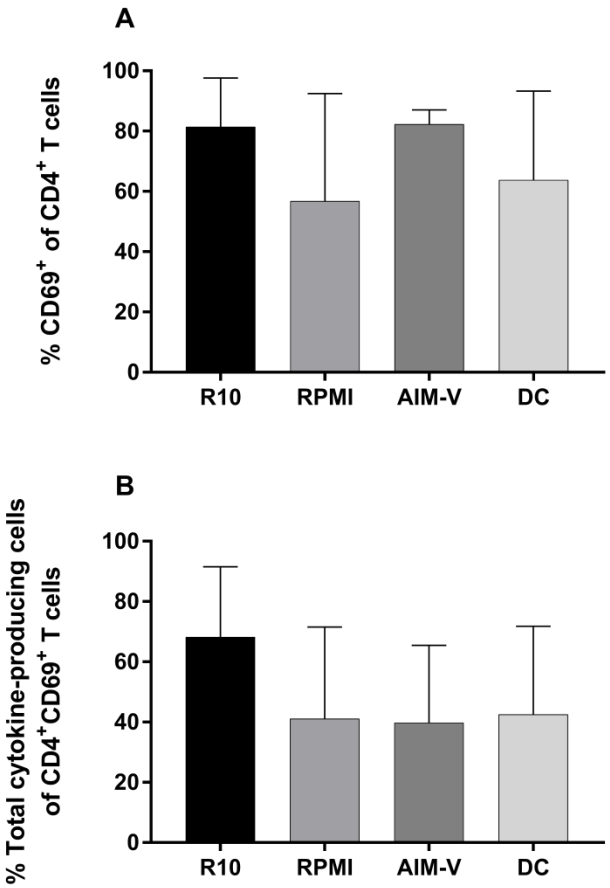

Fig. 2 Activation and cytokine production of anti-CD3/28 expanded $CD4^+$ T cells from different culture conditions. (A) Percentages of CD69 expressing cells and (B) percentages of cytokine producing cells were observed from different cultures in R10 (RPMI1640 with 10% FBS) and media containing 10% human serum including RPMI1640, AIM-V, and DC. All data are presented as mean ± SD (n = 3).

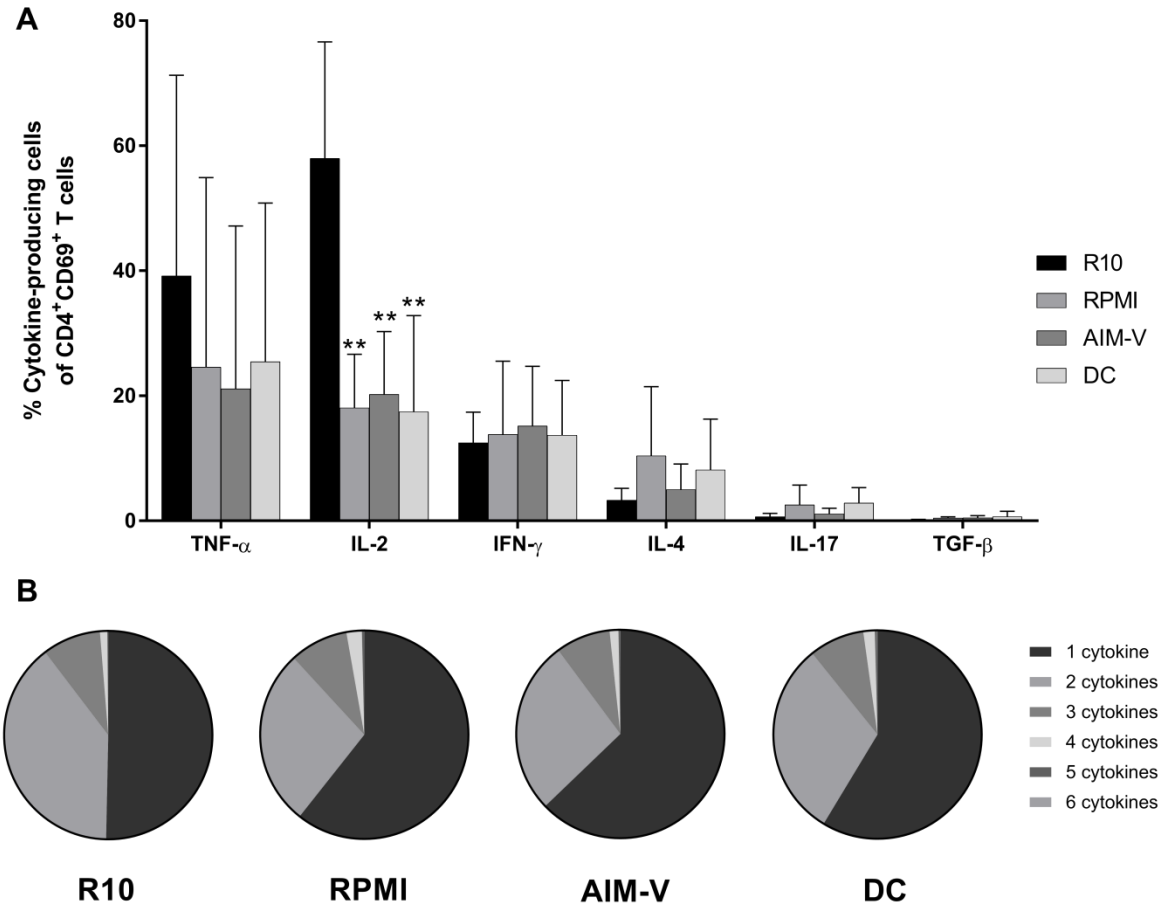

Fig. 3 Cytokine production profiles including TNF-α, IL-2, IFN-γ, IL-4, IL-17, and TGF-β of anti-CD3/28 expanded $CD4^+$ T cells. (A) Percentages (mean ± SD) of different cytokine producing cells and (B) average proportions of polyfunctional cytokine producing cells were observed from different cultures in R10 (RPMI1640 with 10% FBS) and media containing 10% human serum including RPMI1640, AIM-V, and DC. (n = 3, ****p-value < 0.0001).

Manuscript Details

Manuscript number	HIM_2018_29
Title	Differential expression of functional-associated cell surface molecules on anti-CD3/28 expanded CD4+ T cells
Article type	Research Paper

Abstract

CD4+ T cell immunotherapy has potential for treatment in HIV-infected patients. A large number of expanded CD4+ T cells and confirmation of functional-related phenotypes are required for ensuring the successful outcomes of treatment. Freshly isolated CD4+ T cells were activated with anti-CD3/28 coated magnetic beads at different bead-to-cell ratios and cultured in the absence and presence of IL-2 supplementation for three weeks. Fold expansion, cell viability, growth kinetic and lymphocyte subset identities were determined. Data demonstrated that a 1:1 bead-to-cell ratio rendered the highest expansion of 1,044 –fold with 88% viability and 99.5% purity followed by the 2:1 and 0.5:1 ratios. No significant difference in proliferation and phenotypes was found between non-IL-2 and IL-2 supplementation groups. Several specific surface molecule expressions of the expanded cells including chemokine receptors, adhesion molecules, co-stimulatory molecules, activation molecules, maturation markers, cytokine receptors and other molecules were altered when compared to the unexpanded cells. This optimized expansion protocol using the 1:1 bead-to-cell ratio of anti-CD3/28 coated magnetic beads and culture condition without IL-2 supplementation provided the satisfied yield with good reproducibility. Specific surface molecule expressions of the expanded cells presented potential roles in proliferation, differentiation, homeostasis, apoptosis and organ-homing.

Keywords	CD4+ T lymphocytes, cell surface molecules, immunotherapy, anti-CD3/28 coated beads, HIV.

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Dear Editor-in-Chief,

My colleagues and I would like to submit a manuscript for consideration as an original article in the Human Immunology entitled "Differential expression of functional-associated cell surface molecules on anti-CD3/28 expanded $CD4^+$ T cells" by authors, Premrutai Thitilertdecha, Poonsin Poungpairoj, Varangkana Tantithavorn, Palanee Ammaranond, and Nattawat Onlamoon.

This manuscript highlights optimization of the expansion protocol using anti-CD3/28 coated magnetic beads in terms of bead quantity and medium supplementation for $CD4^+$ expansion in order to achieve the satisfied yield for clinical uses. This study also firstly provide the whole range of specific cell surface molecule expressions of the expanded $CD4^+$ T cells including chemokine receptors, adhesion molecules, co-stimulatory molecules, activation molecules, maturation markers, cytokine receptors and other molecules related to apoptosis and cell exhaustion. Results show that a 1:1 bead-to-cell ratio of anti-CD3/28 coated beads and autocrine cytokines produced by the expanded cells were sufficient to maintain the culture and provided the good yield. Phenotypic profiles of the expanded cells also suggested that the expression of markers for activation, proliferation, differentiation, homeostasis and apoptosis revealed certain functions of the expanded cells. Interestingly, the expanded cells presented advanced capabilities in HIV resistance and migration to gut-associated lymphoid tissues. Therefore, the expanded cells from our optimized culture method are promising to use in adoptive immunotherapy for HIV-infected patients.

This manuscript has been seen and approved by all authors and has not been submitted elsewhere.

Yours sincerely,

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Differential expression of functional-associated cell surface molecules on anti-CD3/28 expanded $CD4^+$ T cells

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ABSTRACT

$CD4^+$ T cell immunotherapy has potential for treatment in HIV-infected patients. A large number of expanded $CD4^+$ T cells and confirmation of functional-related phenotypes are required for ensuring the successful outcomes of treatment. Freshly isolated $CD4^+$ T cells were activated with anti-CD3/28 coated magnetic beads at different bead-to-cell ratios and cultured in the absence and presence of IL-2 supplementation for three weeks. Fold expansion, cell viability, growth kinetic and lymphocyte subset identities were determined. Data demonstrated that a 1:1 bead-to-cell ratio rendered the highest expansion of 1,044 –fold with 88% viability and 99.5% purity followed by the 2:1 and 0.5:1 ratios. No significant difference in proliferation and phenotypes was found between non-IL-2 and IL-2 supplementation groups. Several specific surface molecule expressions of the expanded cells including chemokine receptors, adhesion molecules, co-stimulatory molecules, activation molecules, maturation markers, cytokine receptors and other molecules were altered when compared to the unexpanded cells. This optimized expansion protocol using the 1:1 bead-to-cell ratio of anti-CD3/28 coated magnetic beads and culture condition without IL-2 supplementation provided the satisfied yield with good reproducibility. Specific surface molecule expressions of the expanded cells presented potential roles in proliferation, differentiation, homeostasis, apoptosis and organ-homing.

Keywords: $CD4^+$ T lymphocytes, cell surface molecules, immunotherapy, anti-CD3/28 coated beads, HIV.

1 INTRODUCTION

Human immunodeficiency virus (HIV) infection causes a progressive decrease of $CD4^+$ T lymphocytes and an increase of HIV viral load (or HIV RNA level), leading to higher susceptibility to opportunistic infections which can further develop to acquired immune deficiency syndrome (AIDS) [1]. HIV enters target cells through the binding of viral envelope glycoproteins to CD4 receptors along with CCR5 and CXCR4 co-receptors markedly expressed on the target $CD4^+$ T lymphocytes [2-4]. Although highly active antiretroviral therapy (HAART) succeeds to control the HIV viral load into an undetectable level and recovers the CD4 counts in HIV-infected patients, the latent reservoir of virus still exists [5] and the immune restoration is incomplete [6-9]. A life-long treatment of HAART has also feasible consequences in cumulative drug toxicities, emergent drug-resistant viruses and unaffordable costs due to more complicated regimens. Moreover, some patients who have discordant immune responses (DIR) to HAART fail to achieve target CD4 count levels despite accomplished virological control, suggesting a higher risk in mortality [10].

An alternative approach, such as adoptive transfer of autologous activated $CD4^+$ T lymphocytes, has been proposed to be a potential treatment for the benefit of both virological control and direct immune reconstitution. Its effectiveness and safety have been confirmed by *in vivo* studies in both simian deficiency virus (SIV)-infected rhesus macaques and HIV-infected patients [11-15]. To expand $CD4^+$ T cells *in vitro*, anti-CD3/28 coated magnetic beads are widely used for stimulation. The anti-CD3/28 activated $CD4^+$ T cells showed intrinsic resistance to macrophage (M)-tropic isolates of HIV-1 infection [16-18] and promoted expression of RANTES, MIP-1α and MIP-1β as well as reduced expression of CCR5 [11,13,14,16]. Furthermore, the expanded $CD4^+$ T cells induced interferon (IFN)-γ production which is associated to type 1 T helper (Th1) cell function and increased the density of variable beta (Vβ) chain T cell receptor (TCR) repertoires [14] together with telomerase activity, resulting in a longer survival of the cells [11].

With respect to the clinical uses, a large number of $CD4^+$ T cells expanded *in vitro* was required for reinfusion in HIV-infected patients [14,15]; therefore, optimization of expansion protocols is warranted. There have been established *in vitro* culture methods for anti-CD3/28 stimulated $CD4^+$ T lymphocytes providing different yields [19-21] which can be related to different cell isolation methods, bead-to-cell ratios used for stimulation, and medium supplementation. More importantly, functional-associated phenotypic characters of the

expanded cells are essential which are not only related to cell characterization but also maturation and activation stages as well as cell migration. Even so, there is limited information concerning specific cell surface molecule expressions of the expanded $CD4^+$ T cells, such as chemokine receptors and maturation markers.

This study thus purposed to investigate the optimum bead-to-cell ratios and supplementation used for $CD4^+$ T cell expansion as well as to explore the whole series of surface molecule expressions of the expanded cells including chemokine receptors, adhesion molecules, co-stimulatory molecules, activation molecules, maturation markers, cytokine receptors, and other functional-specific molecules.

2 MATERIALS AND METHODS

2.1 Sample collection

Three healthy volunteers aged 26-30 years were enrolled in this study. The protocol was approved by the Institutional Review Board of the Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. Written informed consent was obtained from each subject prior to sample collection.

2.2 Characterization of lymphocyte subsets and specific surface molecule expressions

Monoclonal antibodies (mAbs) and their conjugated fluorochromes were obtained from Becton Dickinson Biosciences (BDB: San Jose, CA) and used at the concentrations recommended by the manufacturer. The fluorescent-labeled mAbs used for phenotypic characterization of the cells were 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. The fluorescent-labeled mAbs used for identification of specific surface molecule expression were anti-CD4 PerCP, anti-CD3 FITC, anti-CD45RO FITC, anti-CD45RA FITC, anti-CD57 FITC, anti-CD27 FITC, anti-CCR7 PE, anti-CD62L PE, anti-CD11a PE, anti-CD11b PE, anti-CD11c PE, anti-CD126 PE, anti-CD127 PE, anti-CD95 PE, anti-CD95L PE, anti-CD154 (CD40L) PE, anti-CD40 PE, anti-CD134 (OX40) PE, anti-CD278 (ICOS) PE, anti-CD71 PE, anti-HLA-DR PE, anti-GITR PE, anti-CD28 PE, anti-CD103 PE, anti-CD38 PE, anti-CD69 PE, anti-CD25 PE, anti-CD184 (CXCR4) PE, anti-CD183 (CXCR3) PE, anti-CCR10 PE, anti-CD195 (CCR5) PE, anti-PD-1 PE, anti-CXCR5 PE, anti-CCR6 PE, anti-CCR4 PE and anti-$\alpha 4\beta 7$ PE.

2.3 *CD4$^+$ T lymphocyte isolation using immunorosettes formation method*

CD4$^+$ T lymphocytes can be directly isolated from whole blood by an immunorosettes formation method using RosetteSep® human CD4$^+$ T cell enrichment cocktail (STEMCELL Technologies, Vancouver, BC, Canada). Briefly, CD4$^+$ T lymphocytes were isolated from 5 mL of whole blood by adding 250 µL of RosetteSep® human CD4$^+$ T cell enrichment cocktail. After that, the samples were thoroughly mixed and incubated at room temperature for 20 minutes. The samples were then diluted with an equal volume of phosphate buffered saline (PBS) containing 2% fetal bovine serum (FBS) and gently mixed. The diluted blood samples were carefully layered on top of LSM® lymphocyte separation medium and centrifuged at 1200g with no break at room temperature for 20 minutes. After centrifugal separation, the samples were divided into four layers including plasma, enriched CD4$^+$ T cells, LSM® lymphocyte separation medium and red blood cells (from top to bottom). Pasteur pipettes were used to remove the plasma layer and collect enriched CD4$^+$ T cells from the layer interface. The collected CD4$^+$ T cells were then washed with 10 mL of PBS containing 2% FBS and centrifuged twice at 1400 rpm at room temperature for 5 minutes. The cell pellets were collected and re-suspended with a complete medium (RPMI1640 with 10% FBS, 50 µg/mL pencillin-streptomycin and 2 mM L-glutamine). Cell number and viability of the enriched CD4$^+$ T cells were determined by trypan blue exclusion using a hemacytometer.

2.4 *Expansion of isolated CD4$^+$ T lymphocytes using anti-CD3/28-coated beads*

The isolated CD4$^+$ T cells from the immunorosettes formation method were stimulated with anti-CD3/28 coated beads. Dynabeads® human T–activators (Invitrogen Dynal, Oslo, Norway) were used in this study. The expansion procedures of CD4$^+$ T lymphocytes were divided into two steps including bead washing for elimination of preservatives and cell activation. The bead number was calculated for 0.5:1, 1:1, and 2:1 bead-to-cell ratios to use for the expansion. Anti-CD3/CD28 coated beads with calculated amounts were then transferred into the tube and washed with 2 mL PBS to an original volume of beads. After that, the tube was placed on the magnet for 1 minute in order to remove the washing solvent. The washed beads remaining in the tube were re-suspended in the complete medium with an equal volume to the initial volume of beads.

For bead-to-cell ratio comparison, 1x10^6 enriched CD4$^+$ T cells were stimulated with anti-CD3/CD28 coated beads in the absence of exogenous interleukin (IL)-2. The stimulated CD4$^+$ T cells were then expanded in the complete medium at a concentration of 0.5x10^6

cells/mL and incubated at 37 °C and 5% CO_2 humidification before reactivation on day 7. The cells were expanded for a total of 3-week culture period and the medium was replenished with calculated amounts of fresh media on days 4, 7, 11, 14, and 17 to maintain the cell suspension concentration at $0.5x10^6$ cells/mL before transferring to appropriate culture vessels. Cell numbers and viability were observed on days 4, 7, 11, 14, 17 and 21 by using trypan blue exclusion and a hematocytometer. Lymphocyte subset characters were analyzed by a flow cytometer on days 0, 14 and 21.

With respect to IL-2 supplementation comparison, the similar activation and culture protocols were conducted by using only the 1:1 bead-to-cell ratio and cultures in the absence and in the presence of exogenous interleukin (IL)-2 at the low concentration of 20 units/mL (Prospec, Ness-Ziona, Israel). Cell numbers and viability were observed on days 4, 7, 11, 14, 17 and 21 by using trypan blue exclusion and a hematocytometer. Lymphocyte subset characters were analyzed by a flow cytometer on days 0, 14 and 21.

2.5 Immunofluorescent staining

Whole blood, purified and expanded $CD4^+$ T cells were stained with fluorochrome-conjugated mAbs and incubated for 15 minutes before adding 1X lysing solution for red blood cell lysis. The stained cells were then washed with PBS containing 2% FBS prior to centrifugation at 1,400 rpm at 25 °C for 5 minutes. Subsequently, the stained cells were re-suspended in PBS containing 1% paraformaldehyde. The stained cells were finally acquired by a BD FACSCalibur flow cytometer (BDB, San Jose, CA) and the data were analyzed by using FlowJo Software (Tree Star, San Carlos, CA).

2.6 Flow cytometric analysis

Six-parameter analysis including forward scatter (FSC), side scatter (SSC), FITC, PE, PerCP, and APC was performed using FlowJo Software (Tree Star, San Carlos, CA). The stained cells were gated using lymphogate (FSC/SSC) to determine a viable lymphocyte population. After that, lymphocyte subsets were defined using two-dimensional dot plots between CD45/SSC, CD45/CD3 and CD4/CD8 or CD19/CD16+CD56. Therefore, the lymphocyte subsets were detected into $CD4^+CD8^-$, $CD4^-CD8^+$, $CD4^+CD8^+$, $CD19^-CD16^+CD56^+$ and $CD19^+CD16^-CD56^-$ populations.

2.7 Statistical analysis

All statistical analyses were performed using GraphPad Prism® version 7.02 (GraphPad Software Inc., CA, USA). Datasets were expressed as mean ± standard deviation (S.D.) and compared for statistical significance at p-value ≤ 0.05 with 2-way ANOVA followed by Bonferroni's multiple comparisons test.

3 RESULTS

3.1 Bead-to-cell ratio comparison for anti-CD3/28 $CD4^+$ T cell expansion

To achieve satisfied yields of the expanded cells, it is important to determine the optimum bead-to-cell ratio used for stimulation. In this study, three healthy volunteers were recruited for blood collection. Isolated $CD4^+$ T cells were activated with anti-CD3/28 coated magnetic beads at different bead-to-cell ratios (i.e., 0.5:1, 1:1 and 2:1) and cultured in the absence of IL-2 for 21 days. Fold expansion, cell viability, growth kinetic and phenotypic characters were observed for proliferation efficiency of the expanded $CD4^+$ T cells on days 0, 4, 7, 11, 14, 17 and 21.

There was no difference in fold expansion among 3 different bead-to-cell ratios during the first 14 days of culture, however, the fold expansion number of $CD4^+$ T cells expanded with the 1:1 bead-to-cell ratio on day 17 showed remarkably higher than the others (Fig. 1a). On day 21 of culture, it was obvious that stimulation with the 1:1 bead-to-cell ratio provided the highest yield of the anti-CD3/28 expanded $CD4^+$ T cells followed by the 2:1 and 0.5 bead-to-cell ratios (1,044 ± 259 –, 629 ± 457 –, and 301 ± 167 – fold, respectively). Cell viabilities of the expanded cells from the 3 different ratios were comparable with over 90% throughout the 3-week culture period (Fig. 1b). There were only slightly decreases in viable cells at the end of the culture for the 1:1 and 2:1 ratios (88 ± 7% and 83 ± 15%, respectively).

Lymphocyte subset characters of the anti-CD3/28 stimulated $CD4^+$ T cells were analyzed by a flow cytometer (Table 1). It was clearly demonstrated that the major population of the expanded cells was $CD3^+$ T cells (> 99% of lymphocytes) with the dominant subset of $CD3^+CD4^+CD8^-$ for all bead-to-cell ratio groups over the culture period (> 98% of lymphocytes). The purity of the expanded cells was also confirmed with low frequencies of $CD3^+CD4^-CD8^+$, $CD3^+CD4^+CD8^+$, $CD3^-CD19^+$, $CD3^-CD16^+CD56^+$, $CD3^+CD16^+CD56^+$, and $CD3^+CD19^+$ (< 3.5% of expanded cells).

3.2 Effects of IL-2 supplementation on cell expansion

IL-2 supplementation has been generally used to promote cell proliferation in addition to the autocrine/paracrine IL-2 production by the activated T cells. High concentrations of IL-2 (100 and 300 IU/mL) have been reported to predominantly affect $CD8^+$ T cell development [22]. This study, therefore, used a low concentration of IL-2 at 20 IU/mL to support cell expansion and compared this expansion effect of IL-2 to the autocrine/paracrine IL-2 production (i.e., cell culture in the absence of IL-2). Fold expansion, cell viability, growth kinetic and lymphocyte subset characters were observed for proliferation efficiency of the expanded $CD4^+$ T cells on days 0, 4, 7, 11, 14, 17 and 21.

Data showed that fold expansion numbers between the culture without and with IL-2 supplementation were similar throughout the 21-day culture period (Fig. 2a). Only the expanded cells cultured in the absence of IL-2 supplementation on day 17 proliferated significantly higher than those in the presence of IL-2 supplement (582 ± 166 – and 455 ± 125 – fold, respectively). At the end of the culture, there was no significant difference in proliferation between the two culture groups. With respect to cell viability, the cultures without or with IL-2 supplementation maintained great numbers of viable cells with over 90% throughout the 3-week culture period (Fig. 2b).

Predominant phenotypes of the expanded cells from both culture groups were $CD3^+$ T cells (> 97% of expanded cells) with the major $CD3^+CD4^+CD8^-$ subset (> 94% of expanded cells) as presented in Table 2. The minor cell populations including $CD3^+CD4^-CD8^+$, $CD3^+CD4^+CD8^+$, $CD3^-CD19^+$, $CD3^-CD16^+CD56^+$, $CD3^+CD16^+CD56^+$ and $CD3^+CD19^+$ were also found with very low frequencies (< 2% of expanded cells), suggesting a specific expansion of purified $CD4^+$ T cells.

3.3 Specific cell surface molecule expressions of anti-CD3/28 $CD4^+$ T cells

As surface molecule expressions are related to cell maturation, activities and functions, this study thus explored several cell surface molecule expressions of anti-CD3/28 expanded $CD4^+$ T cells which were divided into seven groups according to the molecules' functions and roles. The cell surface molecules included (i) chemokine receptors: CCR4, CCR5, CCR6, CCR7, CCR10, CXCR3, CXCR4 and CXCR5; (ii) adhesion molecules: CD11a, CD11b, CD11c, CD103 and $\alpha4\beta7$; (iii) co-stimulatory molecules: CD27, CD28, CD40, CD40L, CD134, PD-1 and ICOS; (iv) activation molecules: CD25, CD38, CD69, CD71 and HLA-DR; (v)

maturation markers: CD45RO, CD45RA and CD62L; (vi) cytokine receptors: CD126 and CD127; and (vii) other molecules: CD57, CD95, CD95L, and GITR. Frequencies of the expanded cells expressing these surface molecules in the cultures without and with IL-2 supplementation on day 21 were observed and compared to those of whole blood on day 0 as a baseline control.

For chemokine receptors, the expanded cells from cultures whether IL-2 supplementation or not exhibited significant lower expressions of CCR6, CCR7 and CXCR4 and dramatic higher expression of CXCR3 when compared to the unexpanded cells (i.e., whole blood) as seen in Fig. 3a. Other molecules, CCR4, CCR5, CCR10, CXCR5, remained similar after the expansion. The expanded cells also had marked increases in expressions of adhesion molecules, CD11b, CD11c and $\alpha 4\beta 7$ when compared to the unexpanded cells (Fig. 2b). There was also no change in expressions of CD11a and CD103 between the expanded and unexpanded cells.

With respect to co-stimulatory molecules, only frequencies of CD40L of the expanded cells from both expansion groups were significantly increased when compared to the unexpanded cells (Fig. 3c). All other molecules including CD27, CD28, CD40, CD134, PD-1 and ICOS remained unchanged after the expansion. Furthermore, expressions of all activation molecules, except CD69, on the expanded cells were significantly upregulated when compared to those of the unexpanded cells (Fig. 3d). The expanded cells also showed significant lower frequencies of CD45RO and higher frequencies of CD45RA, while their CD62L expression was similar to the unexpanded cells (Fig. 3e).

The expanded cells also exhibited notable downregulation of cytokine receptor, CD126, and upregulation in CD127 when compared to the unexpanded cells (Fig. 3f). Expressions of other molecules including CD57, CD95 and GITR were dramatically raised after the expansion, whereas there were slight increases in CD95L (Fig. 3g). Moreover, no significant difference in numbers of any chemokine receptors, adhesion molecules, co-stimulatory molecules, activation molecules, maturation markers, cytokine receptors and other molecules was found between the two culture groups with and without IL-2 supplementation (Figs. 3a-3g).

4 DISCUSSION

In this report, we have described effects of bead-to-cell ratio of anti-CD3/28 coated magnetic beads for stimulation and IL-2 supplementation on the growth of expanded $CD4^+$ T cells as well as their specific surface molecule expression changes after the expansion. Ones of important concerns are quantity of anti-CD3/28 coated magnetic beads used for expansion and medium supplementation. Levine *et al.* (1997) used immobilized anti-CD3/28 coated beads at 1 - 3 beads/cell for a long-term proliferation of polyclonal $CD4^+$ T cells from normal donors. The expanded cells were able to maintain the exponential growth of 2.3×10^5 – fold over 40 days of culture without exogenous cytokines or feeder cells added to the culture. However, the fold expansion was dramatically increased to 10^9 – to 10^{11} – fold when supplemented with IL-2 at the late culture (~ days 28 – 106) [20]. Levine *et al.* (1998) also implemented the previous method for a large-scale production of $CD4^+$ T cells from HIV-infected patients by using anti-CD3/28 coated beads at 3 beads/cell for stimulation and the culture method without IL-2 supplementation. The cell numbers were increased by 37 – fold after a 14-day culture [21]. Later, our previous study showed that $CD4^+$ T cell stimulation with reduced numbers of anti-CD3/28 coated magnetic beads to a 1:1 bead-to-cell ratio and culture in the absence of IL-2 were able to prolong the cell expansion throughout a 3-week culture with the good yield of 1,000 – fold [19]. During the cell harvest, the higher number of beads used for expansion, the higher number of beads remaining in the final products which leads to the bead accumulation in the body after the cell reinfusion tentatively causing toxicity. It is then warranted to compromise between the bead quantity for the highest activation efficiency and the treatment safety for patients.

We then compared different bead-to-cell ratios (i.e., 0.5:1, 1:1, and 2:1) to minimize the number of beads used for expansion without sacrificing their promotion for the suitable yield. Our data showed that the 1:1 bead-to-cell ratio provided the highest fold expansion (1,044 ± 259 – fold) which was 1.7 and 3.5 times greater than the 2:1 and 0.5:1 ratios, respectively. Hence, cell stimulation at the 1:1 bead-to-cell ratio was sufficient to achieve the satisfied yield with good viability (88 ± 7%) and high purity (> 98% of lymphocytes) within the reasonable time (i.e., a 3-week culture). Autocrine cytokines produced from the expanded cells themselves were also adequate to supply the proliferation throughout the culture period. This was confirmed by our comparison between the cultures without and with IL-2 supplementation. No significant difference in fold expansion and cell viability was found between the two groups.

Changes in specific cell surface molecule expressions of the anti-CD3/28 $CD4^+$ T cells were also observed in this study. Several chemokine receptors have been identified as coreceptors for the HIV entry, such as CCR5, CXCR4, CCR4, CCR6 and CCR10. The *in vitro* expanded $CD4^+$ T cells with anti-CD3/28 activation were proved to be resistant to HIV-1 infection via the reduction in frequencies and densities of CCR5 molecules [18,23]. Our expanded cells also rendered a low frequency in CCR5 (< 15% of $CD4^+$ T cells) which presumably maintain at this low level because the recovery of CCR5 expression was low when activation with anti-CD3/28 coated beads compared to stimulation with anti-CD3/28 immobilized on the surface of a tissue culture plate [23]. Our anti-CD3/28 activation protocol also rendered the expanded $CD4^+$ T cells with twice as less CXCR4 expression than the unexpanded cells. Although a high number of the expanded cells still expressed CXCR4 (52% of $CD4^+$ T cells), the chance of viral entry when switching coreceptor usage from CCR5 to CXCR4 [24] will feasibly diminished when compared to the unexpanded cells.

CCR4, CCR6 and CCR10 were also reported to be other HIV-1 coreceptors of primary HIV-1 isolates [25-27]. Our expanded cells showed that CCR4 was highly expressed throughout the expansion period (84% of $CD4^+$ T cells), whereas a significant downregulation in CCR6 by 2.5 – fold and low frequency of CCR10 (12% of $CD4^+$ T cells) were found. Although CCR4 expression was high, soluble viral protein gp120 had greater affinity to CCR5 than CCR4 [27]. $CCR4^+$ T cells are also able to be defined as Th2 cells, while Th1 cells are classified by $CXCR3^+$ [28]. In our study, the frequency of $CXCR3^+$ T cells was 2-fold higher than that of the unexpanded cells (~83% of $CD4^+$ T cells) which was almost equal to $CCR4^+$ T cells. However, we cannot specify that our $CXCR3^+$ T cells are absolutely purified Th1 cells as they need to be further characterized with expressions of $CCR4^-$, $CCR5^+$, and $CXCR6^+$ [28]. These evidences clearly demonstrate that our expanded cells are suitable for reinfusion due to their highly feasible ability for HIV-1 resistance *in vivo*.

As the gut compartment is a major portal for HIV entry, the considerable depletion in mucosal $CD4^+$ T cells was observed during acute infection [29]. Our study shows that the expanded cells had a pretentious increase in $\alpha4\beta7$ expression to 82% of $CD4^+$ T cells. In contrary, the level of another gut mucosal specific adhesion molecule, CD103 [30], was low at the end of the culture (10% of $CD4^+$ T cells) even though it was 5 – fold higher expressed in the expanded cells. It is then worth proposing that these cells feasibly migrate to the site of depletion and improve immune response at the gut-associated lymphoid tissues.

Other surface adhesion molecules on T lymphocytes are β2 integrins (CD11/CD18 family) including CD11a, CD11b, and CD11c. These molecules facilitate the T cell engagement with target cells and endothelial cells. CD11a is generally highly expressed in $CD4^+$ T cells, whereas CD11b and CD11c are more prevalent in $CD8^+$ T cells. [31]. Our results affirm this evidence as the unexpanded $CD4^+$ T cells exhibited high expression of CD11a (100% of $CD4^+$ T cells) and low expressions of CD11b and CD11c (3% and 12% of $CD4^+$ T cells, respectively). Interestingly, after a-3 week culture, the expression of CD11a on the expanded cells remained high. Results also showed significant upregulation in CD11b (14.6 – fold) and CD11c (5.3 – fold). These expanded cells with the notable high CD11a expression are suitable for survival due to ability for T cell localization to areas rich in cytokines for promoting their homeostasis [32].

With respect to co-stimulatory molecules, a high frequency of CD28, in our study remained unchanged during the expansion (99% of $CD4^+$ T cells). This is in agreement with the previous report showing the constitutive expression of CD28 on $CD4^+$ T cells [33], suggesting that the function for an effective antigen-specific immune response is still active. However, our results are in contrary to another study reporting that its expression was transiently down-regulated following T-cell activation and progressively declined due to *in vitro* senescence [34]. For CD40 expression, it is not surprisingly to find its low level on the expanded $CD4^+$ T cells (9% of $CD4^+$ T cells) as they are commonly identified and functionally characterized on B cells [35]. On the other hand, CD40L is only expressed on activated $CD4^+$ T cells [35] which is in accordance with our results presenting a significantly increased CD40L expression by 3.4 – fold, proposing that the expanded $CD4^+$ T cells can probably promote isotype switching, maturation and survival of B cells [36].

We also found that the expression of CD134 (OX40) was slightly increased after the expansion (18% of $CD4^+$ T cells), possibly promoting survival of T cell numbers and accumulation of developed memory $CD4^+$ T cells over time [37]. For ICOS, it was highly expressed and comparable to that of the unexpanded cells (68% of $CD4^+$ T cells), even though this ICOS has to be *de novo* induced on the T-cell surface [33] and augmented by CD28 co-stimulation together with TCR engagement [38]. It was also reported to be matching CD28 in potency and enhancing all basic T-cell responses [33] as well as T-cell-dependent B-cell help [39]. Overall, all co-stimulatory molecules of the anti-CD3/28 $CD4^+$ T cells were endurable over the expansion period. Only CD40L was markedly higher expressed in the expanded cells. It is then suggested that these *in vitro* expanded cells with consistent co-

stimulatory molecules will be able to have normal proliferation and differentiation *in vivo* when reinfusion to HIV-infected patients in which some co-stimulatory molecules are dysregulated following the disease progression.

Expression kinetics of the CD25, CD69, CD38, CD71 and HLA-DR were previously described [40,41]. The results showed that while CD25 and CD69 expression on $CD4^+$ T cells reached over 90% at 24 hours after soluble anti-CD3/28 stimulation, whereas CD38 and CD71 reached their maximum levels at 72 hours [41]. Our activation molecules including CD25, CD38, CD71 and HLA-DR were also dramatically increased (80%, 100%, 75%, and 63% of $CD4^+$ T cells, respectively), while CD69 was slightly increased to 32% of $CD4^+$ T cells. It is then indicating that our expanded cells expressed full ranges of activation molecules in both early- (i.e., CD25 and CD69) and late-activation markers (i.e., CD38, CD71 and HLA-DR). Interestingly, the previous study also demonstrated that CD25 expression maintained at high level, whereas CD69 was significantly dropped to ~50% after 72 hours [41]. The low level of CD69 expression on our expanded cells revealed similar expression kinetics since the data was observed 2 weeks after re-stimulation.

Maturation stage of $CD4^+$ T cells subsets including naïve, effector, effector memory, and central memory cells is generally based on expression of CD45RA with CD62L or CD45RA with CCR7 [42]. We found that the unexpanded cells had equal numbers of naïve ($CD4^+CD45RA^+$) and memory ($CD4^+CD45RO^+$) subsets with ~50% of $CD4^+$ T cells. After a 3-week culture, $CD45RA^+$ cells were notably declined by ~2.7 – fold, whereas $CD45RO^+$ cells were 2-fold increased (84% of $CD4^+$ T cells). Furthermore, there were contradict expressions between CD62L and CCR7 which were supposed to be correlated each other. CD62L remained highly expressed (97% of $CD4^+$ T cells), whereas CCR7 was prominently decreased to 9% of $CD4^+$ T cells. However, this study had a limitation in T cell subset characterization due to those molecules were not determined simultaneously. Therefore, we cannot precisely specify T cell subsets. Even so, our results can assume that the expanded cells were probably in the transition towards memory cells either central memory ($CD45RA^-CCR62L^+$ or $CD45RA^-CCR7^+$) or effector memory ($CD45RA^-CCR62L^-$ or $CD45RA^-CCR7^-$) cells. A great number of $CD27^+$ T cells (84% of $CD4^+$ T cells) were also found, indicating that the cells have not been differentiated into effector cells which finally lose this CD27 expression [43].

Additionally, the expression of CXCR5 on CD4+ T cells indicates a distinct memory T cell subset with B cell helper function (i.e., follicular B helper T cells (T_{FH})) [44]. Our data exhibited a very low frequency of $CXCR5^+$ T cells (1.7% of $CD4^+$ T cells), revealing that our expanded cells are not T_{FH}. A low frequency of $GITR^+$ cells designated as regulatory T (T_{reg}) cells was also significantly increased but still less than 20% of $CD4^+$ T cells. This small augmented population of T_{reg} may help preventing an aberrant HIV-induced chronic T-cell hyperactivation, leading to retardation of disease progression [45]. Therefore, it is then suggested that our expanded cells may pose a regulatory function.

According to cytokine receptors, CD126 expression was markedly low in our expanded $CD4^+$ T cells compared to the unexpanded cells (7% vs 93% of $CD4^+$ T cells). This downregulation might be resulted from TCR cross-linking *in vitro*, indicating a non-naïve stage of T-cell differentiation [46]. This supports our cell maturation stage discussed previously. Our study also shows that CD127 was markedly decreased over the expansion by 1.5 – fold (from 92% to 60% of $CD4^+$ T cells). The reduced expression possibly cause from prolonged CD127 suppression via TCR stimulation [47].

During the expansion, activation-induced cell death of $CD4^+$ T lymphocytes can occur via Fas-dependent apoptosis by triggering Fas (CD95) with its ligand (FasL or CD95L) [48]. We found that the expanded cells expressing $CD95^+$ were significantly increased to 100% of $CD4^+$ T cells, whereas $CD95L^+$ cells remained at low level (14% of $CD4^+$ T cells). It is then assumed that the apoptosis following cell-cell contact between $CD95^+$ and $CD95L^+$ $CD4^+$ T cells will be limited. We also investigated the expression of CD57 which is associated to functions in termination of cell differentiation and submission to apoptosis [49]. A significant increase in $CD57^+$ cells was found but the level was low at 17% of $CD4^+$ T cells, indicating some of the expanded cells may be in the exhaustion stage. However, the expression of PD-1 which is also a critical mediator for T-cell exhaustion [50] was not changed during the expansion (33% of $CD4^+$ T cells). Our expanded cells thus possibly retain normal function in proliferation.

Moreover, a low concentration of IL-2 supplementation did not affect surface molecule expressions of any chemokine receptors, adhesion molecules, co-stimulatory molecules, activation molecules, maturation markers, cytokine receptors and other molecules of the expanded cells. These findings then support the previous experiments from Onlamoon *et al.* (2013).

This study demonstrated that the 1:1 bead-to-cell ratio of anti-CD3/28 coated magnetic beads for $CD4^+$ T cell expansion was the most optimum bead quantity to achieve the satisfied yield of the expanded cells. The autocrine cytokines, mainly IL-2, produced by the expanded cells themselves are also adequate for a 3-week proliferation without additional IL-2 supplementation. After the expansion, phenotypic profiles of the expanded cells were changed. The expanded cells likely become more resistant to HIV-1 via downregulation of dominant coreceptors for HIV entry, CCR5 and CXCR4, as well as migrate to the site of depletion and improve immune response at the gut-associated lymphoid tissues due to higher expressions of gut-homing molecules, $\alpha 4\beta 7$ integrin. Furthermore, other specific surface molecule expressions related to activation, proliferation, differentiation, homeostasis and apoptosis revealed certain functions of the expanded cells. It is thus worth suggesting that these expanded cells following our optimized protocol are suitable for $CD4^+$ T cell immunotherapy used in HIV-infected patients, even though further investigation on $CD4^+$ T cells from HIV-infected patients and a large-scale production are required.

5 LIST OF ABBREVIATIONS

ACT	Actin
AIDS	Acquired immune deficiency syndrome
APC	Allophycocyanin
CCR	Chemokine receptor
CXCR	CXC chemokine receptor
DIR	Discordance immune response
FBS	Fetal bovine serum
FSC	Forward scatter
FITC	Fluorescein isothiocyanate
GITR	Glucocorticoid-induced TNFR (tumor necrosis factor receptor) family related gene
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HLA-DR	Human leukocyte antigen – antigen D related
ICOS	Inducible costimulatory molecule
IFN	Interferon

IL	Interleukin
M	Macrophage
mAbs	Monoclonal antibodies
MIP	Macrophage inflammatory protein
PBS	Phosphate buffered saline
PD-1	Programmed cell death protein 1
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
RANTES	Regulated on activation, normal T cell expressed and secreted
RNA	Ribonucleic acid
SIV	Simian deficiency virus
SSC	Side scatter
STI	Structured treatment interruption
TCR	T cell receptor
T_{FH}	Follicular B helper T cells
Th1	Type 1 T helper cells
T_{reg}	Regulatory T cells
Vβ	Variable beta

6 DECLARATIONS

6.1 Ethical approval and consent to participate:

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable standards. The study was approved by the Institutional Review Board of the Faculty of Medicine Siriraj Hospital at Mahidol University. Informed consent was obtained from all individual participants recruited in the study.

6.2 Consent for publication:

Written informed consent was obtained from the patients and participants for publication of their individual details and accompanying images in this manuscript. The consent form is held by the authors and is available for review by the Editor-in-Chief.

6.3 Availability of data and material:

All data generated or analyzed during this study are included in this published article.

6.4 Competing interests:

The authors declare that they have no conflict of interest.

6.5 Funding:

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6.6 Authors' contributions:

PT: performed the experiment, data analysis and manuscript writing; PP, VT, PA, and SL: performed the experiment and data analysis; NO: research idea formation, research monitoring, and manuscript editing. All authors read and approved the final manuscript.

6.7 Acknowledgement:

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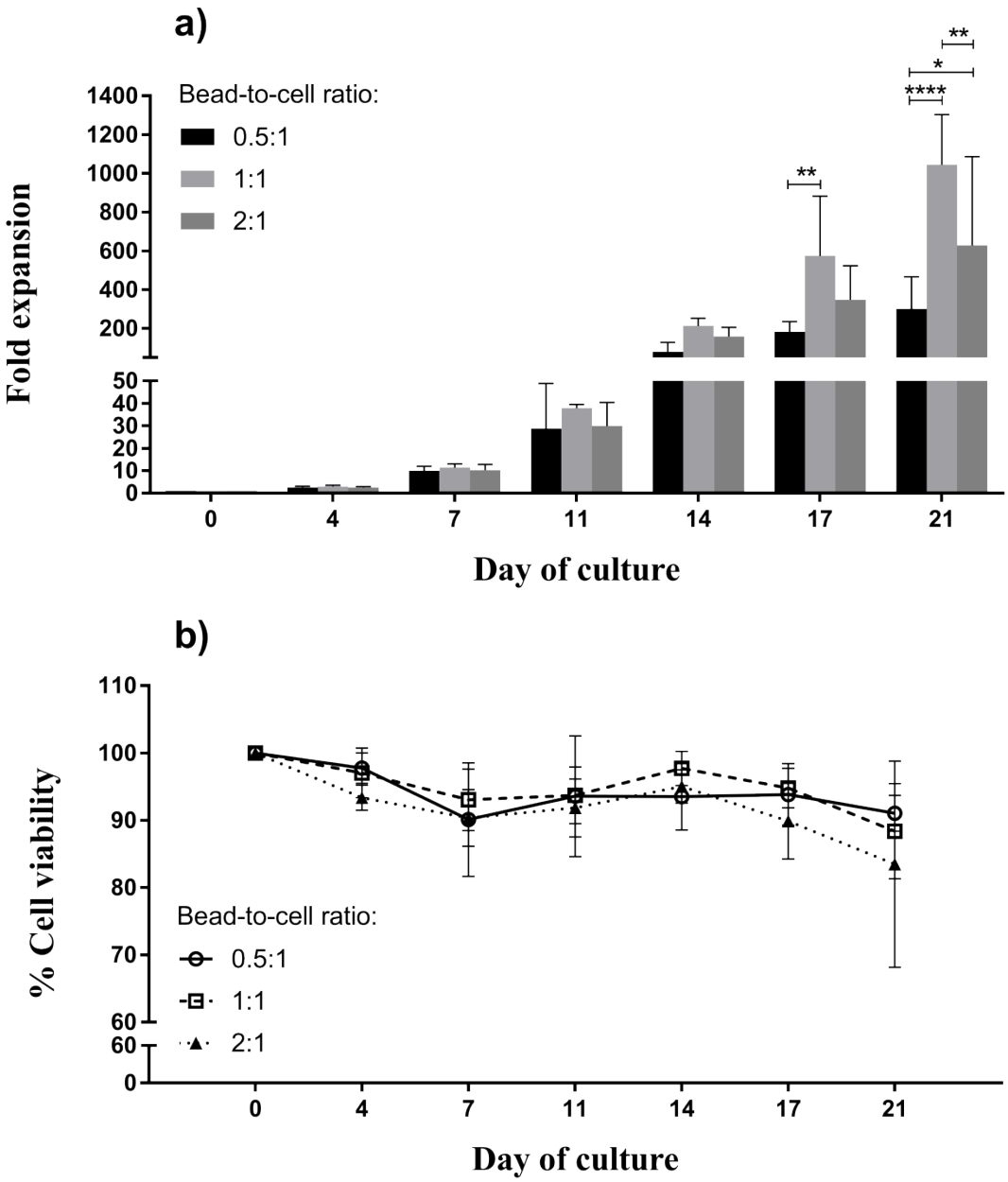

Fig. 1 Growth kinetics of anti-CD3/28 expanded $CD4^+$ T cells when using different bead-to-cell ratios for stimulation. Fold expansion (a) and cell viability (b) of the expanded cells at different bead-to-cell ratios (i.e., 0.5:1, 1:1, and 2:1) were observed over the 21-day culture. All data are presented as mean ± SD (n = 3, *p-value < 0.05, **p-value < 0.01 and ****p-value < 0.0001).

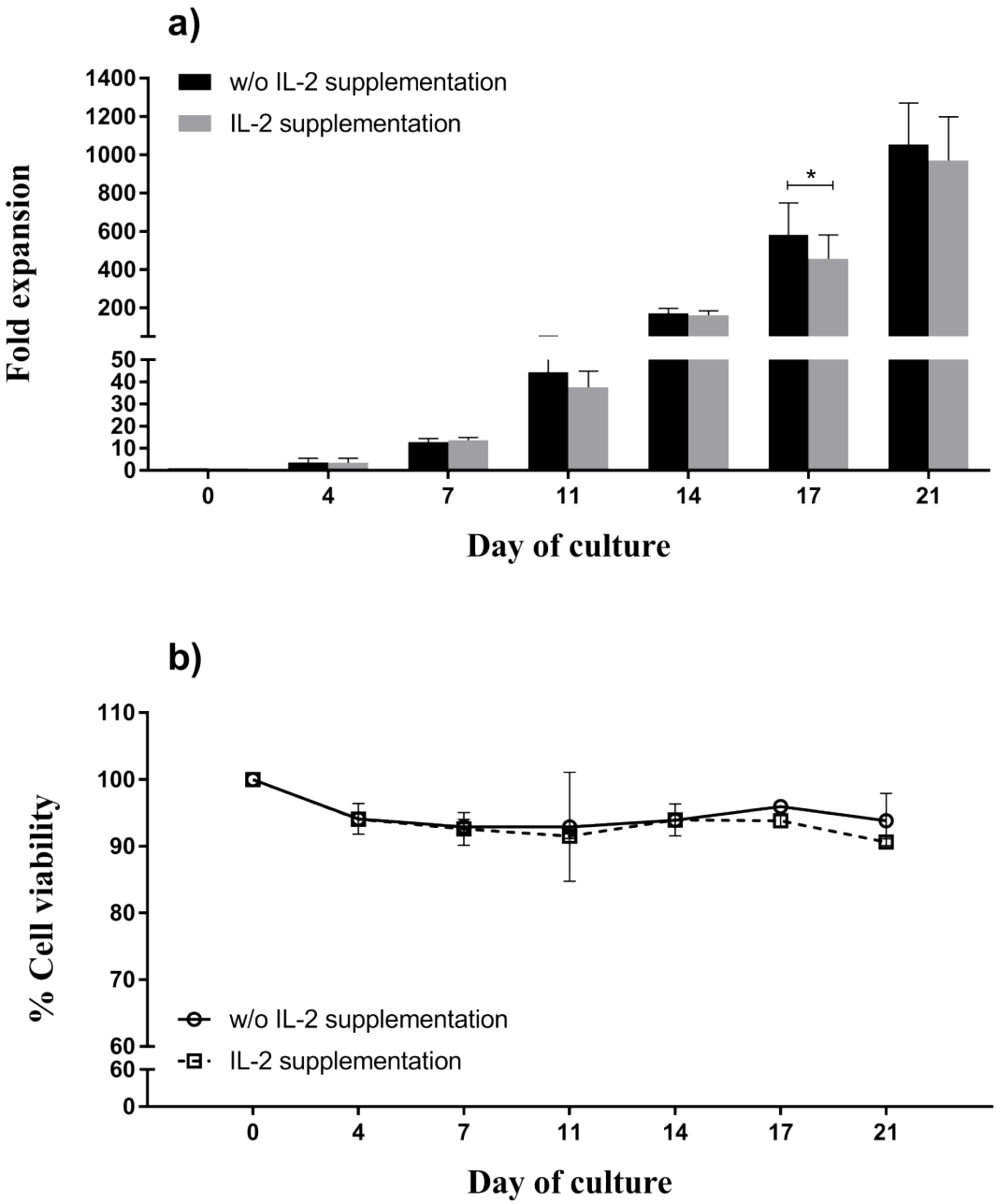

Fig. 2 Growth kinetics of anti-CD3/28 expanded $CD4^+$ T cells when cultured without and with IL-2 supplementation. Fold expansion (a) and cell viability (b) of the expanded cells when cultured without (w/o) and with IL-2 supplementation at the concentration of 20 units/mL were observed over 21 days. All data are presented as mean ± SD (n = 3 and *p-value < 0.05).

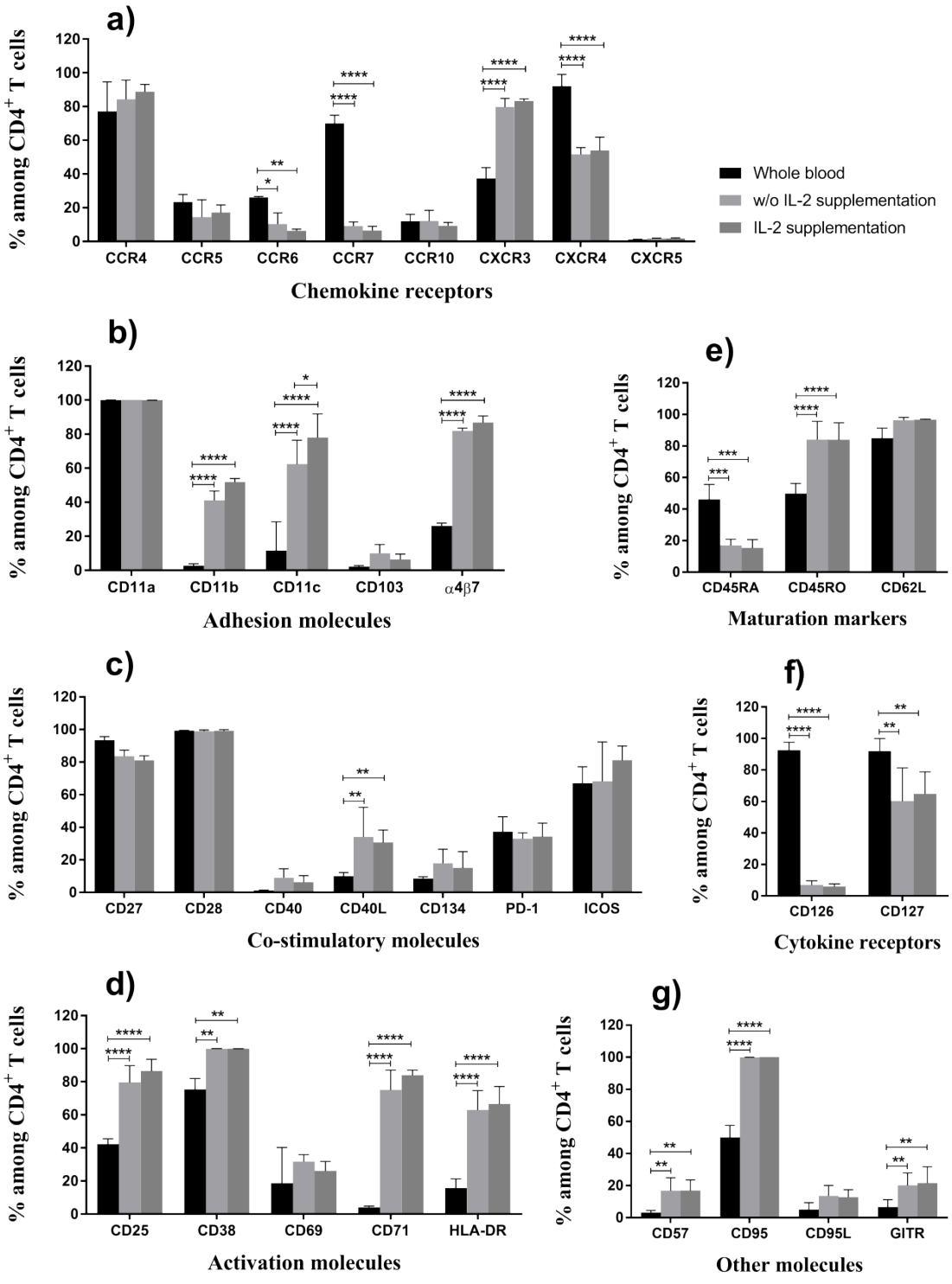

Fig. 3 Specific cell surface molecule expressions of anti-CD3/28 expanded CD4+ T cells. Expressions of a) chemokine receptors, b) adhesion molecules, c) co-stimulatory molecules, d) activation molecules, e) maturation markers, f) cytokine receptors and g) other molecules of the expanded cells when cultured without (w/o) and with IL-2 supplementation were observed on day 21 of the culture and compared to the expressions of whole blood observed on day 0. All data are presented as mean ± SD (n = 3, *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001 and ****p-value < 0.0001).

Table 1 Characterization of anti-CD3/28 stimulated $CD4^+$ T lymphocytes at different bead-to-cell ratios over the 21-day culture. Frequencies of all cell populations are represented as percentages of lymphocytes (mean ± SD, n = 3).

Cell population	Day of culture	Bead-to-cell ratio		
		0.5:1	**1:1**	**2:1**
$CD3^+$	0	99.3 ± 0.4	99.3 ± 0.4	98.8 ± 1.2
	14	99.8 ± 0.2	99.9 ± 0.1	99.9 ± 0.1
	21	99.6 ± 0.5	99.9 ± 0.1	99.9 ± 0.2
$CD3^+CD4^+CD8^-$	0	97.8 ± 1.3	98.1 ± 1.4	97.5 ± 1.7
	14	99.1 ± 0.6	99.8 ± 0.1	99.7 ± 0.1
	21	98.7 ± 1.5	99.5 ± 0.3	99.3 ± 0.4
$CD3^+CD4^-CD8^+$	0	0.0	0.0	0.0
	14	0.0 ± 0.1	0.0	0.0
	21	0.0	0.0	0.0
$CD3^+CD4^+CD8^+$	0	0.0	0.0	0.0
	14	0.0	0.0	0.0
	21	0.0 ± 0.1	0.0 ± 0.1	0.0
Others[a]	0	0.9 ± 0.5[b]	0.7 ± 0.4[b]	1.1 ± 0.9[b]
	14	1.7 ± 0.4[b]	3.5 ± 1.5[b]	2.4 ± 0.3[b]
	21	1.3 ± 0.7[b]	1.3 ± 0.4[b]	1.2 ± 0.4[b]

[a]Other cell populations include $CD3^-CD19^+$, $CD3^-CD16^+CD56^+$, $CD3^+CD16^+CD56^+$, and $CD3^+CD19^+$.

[b]A sum of average percentages of frequencies of the other cell populations.

Table 2 Characterization of anti-CD3/28 stimulated $CD4^+$ T lymphocytes when cultured without (w/o) and with IL-2 supplementation over 21 days. Frequencies of all cell populations are represented as percentages of lymphocytes (mean ± SD, n = 3).

Cell population	Day of culture	Supplementation	
		w/o IL-2	IL-2
$CD3^+$	0	99.5 ± 0.2	99.2 ± 0.7
	14	96.7 ± 5.7	99.8 ± 0.1
	21	99.9 ± 0.1	99.9 ± 0.0
$CD3^+CD4^+CD8^-$	0	98.3 ± 0.6	97.9 ± 1.7
	14	94.4 ± 5.2	99.6 ± 0.1
	21	99.2 ± 0.1	99.0 ± 0.9
$CD3^+CD4^-CD8^+$	0	0.0	0.0
	14	0.0	0.0
	21	0.1 ± 0.1	0.0
$CD3^+CD4^+CD8^+$	0	0.0	0.0
	14	1.4 ± 2.4	0.1 ± 0.1
	21	0.0	0.0
Others[a]	0	0.7 ± 0.4[b]	0.8 ± 0.6[b]
	14	2.0 ± 1.0[b]	1.9 ± 0.6[b]
	21	1.3 ± 0.4[b]	0.6 ± 0.4[b]

[a]Other cell populations include $CD3^-CD19^+$, $CD3^-CD16^+CD56^+$, $CD3^+CD16^+CD56^+$, and $CD3^+CD19^+$.

[b]A sum of average percentages of frequencies of the other cell populations.

มหาวิทยาลัยมหิดล
คณะแพทยศาสตร์ศิริราชพยาบาล

รายงานวิจัยฉบับสมบูรณ์

โครงการการประเมินกระบวนการสร้าง anti-CD3/28 expanded CD4+ T cell สำหรับการพัฒนาวิธีการรักษาด้วยภูมิคุ้มกันในผู้ป่วยโรคติดเชื้อเอชไอวี (Evaluation of anti-CD3/28 expanded CD4+ T cell production strategy for a development of an immune based therapy in HIV-1 infected patients)

โดย
รศ.ดร.ณัฐวัฒน์ อ่อนลมูล

มิถุนายน 2561

สัญญาเลขที่ RSA5880020

รายงานวิจัยฉบับสมบูรณ์

โครงการการประเมินกระบวนการสร้าง anti-CD3/28 expanded CD4+ T cell สำหรับการพัฒนาวิธีการรักษาด้วยภูมิคุ้มกันในผู้ป่วยโรคติดเชื้อเอชไอวี (Evaluation of anti-CD3/28 expanded CD4+ T cell production strategy for a development of an immune based therapy in HIV-1 infected patients)

ผู้วิจัย รศ.ดร.ณัฐวัฒน์ อ่อนลมูล
สังกัด คณะแพทยศาสตร์ศิริราชพยาบาล
มหาวิทยาลัยมหิดล

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและ
มหาวิทยาลัยมหิดล

บทคัดย่อ

รหัสโครงการ : RSA5880020

ชื่อโครงการ : การประเมินกระบวนการสร้าง anti-CD3/28 expanded CD4+ T cell สำหรับการพัฒนาวิธีการรักษาด้วยภูมิคุ้มกันในผู้ป่วยโรคติดเชื้อเอชไอวี

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การใช้ยาต้านไวรัสในผู้ป่วยโรคติดเชื้อเอชไอวีช่วยให้เกิดประสิทธิผลสูงสุดในการลดจำนวนไวรัส อย่างไรก็ตามการใช้ยาต้านไวรัสดังกล่าวช่วยเพียงควบคุมปริมาณไวรัสและฟื้นฟูความสามารถของภูมิคุ้มกันร่างกายเพียงบางส่วนโดยที่มีการบูรณะภูมิคุ้มกันได้อย่างเชื่องช้าและไม่สมบูรณ์ การศึกษาก่อนหน้าได้แสดงการใช้เม็ดแม่เหล็กเคลือบด้วยแอนติบอดีต่อซีดี3และซีดี28เพื่อการเพิ่มจำนวนเซลล์ในห้องปฏิบัติการซึ่งช่วยให้เซลล์สามารถควบคุมการแบ่งตัวของไวรัส รวมทั้งการถ่ายโอนเซลล์ซีดี4ที่ถูกเพิ่มจำนวนขึ้นกลับไปยังผู้ป่วยที่ได้รับยาต้านไวรัสยังช่วยให้มีการเพิ่มจำนวนทีเซลล์ชนิดซีดี4 จากผลการศึกษาเบื้องต้นดังกล่าวทำให้คณะผู้วิจัยได้พัฒนาวิธีการเพิ่มจำนวนเซลล์เพื่อการทดสอบระดับคลินิกรวมทั้งทำการจำแนกลักษณะเฉพาะและคุณสมบัติการทำงาน ในการศึกษานี้ การเพิ่มจำนวนเซลล์ปริมาณมากสามารถทำได้โดยการเลี้ยงเซลล์ในถุงสำหรับเลี้ยงเซลล์ที่ได้มาตรฐานการผลิตในระบบปิดร่วมกับการกระตุ้นโดยใช้เม็ดแม่เหล็กเคลือบด้วยแอนติบอดีต่อซีดี3และซีดี28 นอกจากนี้แล้วการเพิ่มจำนวนเซลล์โดยใช้อาหารเลี้ยงเซลล์ที่ได้มาตรฐานการผลิตร่วมกับการเสริมด้วยซีรั่มมนุษย์ยังทำให้ได้การแบ่งตัวเซลล์ในระดับที่น่าพอใจ การประเมิณโมเลกุลแบบจำเพาะบนผิวเซลล์และความสามารถในการสร้างสารไซโตไคน์แสดงให้เห็นรูปแบบของโมเลกุลบนผิวเซลล์ที่จำเพาะและลักษณะของทีเฮลเปอร์แบบที่1 โดยสรุป ผลการศึกษานี้แสดงถึงกระบวนการผลิตเซลล์ที่มีประสิทธิภาพสำหรับการนำไปใช้เพื่อการศึกษาทางคลินิกต่อไป

คำหลัก : ซีดี4 ทีลิมโฟไซต์, การเพิ่มจำนวนเซลล์, เม็ดแม่เหล็กเคลือบแอนติบอดีต่อซีดี3และซีดี28, การรักษาแบบภูมิคุ้มกันบำบัด, โรคติดเชื้อเอชไอวี

Abstract

Project Code : RSA5880020

Project Title : Evaluation of anti-CD3/28 expanded CD4+ T cell production strategy for a development of an immune based therapy in HIV-1 infected patients

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Project Period : 1 July 2015 - 30 June 2018

The use of antiretroviral therapy (ART) in human immunodeficiency virus (HIV) infected patients has become highly effective in lowering viral burden. However, while ART could control viremia and restore some level of immune competency, the immune reconstitution is slow and never complete. Previous studies showed that in vitro expansion of CD4+ T cells from HIV infected patients by anti-CD3/28 coated beads provided intrinsic control of viral replication and transfusion of autologous expanded CD4+ T cells showed increase in CD4+ T cells in antiretroviral treated HIV infected patients. From this preliminary finding, a clinical grade cell expansion protocol with phenotypic and functional characterization was developed. In this study, a large number of expanded CD4+ T lymphocytes can be achieved with the developed closed-culture system using Good Manufacturing Practice (GMP)-grade culture bags and anti-CD3/28 coated bead stimulation. Moreover, cell expansion using GMP-grade media with human serum supplementation rendered satisfied proliferation rates. Evaluation of specific surface molecule expressions and cytokine producing ability presented a specific surface molecule expression pattern with T helper 1-like phenotype. In conclusion, this result presented the effective cell manufacturing method for further uses in clinical trials.

Keywords : CD4+ T lymphocyte, cell expansion, anti-CD3/28 coated beads, immunotherapy, HIV infection

Executive summary

CD4 immunotherapy is potentially useful in immune reconstitution of CD4+ T cells for HIV-infected patients. Transfusion of anti-CD3/28 expanded CD4+ T cells is also proved to be safe and effective in both SIV-infected macaques and HIV-infected patients. However, there is no such standardized and practical protocol available for cell production in order to use in clinics. This study thus aimed to develop a closed-culture system for in vitro CD4+ T lymphocyte expansion by using a commercially available GMP-grade culture bag and anti-CD3/28 activation. The expansion rates and yields of expanded CD4+ T cells by using commercially available GMP-grade culture media with human serum (HS) supplementation were also determined. Moreover, a confirmation of functional-related phenotypes was assessed for ensuring the successful outcomes of treatment.

Freshly isolated CD4+ T cells by immunorosette formation from healthy donors and cryopreserved CD4+ T cells from HIV-infected patients with CD4 count over 500 cells/μL were stimulated with anti-CD3/28 coated beads. The activated cells were then expanded in conventional culture flasks and GMP-grade culture bags for three weeks. Fold expansion, cell viability, growth kinetic and phenotypic characters were observed. Results revealed that purified CD4+ T cells from healthy individuals cultured in flasks showed better expansion than those cultured in bags (797-fold and 331-fold, respectively), whereas, their cell viability, growth kinetic and expanded CD4+ T cell purity were almost similar. A large-scale production was also conducted and supported consistency of cell proliferation in the closed-culture system. Frozen CD4+ T lymphocytes from the patients were able to remain their growth function and well expanded with a good yield of 415-fold, 85% viability and 96% purity of CD4+ T cells at the end of a 3-week culture in bags.

Isolated CD4+ T cells from healthy donors were also expanded in different media supplemented with either HS or fetal bovine serum (FBS) for 3 weeks. Results showed that the expanded cells from the cultures using AIM-V and DC media with 10% HS were well expanded (~255-fold) and had similar polyfunctional patterns, however, they showed poorer proliferation rates than those from the cultures using RMPI1640 with 10% FBS (R10; ~637-fold). Moreover, cell viability of the expanded cells from R10 was also significantly higher than the others after a 3-week culture. High purity with CD3+CD4+CD8- phenotype of the expanded cells from all cultures was also observed. The expanded cells from all conditions were found predominantly producing TNF-α, IL-2 and IFN-γ and frequency of IL-2 producing cells in R10 was the highest.

As surface molecule expressions are related to cell maturation, activities and functions, this study thus explored several cell surface molecule expressions of anti-CD3/28 expanded CD4+ T cells which were divided into seven groups according to the molecules' functions and roles. The cell surface molecules included (i) chemokine receptors: CCR4, CCR5, CCR6, CCR7, CCR10, CXCR3, CXCR4 and CXCR5; (ii) adhesion molecules: CD11a, CD11b, CD11c, CD103 and $\alpha 4\beta 7$; (iii) co-stimulatory molecules: CD27, CD28, CD40, CD40L, CD134, PD-1 and ICOS; (iv) activation molecules: CD25, CD38, CD69, CD71 and HLA-DR; (v) maturation markers: CD45RO, CD45RA and

CD62L; (vi) cytokine receptors: CD126 and CD127; and (vii) other molecules: CD57, CD95, CD95L, and GITR. Changes in specific cell surface molecule expressions of the anti-CD3/28 CD4+ T cells were observed in this study. Our expanded cells also rendered a low frequency in CCR5 and twice as less CXCR4 expression than the unexpanded cells. Furthermore, our study shows that the expanded cells had a pretentious increase in $\alpha 4\beta 7$ expression suggesting that that these cells feasibly migrate to the site of depletion and improve immune response at the gut-associated lymphoid tissues.

The developed closed-culture system using culture bags and anti-CD3/28 coated beads can achieve a large number of expanded CD4+ T lymphocytes with good reproducibility, suggesting a promising protocol required for adoptive immunotherapy. Our expansion protocol using GMP-grade media, AIM-V and DC with HS supplementation rendered satisfied proliferation rates and yields without differences in polyfunctional patterns, indicating the promising method for further uses in clinical trials. Addition of FBS is also suggested for expansion improvement but under the condition that its source must be free from bovine spongiform encephalopathy. Moreover, specific surface molecule expressions of the expanded cells presented potential roles in proliferation, differentiation, homeostasis, apoptosis and organ-homing.

Introduction

Human immunodeficiency virus (HIV) infection causes a progressive decrease of CD4+ T lymphocytes and an increase of HIV viral load (or HIV RNA level), leading to higher susceptibility to opportunistic infections which can further develop to acquired immune deficiency syndrome (AIDS) [1]. HIV enters target cells through the binding of viral envelope glycoproteins to CD4 receptors along with CCR5 and CXCR4 co-receptors markedly expressed on the target CD4+ T lymphocytes [2-4]. Although highly active antiretroviral therapy (HAART) succeeds to control the HIV viral load into an undetectable level and recovers the CD4 counts in HIV-infected patients, the latent reservoir of virus still exists [5] and the immune restoration is incomplete [6-9]. A life-long treatment of HAART has also feasible consequences in cumulative drug toxicities, emergent drug-resistant viruses and unaffordable costs due to more complicated regimens. Moreover, some patients who have discordant immune responses (DIR) to HAART fail to achieve target CD4 count levels despite accomplished virological control, suggesting a higher risk in mortality [10].

An alternative approach, such as adoptive transfer of autologous activated CD4+ T lymphocytes, has been proposed to be a potential treatment for the benefit of both virological control and direct immune reconstitution. Its effectiveness and safety have been confirmed by in vivo studies in both simian deficiency virus (SIV)-infected rhesus macaques and HIV-infected patients [11-15]. To expand CD4+ T cells in vitro, anti-CD3/28 coated magnetic beads are widely used for stimulation. The anti-CD3/28 activated CD4+ T cells showed intrinsic resistance to macrophage (M)-tropic isolates of HIV-1 infection [16-18] and promoted expression of RANTES, MIP-1α and MIP-1β as well as reduced expression of CCR5 [11,13,14,16]. Furthermore, the expanded CD4+ T cells induced interferon (IFN)-γ production which is associated to type 1 T helper (Th1) cell function and increased the density of variable beta (Vβ) chain T cell receptor (TCR) repertoires [14] together with telomerase activity, resulting in a longer survival of the cells [11].

With respect to the clinical uses, a large number of CD4+ T cells expanded in vitro was required for reinfusion in HIV-infected patients [14,15]; therefore, optimization of expansion protocols is warranted. There have been established in vitro culture methods for anti-CD3/28 stimulated CD4+ T lymphocytes providing different yields [19-21] which can be related to different cell isolation methods, bead-to-cell ratios used for stimulation, and medium supplementation.

Regarding to the usage of culture media, basal culture media are generally supplemented with animal serum (e.g., fetal bovine serum (FBS)) which is essential for cell growth, metabolism and proliferation. The functions of serum in culture media are (i) to provide hormonal factors stimulating cell growth, proliferation and differentiation, (ii) to transport proteins carrying hormones, minerals, trace elements and lipids, (iii) to attach and spread factors, acting as germination points for cell attachment and (iv) to stabilize and detoxify factors needed for maintaining pH as well as to inhibit proteases and other toxic molecules. Nevertheless, the use of animal serum, particularly FBS, has been debated in some serious scientific and ethical concerns in terms of serum production and cell harvesting. Development of serum-free or animal/human protein-free media is then important for safety improvement in biological products for cell therapy and vaccination [22].

FBS can be substituted with defined chemical components in serum-free cell culture. Trickett et al. (2002) demonstrated that the expansion of functional T lymphocytes from HIV-infected patients was good when stimulated with anti-CD3/28 coated microspheres and propagated in serum-free media. However, the greater T cell proliferation was observed when supplemented with FBS in the initial period of cell expansion, whereas, human albumin (AB) serum supplementation failed to increase T cell numbers. Plasma supplementation also provided a low level of CD4+ T cells which was resulted from phenotypic switching of CD8+ T lymphocytes [23]. Carlens et al. (2000) studied expansion rates of anti-CD3 stimulated T lymphocytes in three different serum-free media (i.e., X-VIVO 15, AIM-V and Cellgro SCGM) compared to standard RPMI1640 media with 5% human serum (HS) and 10% FBS. A 3-week activation in serum-free media resulted in a small increase in expansion rates, whereas the culture with serum supplementation rendered better consistency and effectiveness in cell expansion. The additional low level of HS thus supported the T-cell expansion in all culture media types. On the other hands, supplementation with 10% FBS showed inconsistency in cell expansion when compared to that with 5% HS. Increased levels of IFN-γ secretion were detected for all media combinations when compared with serum-free culture. No IL-4 and IL-10 production was found [24].

Although the in vitro cultures with HS supplementation supported better CD4+ T cell expansion than those with serum-free media and more consistency than those with FBS supplementation, the fold expansion numbers (< 267-fold) [24] was not high when compared to our previous method [19]. While GMP-grade culture media are required for cell production, the information about the effect of serum supplementation in cell functions is limited.

More importantly, functional-associated phenotypic characters of the expanded cells are essential which are not only related to cell characterization but also maturation and activation stages as well as cell migration. Even so, there is limited information concerning specific cell surface molecule expressions of the expanded CD4+ T cells, such as chemokine receptors and maturation markers.

Objectives

1. To develop a closed-culture system for *in vitro* CD4+ T lymphocyte expansion by using a commercially available GMP-grade culture bag and anti-CD3/28 activation as well as a large-scale production aiming for adoptive immunotherapy.
2. To assess the expansion rates and yields of anti-CD3/28 expanded CD4+ T cells by using commercially available GMP-grade culture media with HS supplementation in comparison with the established protocol.
3. To investigate the expressions of surface molecule on anti-CD3/28 expanded cells including chemokine receptors, adhesion molecules, co-stimulatory molecules, activation molecules, maturation markers, cytokine receptors, and other functional-specific molecules.

Materials and Methods

1. Samples

Healthy volunteers and HIV-infected patients were recruited and signed informed consents which were approved by the Institutional Review Board of the Faculty of Medicine Siriraj Hospital at Mahidol University. Sixteen to twenty-five milliliters of whole blood were collected into sodium heparin-containing vacutainer tubes and kept at room temperature prior to phenotypic determination of whole blood and CD4+ T lymphocyte isolation. Cryopreserved CD4+ T lymphocytes were obtained from HIV-infected patients with CD4+ T cell count over 500 cells/μL and stored in a liquid nitrogen tank at -196 °C for 1.5 - 2 years.

2. Antibodies

Monoclonal antibodies (mAbs) and their conjugated fluorochromes including anti-CD3 conjugated with fluorescein isothiocyanate (FITC), anti-CD4 conjugated with phycoerythrin (PE), anti-CD8 conjugated with PE, anti-CD19 conjugated with PE, anti-CD4 conjugated with allophycocyanin (APC), anti-CD16 conjugated with APC, anti-CD56 conjugated with APC, anti-CD19 conjugated with allophycocyanin and cyanine dye (APC-Cy7), anti-CD45 conjugated with peridinin chlorophyll protein (PerCP), and anti-CD56 conjugated with phycoerythrin and cyanine dye (PE-Cy7) were purchased from Becton Dickinson Bioscience (BDB, San Jose, CA). In addition, anti-CD3 conjugated with AlexaFluor® (A700), anti-CD4 conjugated with Brilliant Violet™ 605 (BV605), anti-CD8 conjugated with PE/Dazzle™ 594, anti-CD69 conjugated with PerCP/Cy5.5, anti-IL-2 conjugated with BV510, anti-IL-4 conjugated with FITC, anti-IL-17 conjugated with PE, anti-IFN-γ conjugated with APC, anti-TNF-α conjugated with BV650 and anti-TGF-β conjugated with BV421 were obtained from BioLegend (San Diego, CA). The fluorescent-labeled mAbs used for identification of specific surface molecule expression were anti-CD4 PerCP, anti-CD3 FITC, anti-CD45RO FITC, anti-CD45RA FITC, anti-CD57 FITC, anti-CD27 FITC, anti-CCR7 PE, anti-CD62L PE, anti-CD11a PE, anti-CD11b PE, anti-CD11c PE, anti-CD126 PE, anti-CD127 PE, anti-CD95 PE, anti-CD95L PE, anti-CD154 (CD40L) PE, anti-CD40 PE, anti-CD134 (OX40) PE, anti-CD278 (ICOS) PE, anti-CD71 PE, anti-HLA-DR PE, anti-GITR PE, anti-CD28 PE, anti-CD103 PE, anti-CD38 PE, anti-CD69 PE, anti-CD25 PE, anti-CD184 (CXCR4) PE, anti-CD183 (CXCR3) PE, anti-CCR10 PE, anti-CD195 (CCR5) PE, anti-PD-1 PE, anti-CXCR5 PE, anti-CCR6 PE, anti-CCR4 PE and anti- $\alpha 4\beta 7$ PE.

3. CD4+ T lymphocyte isolation

CD4+ T lymphocytes can be directly isolated from whole blood by an immunorosettes formation method using RosetteSep® human CD4+ T cell enrichment cocktail (STEMCELL Technologies, Vancouver, BC, Canada). Briefly, CD4+ T lymphocytes were isolated from 5 mL of whole blood by adding 250 μL of RosetteSep® human CD4+ T cell enrichment cocktail. After that, the samples were thoroughly mixed and incubated at room temperature for 20 minutes. The samples were then diluted with an equal volume of phosphate buffered saline (PBS) containing 2% fetal bovine serum (FBS) and gently mixed. The diluted blood samples were carefully layered on top of LSM® lymphocyte separation medium and centrifuged at 1200g with no break at room temperature for 20 minutes. After centrifugal separation, the samples were divided into four layers including

plasma, enriched CD4+ T cells, LSM® lymphocyte separation medium and red blood cells (from top to bottom). Pasteur pipettes were used to remove the plasma layer and collect enriched CD4+ T cells from the layer interface. The collected CD4+ T cells were then washed with 10 mL of PBS containing 2% FBS and centrifuged twice at 1400 rpm at room temperature for 5 minutes. The cell pellets were collected and re-suspended with a complete medium (RPMI1640 with 10% FBS, 50 µg/mL pencillin-streptomycin and 2 mM L-glutamine). Cell number and viability of the enriched CD4+ T cells were determined by trypan blue exclusion using a hemacytometer.

4. Cell stimulation and expansion for freshly isolated CD4+ T cells

Freshly purified CD4+ T cells of $1x10^6$ cells were stimulated with anti-CD3/28 coated beads (Dynabeads® Human T-Activator CD3/CD28, Invitrogen Dynal, Oslo, Norway) at a bead-to-cell ratio of 1:1. The stimulated CD4+ T cells were then expanded in complete media (RPMI1640 with 10% fetal bovine serum (FBS)), 50 µg/mL pencillin-streptomycin and 2 mM L-glutamine). The expanded cells were incubated at 37°C and 5% CO2 humidification and reactivated on day 7. The cells were expanded for a 3-week culture period. Cell numbers and viability were observed by using trypan blue exclusion and a TC10™ automated cell counter. Phenotypic characters were analyzed by flow cytometry on days 14 and 21.

For an expansion method of flask culture, the stimulated cells of $1x10^6$ cells were placed in a 24-well plate (Costar® 24 well clear TC-treated multiple well plates, sterile, Corning Inc., Life Sciences, NY, USA) on day 0 and expanded at a concentration of 0.5x106 cells/mL before transferring to T25, T75 and T175 plastic tissue culture flasks (Corning® U-shaped canted neck cell culture flask with vent cap, Corning Inc., Life Sciences, NY, USA) on days 4, 7, and 11, respectively. Cell numbers and viability were observed on days 4, 7, 11, 14, 17 and 21 while the media were replenished with calculated amounts of fresh media on days 4, 7, 11, 14, and 17 to maintain the cell suspension concentration at $0.5x10^6$ cells/mL.

With respect to bag culture, the expansion process was similar to flask culture during the first week of cell expansion. The expanded cells were re-stimulated in T25 flasks on day 7 and replenished with calculated amounts of fresh media at a concentration of $0.5x10^6$ cells/mL before transferring to a GMP-grade culture bag (Vuelife® cell culture bags, CellGenix, Freiburg, Germany) with a size of 72c (maximum volume of 72 mL). The culture bag was clamped by half and placed on a steel grating culture stage prior to cell transfer. Fresh media were added to reach maximum volume of the bag (72 mL) on day 11. After that, the expanded cells were transferred to another culture bag with a size of 197c on day 14, filled with media up to 197 mL on day 17, and leaved for growth until day 21. Cell numbers and viability were observed on days 4, 7, 14, and 21.

Large-scale production of freshly isolated CD4+ T cells in a closed-culture system was also perform. Freshly purified CD4+ T cells of $8x10^6$ cells from healthy donors were mixed with anti-CD3/28 coated beads (Dynabeads® Human T-Activator CD3/CD28, Invitrogen Dynal, Oslo, Norway) at a bead-to-cell ratio of 1:1 in a plastic tube (Falcon® high clarity polypropylene centrifuge tube, conical bottom, sterile, Corning Inc., Life Sciences, NY, USA) before injecting into a GMP-grade culture bag (Vuelife® cell culture bags, CellGenix, Freiburg, Germany) with a size of 32c. Complete

media (RPMI1640 with 10% fetal bovine serum, 50 µg/mL pencillin-streptomycin and 2 mM L-glutamine) were added in order to achieve a concentration of $0.5x10^6$ cells/mL. The expanded cells were incubated at 37°C and 5% CO2 humidification. Only $20x10^6$ cells of anti-CD3/28 expanded CD4+ T cells were reactivated on day 7. The expanded cells were consequently transferred to larger culture bags with sizes of 72c and 196c on days 7 and 14. Fresh culture media were added to reach suggested maximum volume of individual bag size on days 4, 11 and 17. Cell numbers and viability were observed on days 7, 14 and 21 by using trypan blue exclusion and a TC10™ automated cell counter. Phenotypic characters were analyzed by flow cytometry on days 14 and 21.

For patient's cell expansion, cryopreserved CD4+ T lymphocytes from HIV-infected patients were removed from a liquid nitrogen tank at -196 °C and then thawed in a sterile water bath at 37°C. Cryopreserved cells of $1x10^6$ cells were activated and expanded following the developed bag culture protocol of freshly isolated CD4+ T cells. In addition, fresh media with IL-2 supplementation at a concentration of 100 U/mL was used on day 7 onwards. Cell numbers and viability were observed on days 4, 7, 14 and 21 by using trypan blue exclusion and a TC10™ automated cell counter. Phenotypic characters were analyzed by flow cytometry on days 14 and 21.

For comparison between culture medium and human serum (HS) supplementation, $1x10^6$ cells of purified CD4+ T cells were expanded in 4 different culture conditions including (i) RPMI1640 supplemented with 10% FBS (Gibco, Paisley, UK), (ii) RPMI1640 supplemented with 10% heat inactivated HS (Gibco, Paisley, UK), (iii) AIM-V® medium CTS™ (Therapeutic grade, Gibco, Paisley, UK) supplemented with 10% heat inactivated HS and (iv) CellGro® GMP serum-free dendritic cell medium (CellGro® DC medium, CellGenix GMBH, Freiburg, Germany) supplemented with 10% heat inactivated HS. Cell expansions were conducted by using the same protocol for flask culture described above.

For bead-to-cell ratio comparison, $1x10^6$ enriched CD4+ T cells were stimulated with anti-CD3/CD28 coated beads in the absence of exogenous interleukin (IL)-2. The bead number was calculated for 0.5:1, 1:1, and 2:1 bead-to-cell ratios to use for the expansion. Cell expansions were conducted by using the same protocol for flask culture described above.

With respect to IL-2 supplementation comparison, the similar activation and culture protocols were conducted by using only the 1:1 bead-to-cell ratio and cultures in the absence and in the presence of exogenous interleukin (IL)-2 at the low concentration of 20 units/mL (Prospec, Ness-Ziona, Israel). Cell numbers and viability were observed on days 4, 7, 11, 14, 17 and 21 by using trypan blue exclusion and a hematocytometer. Lymphocyte subset characters were analyzed by a flow cytometer on days 0, 14 and 21.

5. Immunofluorescent staining and flow cytometric analysis

Whole blood, purified and expanded CD4+ T cells were stained with fluorochrome-conjugated mAbs and incubated for 15 minutes before adding 1X lysing solution for red blood cell lysis. The stained cells were then washed with PBS containing 2% FBS prior to centrifugation at 1,400 rpm at 25 °C for 5 minutes. Subsequently, the stained cells were re-suspended in PBS containing 1% paraformaldehyde. The stained cells were finally acquired by a BD FACSCalibur flow

cytometer or BD FACSVerse™ flow cytometer (BDB, San Jose, CA) and the data were analyzed by using FlowJo Software (Tree Star, San Carlos, CA).

Six-parameter analysis including forward scatter (FSC), side scatter (SSC), FITC, PE, PerCP, and APC was performed using FlowJo Software (Tree Star, San Carlos, CA). The stained cells were gated using lymphogate (FSC/SSC) to determine a viable lymphocyte population. After that, lymphocyte subsets were defined using two-dimensional dot plots between CD45/SSC, CD45/CD3 and CD4/CD8 or CD19/CD16+CD56. Therefore, the lymphocyte subsets were detected into CD4+CD8-, CD4-CD8+, CD4+CD8+, CD19-CD16+CD56+ and CD19+CD16-CD56- populations. The frequencies of anti-CD3/28 expanded CD4+ T Cells that express specific surface molecules were also determined on a population of expanded cells.

6. Cell stimulation, intracellular cytokine staining (ICS) and analysis

Cryopreserved expanded CD4+ T cells from different culture conditions were used to determine different types of cytokine production. Expanded CD4+ T cells at $1x10^6$ cells/mL were stimulated with 25 ng phorbol 12-myristate 13-acetate (PMA) and 1 μg ionomycin (I) in the presence of brefeldin A (BFA) at 10 μg, whereas the expanded CD4+ T cells in the presence of BFA without stimulation were used as a control. The samples were then incubated at 37 °C and 5% CO2 for 4 h. After the incubation, PMA/I stimulated and unstimulated samples were stained with Zombie NIR™ dye (BioLegend, San Diego, CA) at 4 °C for 15 min. A washing buffer (phosphate buffered saline (PBS) with 2% FBS) were added and the samples were washed by centrifugation at 450g for 5 min. The samples were then surface stained with a combination of mAbs including anti-CD3 A700, anti-CD4 BV605, anti-CD8 PE/Dazzle™ 594 and anti-CD69 PerCP/Cy5.5 at 4 °C for 15 min and washed once. The stained samples were fixed and permeabilized in 0.5 mL of BD Cytofix/Cytoperm™ fixation and permeabilization solution (BDB, San Jose, CA) at 4 °C for 20 min. After the incubation, the samples were washed by adding 1X BD Perm/Wash™ buffer (BDB, San Jose, CA) and centrifuged at 500g for 5 min. ICS was performed by staining with a combination of mAbs including anti-IL-2 BV510, anti-IL-4 FITC, anti-IL-17 PE, anti-IFN-γ APC, anti-TNF-α BV650, anti-TGF-β BV421 at 4 °C for 30 min. After staining, the samples were washed with 1X BD Perm/Wash™ buffer (BDB, San Jose, CA) and re-suspended in PBS.

The stained cells of at least 100,000 events were acquired for each analysis by a BD Fortessa™ flow cytometer (BDB, San Jose, CA) and the data was analyzed by using FlowJo Software (Tree Star, San Carlos, CA). Cytokine producing cell subsets were determined from activated populations expressing CD69 and percentages of cytokine producing cell subsets were determined from a total cytokine producing cell population. A Boolean gating strategy was used for the analysis of polycytokine producing cell subsets in order to evaluate cytokine producing cell subsets with ability to simultaneously produce 1, 2, 3, 4, 5 or 6 cytokines.

7. Cell harvesting and quality control

Cell suspensions containing expanded CD4+ T cells from a bag culture of cryopreserved CD4+ T cells from HIV-infected patients were collected on day 21 of cell expansion. Aliquots of cell suspensions were transferred to 50-mL tubes and centrifuged at 450g for 5 min.

Culture supernatants were removed, collected in small aliquots and stored in a -80 $^{\circ}$C freezer prior to thaw for using in quality control. Cell pellets were re-suspended in 10 mL of complete media and transferred to 15-mL tubes.

To remove anti-CD3/28 coated beads, each 15-mL tube was placed in a DynaMag™-15 Magnet (Invitrogen Dynal) for 2 min and the cell suspension was transferred to another 15-mL tube. The same procedure was repeated once and aliquots of cell suspensions were pooled. The centrifugation process was repeated and media was removed. Cell pellets were re-suspended in complete media and pooled into a single tube at a volume of 20 mL. Cell numbers and viability were observed by using trypan blue exclusion and a TC10™ automated cell counter. Aliquots of the expanded CD4+ T cells at 1x10^{7} cells/mL were cryopreserved in RPMI-1640 media containing 20% FBS with 10% DMSO and stored at 180 $^{\circ}$C prior to thaw for using in quality control and intracellular cytokine staining.

For quality control, frozen expanded CD4+ T cells were thawed and re-suspended in PBS containing 5% human albumin. To detect residual bead contamination, samples containing 1x10^{7} cells were mixed with sodium hypochlorite solution and centrifuged at 450g for 5 min. Supernatants were removed and the pellets were re-suspended with PBS. Residual bead counts were determined on a hemacytometer and the total numbers of beads were calculated. For sterility testing, a 14-days United States Pharmacopoeia (USP) sterility testing on aliquots of thawed cells and culture supernatants was performed at Siriraj Hospital Microbiology Laboratory by using fluid thioglycollate medium (for the detection of anaerobic bacteria) and soybean-casein digest medium (for the detection of aerobic bacteria and fungi). Sterile results were identified as no growth of microorganism.

8. Data analysis

Fold expansion number was calculated by using the viable cell number at each indicated time point divided by the viable cell number at the beginning of cell expansion. For a large-scale production experiment, fold expansion numbers were calculated to get an approximated number if a total number of expanded cells on day 7 were expanded until day 21. Fold expansion, cell viability and frequencies of T lymphocytes and their subsets were shown as mean ± SD (standard deviation). Statistical differences of mean values of fold expansion and cell viability of expanded CD4+ T lymphocytes were analyzed by paired t-tests or 2-way ANOVA followed by Bonferroni's multiple comparisons test. Statistical significance was considered when p-value was below 0.05. For cytokine producing cell subset analysis, the percentages of specific-cytokine producing cell subsets were shown as mean ± SD. The proportions of cytokine producing cell subsets were presented as pie-charts. All data were analyzed by using Prism software (GraphPad, La Jolla, CA).

Results

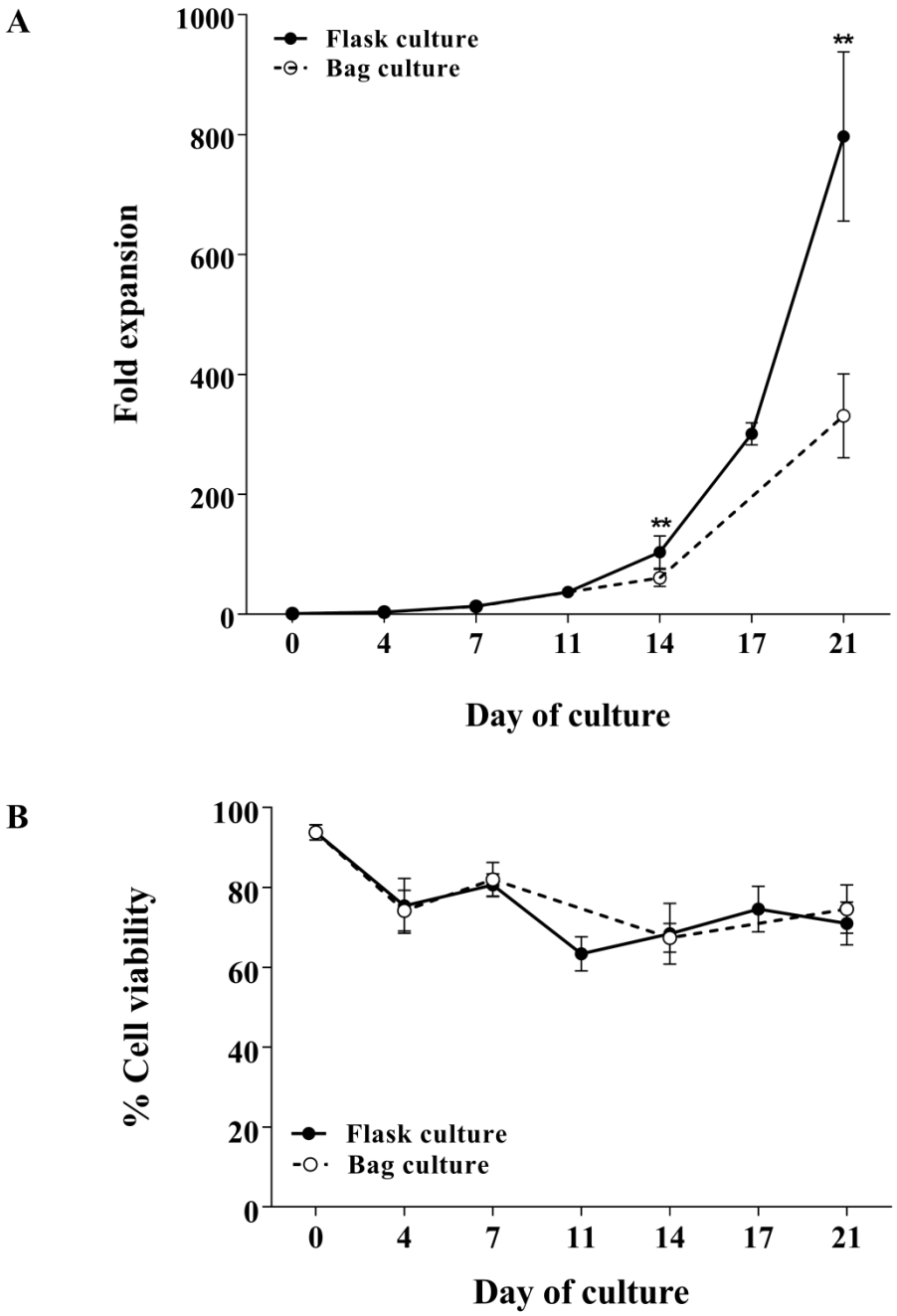
1. Expanded CD4+ T lymphocytes in small-scale plastic flasks and GMP-grade culture bags after anti-CD3/28 coated bead stimulation

To develop a closed-culture system for CD4+ T lymphocytes, GMP-grade culture bags were used for cell expansion and compared to conventional plastic flasks. Freshly isolated CD4+ T cells were obtained from five healthy volunteers for the study. Fold expansion, cell viability, growth kinetic and phenotypic characters were observed for proliferation efficiency of expanded CD4+ T cells in flasks and bags.

Fold expansion numbers of CD4+ T lymphocytes expanded in both flasks and bags were similar at the beginning and started dramatically increasing on day 14 as shown in Fig. 1A. A flask culture exhibited significantly higher fold expansion than a bag culture (103.8 ± 27.2 and 60.9 ± 14.4, respectively; p-value = 0.0075). At the end of the culture period, CD4+ T lymphocytes expanded in flasks were proliferated better than those expanded in bags (796.7 ± 141.2 –fold and 330.9 ± 70.0 –fold, respectively; p-value = 0.0078).

Cell viability of anti-CD3/28 expanded CD4+ T cells from both flask and bag cultures were similar throughout a 3-week expansion period (Fig. 1B). There were slightly decreases of viable cells from day 0 to day 21 (93.8 ± 1.9% to 71.0 ± 5.4% in flasks and 93.8 ± 1.9% to 74.6 ± 6.1% in bags) and no significant difference between the two different culture methods.

Phenotypes of whole blood, isolated CD4+ T lymphocytes, and anti-CD3/28 expanded CD4+ T lymphocytes were determined by a flow cytometer (Fig. 2). As shown in Table 1, phenotypic profiles showed that the immunorosette formation technique was effective and reproducible for CD4+ T cell isolation from whole blood with high frequency of CD3+CD4+CD8- subset (91% of lymphocytes). After a 3-week culture, anti-CD3/28 expanded CD4+ T cells (CD3+CD4+CD8-) in either flasks or bags exhibited high frequencies (> 97% of lymphocytes) on days 14 and 21. The cell purity was also confirmed with low frequencies of CD3+CD4-CD8+ and CD3+CD4+CD8+ populations (<0.3% and < 2%, respectively) throughout the expansion period.



** = p-value < 0.01

Fig. 1 Growth kinetics: (A) fold expansion, (B) cell viability of anti-CD3/28 expanded CD4+ T cells of freshly isolated CD4+ T cells from healthy volunteers expanded in flasks and bags (n = 5).

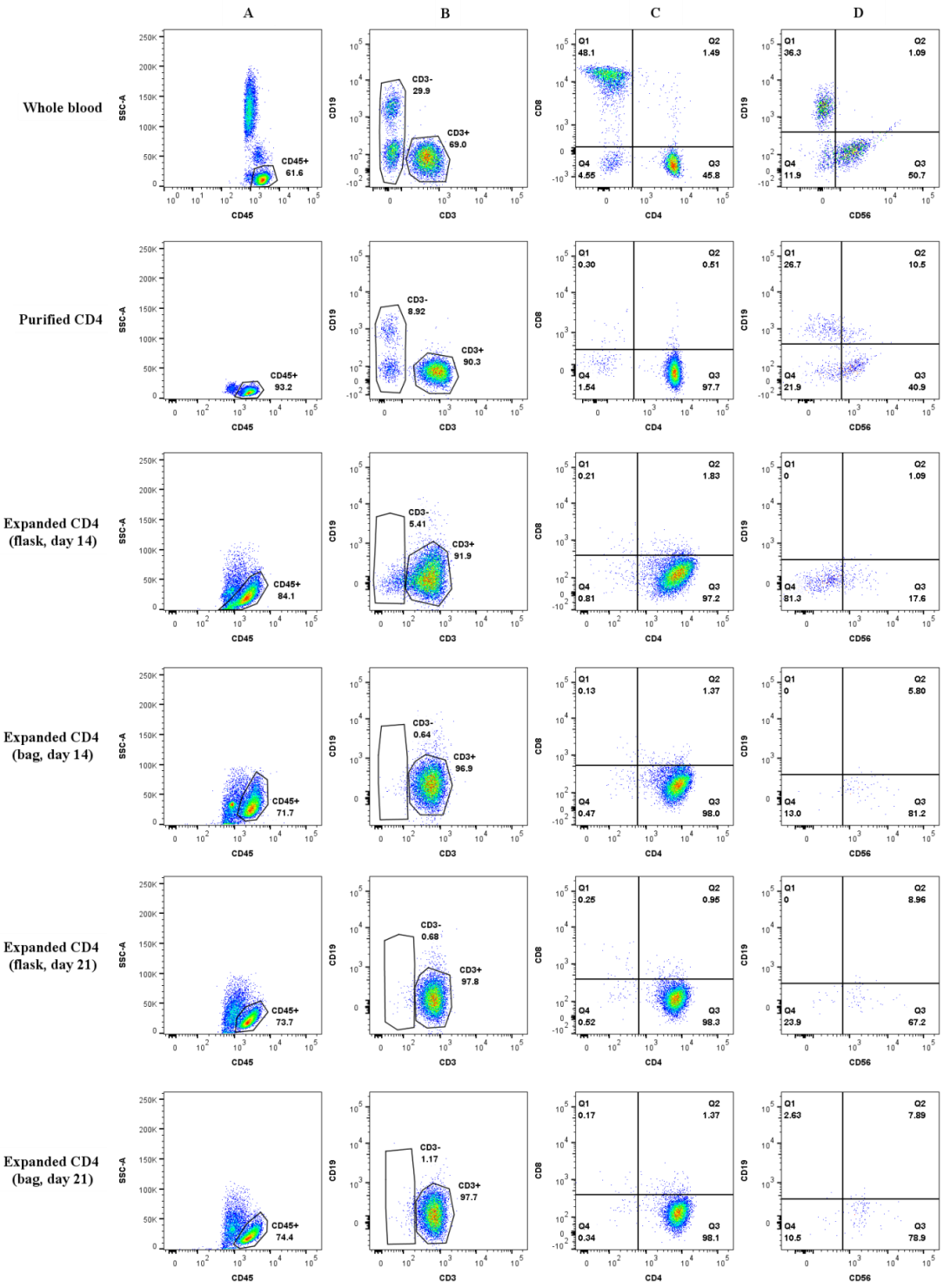


Fig. 2 Phenotype characterization of whole blood, purified CD4+ T cells, expanded CD4+ T cells on days 14 and 21 in flask and bag cultures. Gating identified (A) lymphocytes among leukocyte population, (B) CD3+ and CD3- cells among lymphocyte population, (C) CD4+ and CD8+ T cells among CD3+ cells, and (D) CD19+ B cells and CD56+ NK cells among CD3- cells.

Phenotypes	Whole blood	Isolated $CD4^+$ T cells	Anti-CD3/28 expanded $CD4^+$ T cells[a]			
	Day 0	Day 0	Day 14[b]		Day 21	
			Flasks	Bags	Flasks	Bags
% of lymphocytes						
$CD3^+CD4^-CD8^-$	4.4 ± 3.2	2.2 ± 1.3	0.8 ± 0.5	0.7 ± 0.6	0.8 ± 0.9	0.7 ± 0.4
$CD3^+CD4^+CD8^-$	32.6 ± 4.3	91.0 ± 4.8	98.2 ± 0.4	97.8 ± 1.3	98.3 ± 1.2	97.4 ± 1.5
$CD3^+CD4^-CD8^+$	36.0 ± 2.0	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	0.3 ± 0.3	0.2 ± 0.1
$CD3^+CD4^+CD8^+$	0.5 ± 0.4	2.2 ± 1.3	0.8 ± 0.5	1.4 ± 1.4	0.6 ± 0.5	1.0 ± 0.4
$CD3^-CD19^+CD56^-$	10.9 ± 3.5	1.4 ± 1.6	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^+$	13.5 ± 2.5	0.8 ± 0.8	N/A	N/A	N/A	N/A
$CD3^-CD19^+CD56^+$	0.2 ± 0.3	0.1 ± 0.0	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^-$	2.0 ± 1.4	4.2 ± 3.8	N/A	N/A	N/A	N/A
% of $CD3^+$ T cells						
$CD3^+CD4^-CD8^-$	5.9 ± 4.1	2.3 ± 1.4	N/A	N/A	N/A	N/A
$CD3^+CD4^+CD8^-$	43.9 ± 4.1	97.3 ± 1.3	N/A	N/A	N/A	N/A
$CD3^+CD4^-CD8^+$	49.5 ± 4.1	0.1 ± 0.0	N/A	N/A	N/A	N/A
$CD3^+CD4^+CD8^+$	0.7 ± 0.7	0.3 ± 0.2	N/A	N/A	N/A	N/A
% of $CD3^-$ T cells						
$CD3^-CD19^+CD56^-$	42.3 ± 7.8	24.0 ± 25.9	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^+$	51.1 ± 7.3	10.2 ± 8.5	N/A	N/A	N/A	N/A
$CD3^-CD19^+CD56^+$	0.8 ± 1.2	0.8 ± 0.4	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^-$	7.2 ± 4.9	65.0 ± 27.1	N/A	N/A	N/A	N/A

N/A = Not available; b = 4 subjects (data from one donor was ruled out due to machine error); a = gating of CD45+ lymphocytes was 100% of CD45+CD3+ T cells.

Table 1 Phenotypes of whole blood, isolated CD4+ T cells, and anti-CD3/28 expanded CD4+ T cells from healthy volunteers (mean ± SD, n = 5).

2. Large-scale expansion of CD4+ T lymphocytes in a closed culture system

As a high number of purified CD4+ T lymphocytes are demanded for cell transfusion, a large-scale production of freshly isolated CD4+ T cells from five healthy donors in a closed-culture system was developed in this study. Fold expansion, cell viability, growth kinetic and phenotypic characters were observed for large-scale proliferation.

A large-scale production of CD4+ T cells exhibited a gradual increase of fold expansion from day 0 to day 14 (1.0 ± 0.0 on day 0, 5.5 ± 1.0 on day 7, and 20.4 ± 3.4 on day 14) and a marked increase from day 14 to day 21 (109.1 ± 18.4 on day 21). Growth kinetic of this large-scale

expansion in culture bags also had a similar pattern with that of a small-scale bag culture prescribed earlier. Cell viability of large-scale anti-CD3/28 expanded CD4+ T cells was good with a slight decrease from 91.2 ± 2.6% on day 0 to 77.4 ± 6.3% on day 21.

Again, phenotypic characterization of whole blood and isolated CD4+ T cells was performed for purity confirmation of isolated CD4+ T cells before culture as presented in Table 2. Anti-CD3/28 expanded CD4+ T lymphocytes from a large-scale production showed high frequency of CD3+CD4+CD8- population (> 98%) on days 14 and 21. Low numbers of CD3+CD4-CD8+ and CD3+CD4+CD8+ populations (<0.8% and < 0.5%, respectively) were detected.

Phenotypes	**Whole blood**	**Isolated $CD4^+$ T cells**	**Anti-CD3/28 expanded $CD4^+$ T cells***	
	Day 0	**Day 0**	**Day 14**	**Day 21**
% of lymphocytes				
$CD3^+CD4^-CD8^-$	4.7 ± 1.5	1.3 ± 0.4	0.3 ± 0.1	0.8 ± 0.3
$CD3^+CD4^+CD8^-$	37.5 ± 7.2	97.5 ± 1.2	99.1 ± 0.3	98.0 ± 0.6
$CD3^+CD4^-CD8^+$	28.7 ± 6.7	0.0 ± 0.0	0.1 ± 0.1	0.8 ± 0.4
$CD3^+CD4^+CD8^+$	0.9 ± 0.3	0.1 ± 0.1	0.5 ± 0.3	0.3 ± 0.5
$CD3^-CD19^+CD56^-$	14.4 ± 5.9	0.2 ± 0.2	N/A	N/A
$CD3^-CD19^-CD56^+$	11.4 ± 3.5	0.2 ± 0.1	N/A	N/A
$CD3^-CD19^+CD56^+$	0.3 ± 0.2	0.0 ± 0.0	N/A	N/A
$CD3^-CD19^-CD56^-$	2.0 ± 1.1	0.7 ± 0.7	N/A	N/A
% of $CD3^+$ T cells				
$CD3^+CD4^-CD8^-$	6.8 ± 2.1	1.3 ± 0.4	N/A	N/A
$CD3^+CD4^+CD8^-$	52.1 ± 9.4	98.6 ± 0.5	N/A	N/A
$CD3^+CD4^-CD8^+$	39.9 ± 8.7	0.0 ± 0.0	N/A	N/A
$CD3^+CD4^+CD8^+$	1.2 ± 0.6	0.1 ± 0.1	N/A	N/A
% of $CD3^-$ T cells				
$CD3^-CD19^+CD56^-$	41.0 ± 11.1	20.6 ± 17.6	N/A	N/A
$CD3^-CD19^-CD56^+$	51.2 ± 10.8	17.0 ± 12.7	N/A	N/A
$CD3^-CD19^+CD56^+$	1.0 ± 0.5	1.3 ± 1.9	N/A	N/A
$CD3^-CD19^-CD56^-$	6.8 ± 2.0	61.1 ± 18.8	N/A	N/A

N/A = Not available; * = gating of CD45+ lymphocytes was 100% of CD45+CD3+ T cells.

Table 2 Phenotypes of whole blood, isolated CD4+ T cells, and anti-CD3/28 expanded CD4+ T cells from healthy volunteers expanded in culture bags for large-scale production (mean ± SD, n = 5).

3. Bag culture of anti-CD3/28 expanded CD4+ T cells from HIV-infected patients

Using freshly isolated CD4+ T cells from HIV-infected patients for cell expansion may possibly be inconvenient; therefore, frozen cells become alternative source and more practical for expansion. Cryopreserved CD4+ T lymphocytes from five healthy donors were able to expand in

bags with a similar fold expansion when compared to freshly isolated CD4+ T lymphocytes (data not shown). Cryopreserved CD4+ T lymphocytes from HIV-infected patients were then expanded in the developed close-culture system.

Anti-CD3/28 expanded CD4+ T cells from frozen cells showed the same trend and similar numbers of fold expansion as those from freshly isolated cells (1.0 ± 0.0 on day 0, 3.4 ± 0.4 on day 4, 13.6 ± 3.5 on day 7, 63.6 ± 16.2 on day 14 and 414.9 ± 67.6 on day 21). Cell viability of cryopreserved cells was quite high and remained stable until the end of expansion (76.7 ± 5.8% on day 0 to 85.0 ± 1.7% on day 21).

Purity of isolated CD4+ T cells from cryopreservation was ensued by phenotypic characterization (Table 3). CD3+CD4+CD8- population was detected to be dominant for anti- CD3/28 expanded CD4+ T cells throughout the expansion period (> 98%). This was confirmed by minor populations of CD3+CD4-CD8+ and CD3+CD4+CD8+ T cells (< 0.8% and <0.5%, respectively).

Phenotypes	**Cryopreserved $CD4^+$ T cells**	**Anti-CD3/28 expanded $CD4^+$ T cells***	
	Day 0	**Day 14**	**Day 21**
% of lymphocytes			
$CD3^+CD4^-CD8^-$	2.2 ± 1.1	4.7 ± 2.5	2.5 ± 2.3
$CD3^+CD4^+CD8^-$	93.6 ± 6.6	94.9 ± 2.3	95.9 ± 2.8
$CD3^+CD4^-CD8^+$	0.0 ± 0.0	0.1 ± 0.0	0.5 ± 0.5
$CD3^+CD4^+CD8^+$	0.0 ± 0.0	0.3 ± 0.2	1.1 ± 0.2
$CD3^-CD19^+CD56^-$	0.4 ± 0.5	N/A	N/A
$CD3^-CD19^-CD56^+$	0.5 ± 0.9	N/A	N/A
$CD3^-CD19^+CD56^+$	0.0 ± 0.0	N/A	N/A
$CD3^-CD19^-CD56^-$	3.1 ± 4.3	N/A	N/A

N/A = Not available; * = gating of CD45+ lymphocytes was 100% of CD45+CD3+ T cells.

Table 3 Phenotypes of cryopreserved CD4+ T cells, and anti-CD3/28 expanded CD4+ T cells from cryopreserved HIV-infected samples (CD4 counts ≥ 500 cells/μL) expanded in bags with IL-2 supplementation (mean ± SD, n = 3).

4. Determination of cytokines produced by anti-CD3/28 expanded CD4+ T cells from bag culture of cryopreserved CD4+ T cells

Although cell expansion by using anti-CD3/28 coated beads showed a homogenous population of expanded CD4+ T lymphocytes, the obtained phenotypic character were not able to provide information on a variety of expanded cells in term of function. Since CD4+ T lymphocytes can be divided into subpopulations including T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17), and regulatory T cells (Treg) based on types of cytokine produced by these cells, anti-CD3/28

expanded CD4+ T cells from a bag culture of cryopreserved CD4+ T cells from HIV-infected patients were investigated for their T helper cell subset identity.

CD69 expression was used to determine the level of activated cells after stimulation with PMA/I which is a polyclonal activator. The average percentage of CD69 expressing cells among anti-CD3/28 expanded CD4+ T cells was 95.4 ± 1.4%. Of these activated cells, the average percentage of 76.2 ± 5.0% was cytokine producing cells. Different types of cytokine producing cells including TNF-α, IL-2, IFN-γ, IL-4, IL-17 and TGF-β are shown in Fig. 3A. The major population among total cytokine producing cells was TNF-α producing cells with the average frequency of 81.4 ± 2.5%, followed by IL-2 producing cells (67.3 ± 15.1%). Determination of Th1 cells based on IFN-γ production showed the average percentage of 29.7 ± 11.9%. Low levels of Th2 and Th17 cells were detected based on the production of IL-4 and IL-17 (1.5 ± 0.9% and 1.2 ± 1.2%, respectively). On the contrary, the frequency of Treg cells based on TGF-β production was almost undetectable. When cytokine producing cell populations showing simultaneous production were determined, up to 99% of cytokine producing cells belongs to 3 major populations including single, double and triple cytokine producing cells with the average percentages of 34.3 ± 5.8%, 50.6 ± 3.9% and 14.5 ± 7.4%, respectively (Fig. 3B).

Proportions of specific-cytokine producing cell subsets determined among single, double and triple cytokine producing cells are presented in Fig. 4. For single cytokine producing cells, the majority was TNF-α producing cell with the average percentage of 51.1 ± 12.6%, followed by IL-2 and IFN-γ producing cells (32.8 ± 13.8% and 15.2 ± 4.3%, respectively). With respect of double cytokine producing cells, the highest frequency was observed for TNF-α+ IL-2+ producing cells with the average percentage of 77.7 ± 14.7%, followed by the other 2 main populations including TNF-α+ IFN-γ + (16.8 ± 14.4%) and IFN-γ + IL-2+ (4.1 ± 3.0%). For triple cytokine producing cells, TNF-α+ IL-2+ IFN-γ + producing cells showed the highest frequency with the average percentage of 89.2 ± 7.4%, followed by TNF-α+ IL-2+ IL-4+ (4.6 ± 3.7%), and TNF-α+ IL-2+ IL-17+ (2.4 ± 1.8%).

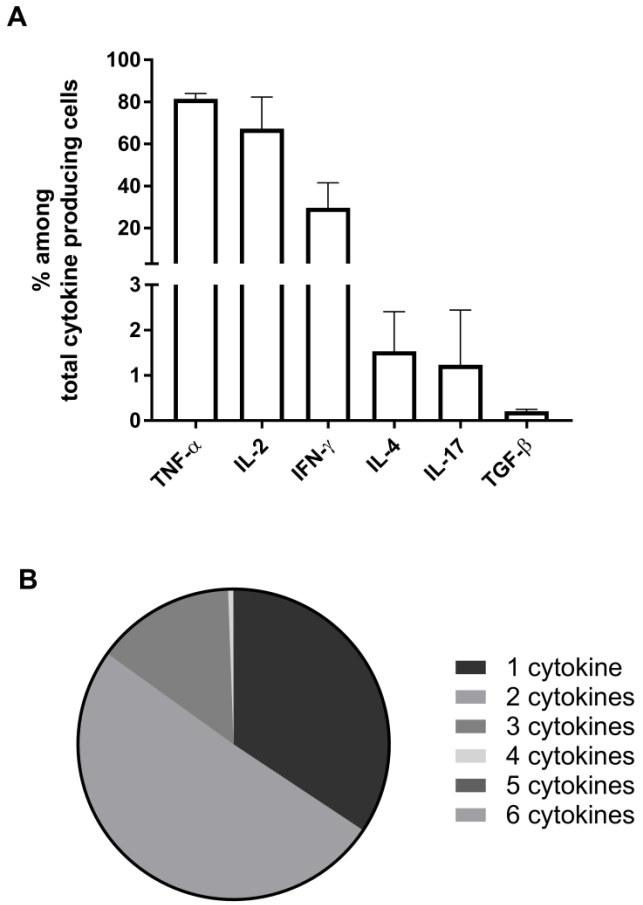


Fig. 3 Cytokine production profiles including TNF-α, IL-2, IFN-γ, IL-4, IL-17, and TGF-β of anti-CD3/28 expanded CD4+ T cells: (A) percentages (mean ± SD) of different cytokine producing cells; (B) average proportions of polyfunctional cytokine producing cells (n = 3).

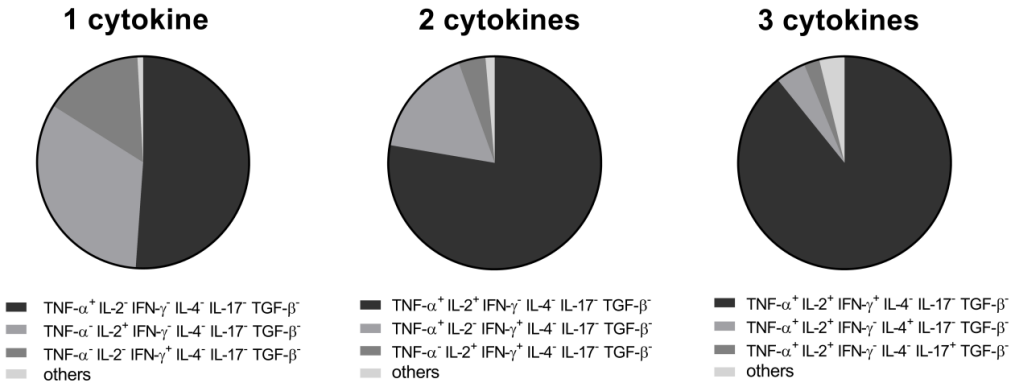


Fig. 4 Average proportions of polyfunctional cytokine producing cell subsets in single, double and triple cytokine producing cells (n = 3).

5. Residual bead detection and sterility testing

To ensure safety and sterility of the final products due to magnetic beads and microorganism contamination, a small-scale bead removal process was performed together with sterility testing on the final cell culture supernatants (day 21) and harvested expanded CD4+ T cells. Since a large volume of culture medium containing expanded CD4+ T cells was harvested, the sample was concentrated by centrifugation before bead removal. By using a small scale depletion method, the average number of $5.5x10^4$ residual beads per 10^7 expanded cell products was measured. Since the average number of $414.9x10^6$ cells was harvested on day 21, the average number of $2.3x10^6$ residual beads may be obtained in the final products. While the average number of beads before harvesting was $13.6x10^6$ beads (average cell number for reactivation on day 7), the result showed 83% depletion of beads. For sterility testing, a 14-day incubation period in specialized media was performed according to USP. The sterile results were presented as the culture supernatant colleting on the final day of cell expansion and expanded cells from cryopreservation were free from any bacterial and fungal contaminations.

6. Expanded CD4+ T lymphocytes in different culture conditions after anti-CD3/28 coated bead stimulation

To assess an optimal cell culture condition for in vitro expansion of CD4+ T lymphocytes by using anti-CD3/28 coated beads, different culture conditions based on serum supplementation and culture media usages were compared. Fold expansion, cell viability, growth kinetic and phenotypic characters from different culture conditions were observed for cell expansion efficiency.

Fold expansion numbers of CD4+ T lymphocytes expanded in all culture conditions were similar during the first 2-week period. After that, the expanded cells cultured in RPMI1640 with 10% FBS (R10) were dramatically increased on day 17 (755.7 ± 337.1-fold), whereas the other cultures maintained similar expansion rates (Fig. 5A). At the end of the culture period (day 21), slight decreases were found in every culture media. The culture in R10 showed the highest fold expansion (637.1 ± 265.3 –fold) which was approximately 2.5-fold higher than the other culture conditions with 10% HS (< 255-fold). No significant difference was observed among 10% HS supplemented media including GMP-grade media (i.e., AIM-V and DC) and standard media (i.e., RPMI1640).

Gradual declines in viable cells were observed throughout the 21-day culture period (Fig. 5B). All culture conditions exhibited a similar pattern in cell viability at over 70% during the first two weeks. Only the culture in R10 still maintained high numbers of viable cells on day 17 (75.9 ± 11.0%) before markedly decreasing on day 21 (54.8 ± 5.8%). On the other hand, the other culture media including RPMI1640, AIM-V and DC supplemented with 10% HS showed notable decreases since day 17 (47.1 ± 7.7%, 56.0 ± 6.4% and 52.6 ± 5.5%, respectively) and lowered to less than 36% on day 21.

Immunophenotypes of whole blood, isolated CD4+ T lymphocytes, and anti-CD3/28 expanded CD4+ T lymphocytes were determined by a flow cytometer (Table 4). Phenotypic profiles

revealed that purification of CD4+ T cells from whole blood by using the immunorosette formation technique provided high frequency of CD3+CD4+CD8- subset (> 95% of lymphocytes). After a 3-week culture, anti-CD3/28 expanded CD4+ T cells (CD3+CD4+CD8-) from R10 and DC with 10% HS exhibited high frequencies (> 90% of lymphocytes), whereas slightly lower frequencies were observed for RPMI1640 and AIM-V with 10% HS. The cell purity was also confirmed with low frequencies of CD3+CD4-CD8+ and CD3+CD4+CD8+ populations at the end of the expansion period.

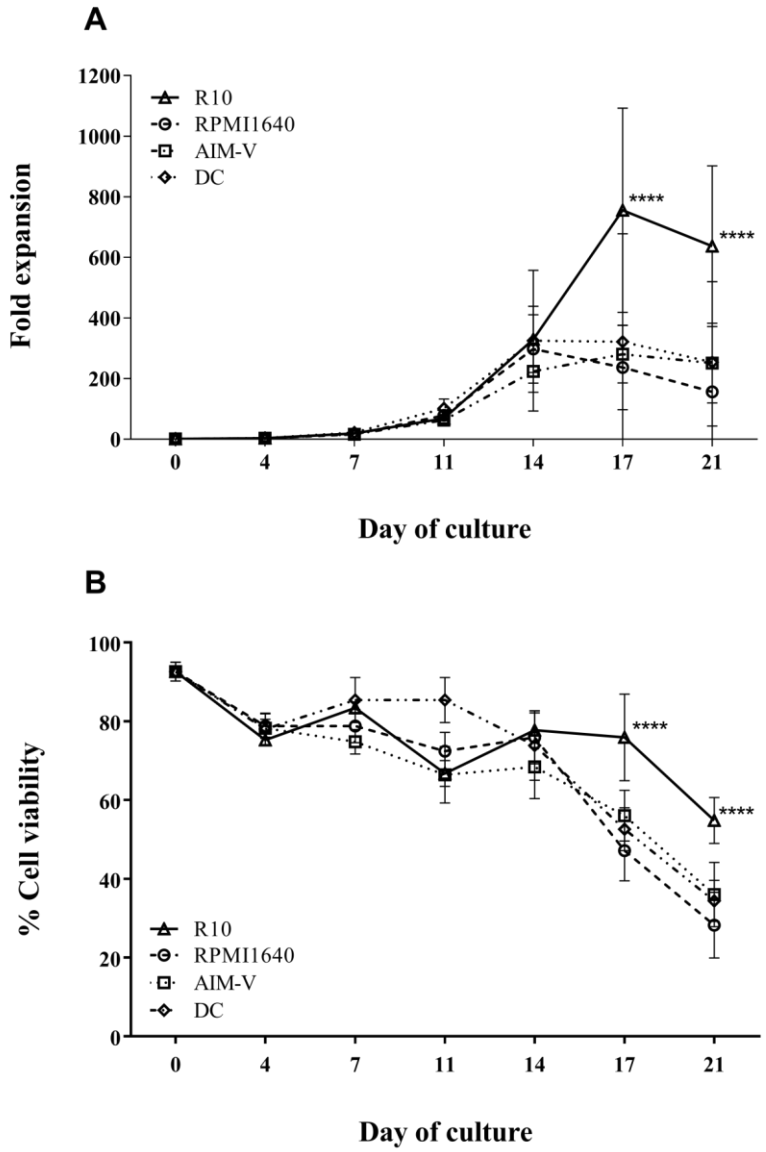


Fig. 5 Growth kinetics of anti-CD3/28 expanded CD4+ T cells from different culture conditions. (A) Fold expansion and (B) cell viability of the expanded cells were observed from different cultures in R10 (RPMI1640 with 10% FBS) and media containing 10% human serum including RPMI1640, AIM-V, and DC. All data are presented as mean ± SD (n = 5, ****p-value < 0.0001).

Phenotypes	Whole blood	Isolated $CD4^+$ T cells	Anti-CD3/28 expanded $CD4^+$ T cells			
			10% FBS	10% HS		
			RPMI1640*	RPMI1640	AIM-V	DC
% of lymphocytes						
$CD3^+CD4^-CD8^-$	6.1 ± 1.9	1.6 ± 0.6	5.7 ± 7.9	7.4 ± 9.3	10.0 ± 6.4	3.5 ± 2.1
$CD3^+CD4^+CD8^-$	34.7 ± 6.2	96.0 ± 2.6	91.9 ± 8.1	89.0 ± 11.3	84.8 ± 7.5	94.1 ± 3.9
$CD3^+CD4^-CD8^+$	30.3 ± 7.2	0.0 ± 0.0	1.0 ± 0.8	2.5 ± 2.2	3.6 ± 1.8	1.2 ± 1.2
$CD3^+CD4^+CD8^+$	0.5 ± 0.3	0.1 ± 0.1	1.4 ± 1.0	0.9 ± 0.5	1.5 ± 1.3	1.0 ± 0.9
$CD3^-CD19^+CD56^-$	11.2 ± 5.1	0.5 ± 0.8	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^+$	15.8 ± 7.4	0.6 ± 0.7	N/A	N/A	N/A	N/A
$CD3^-CD19^+CD56^+$	0.3 ± 0.3	0.0 ± 0.1	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^-$	1.1 ± 0.2	1.0 ± 1.2	N/A	N/A	N/A	N/A

*RPMI1640 with 10% FBS is R10

Table 4 Lymphocyte subset analyses of whole blood, isolated CD4+ T cells, and anti-CD3/28 expanded CD4+ T cells (mean ± SD, n = 5).

7. Determination of cytokines produced by anti-CD3/28 expanded CD4+ T cells from different culture conditions

Although a homogenous population of expanded CD4+ T lymphocytes was observed in all culture conditions with different degree of expansion ability, it remains unclear whether there are any functional changes induced by different culture conditions. Anti-CD3/28 expanded CD4+ T cells from various culture conditions were then investigated in this study for their functional variances based on types of cytokines produced by these cells.

To determine the levels of activated cells among anti-CD3/28 expanded CD4+ T cells after stimulation with PMA/I, the CD69 expression levels were assessed. There was no significant difference in frequencies of CD69 expressing cells among different culture conditions (Fig. 6A). These CD69 expressing cell populations were also consequently determined for cytokine producing cells in order to ensure the presence of cytokine production in the activated cells. While total cytokine producing cells (i.e., a combination of all types of cytokine producing cells) from all culture conditions were not significantly different, the expanded cells from R10 exhibited higher percentages of total cytokine producing cells than the others (Fig. 6B).

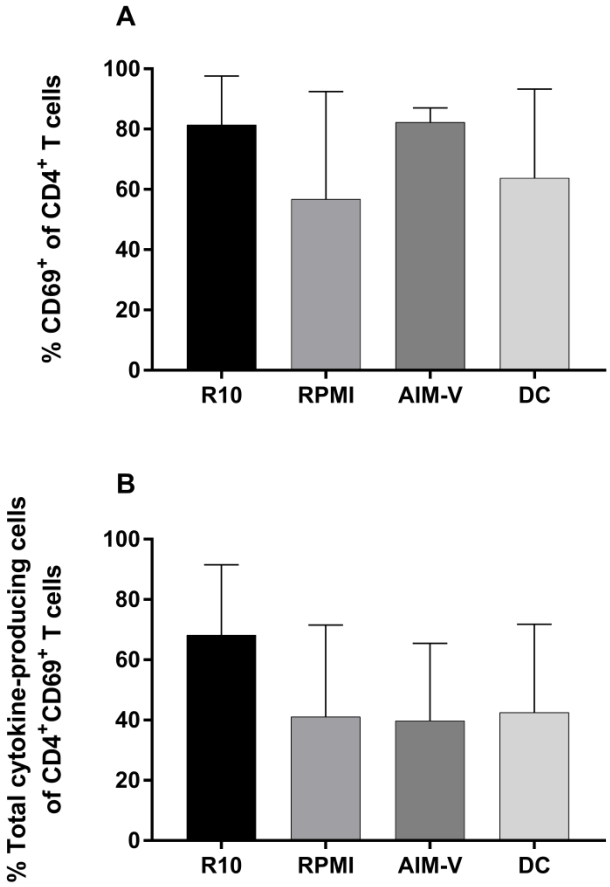


Fig. 6 Activation and cytokine production of anti-CD3/28 expanded CD4+ T cells from different culture conditions. (A) Percentages of CD69 expressing cells and (B) percentages of cytokine producing cells were observed from different cultures in R10 (RPMI1640 with 10% FBS) and media containing 10% human serum including RPMI1640, AIM-V, and DC. All data are presented as mean ± SD (n = 3).

When different cytokine-producing cell populations including TNF-α, IL-2, IFN-γ, IL-4, IL-17 and TGF-β were determined, the major population among total cytokine-producing cells was TNF-α producing cells (Fig. 7A). No difference in all types of cytokine-producing cells was observed among different culture conditions, except IL-2 producing cells with a significant high percentage (58.0 ± 18.6 % of CD4+CD69+ T cells) from the R10 culture. Ability in simultaneous production of cytokines was also determined. Results demonstrated that up to 99% of cytokine-producing cells from all culture conditions belong to 3 main populations including single, double and triple cytokine-producing cells in which a single cytokine-producing cell population showed the highest percentages of over 50% of total cytokine-producing cells (Fig. 7B).

The 3 main populations of single, double and triple cytokine-producing cells were then evaluated for specific-cytokine producing cell subsets. IL-2 producing cells were predominantly found in the single cytokine-producing cells from all culture conditions. For the double cytokine-producing cells, TNF-α+ IL-2+ producing cells showed the highest frequency for all culture conditions, except

the DC culture presenting the highest proportion of IFN-γ+ IL-2+ producing cells instead. The triple cytokine-producing cells in all media supplemented with 10% HS exhibited TNF-α+ IL-2+ IFN-γ+ producing cells as a majority, whereas TNF-α+ IL-2+ IL-4+ producing cells were found predominant in the R10 culture (data not shown).

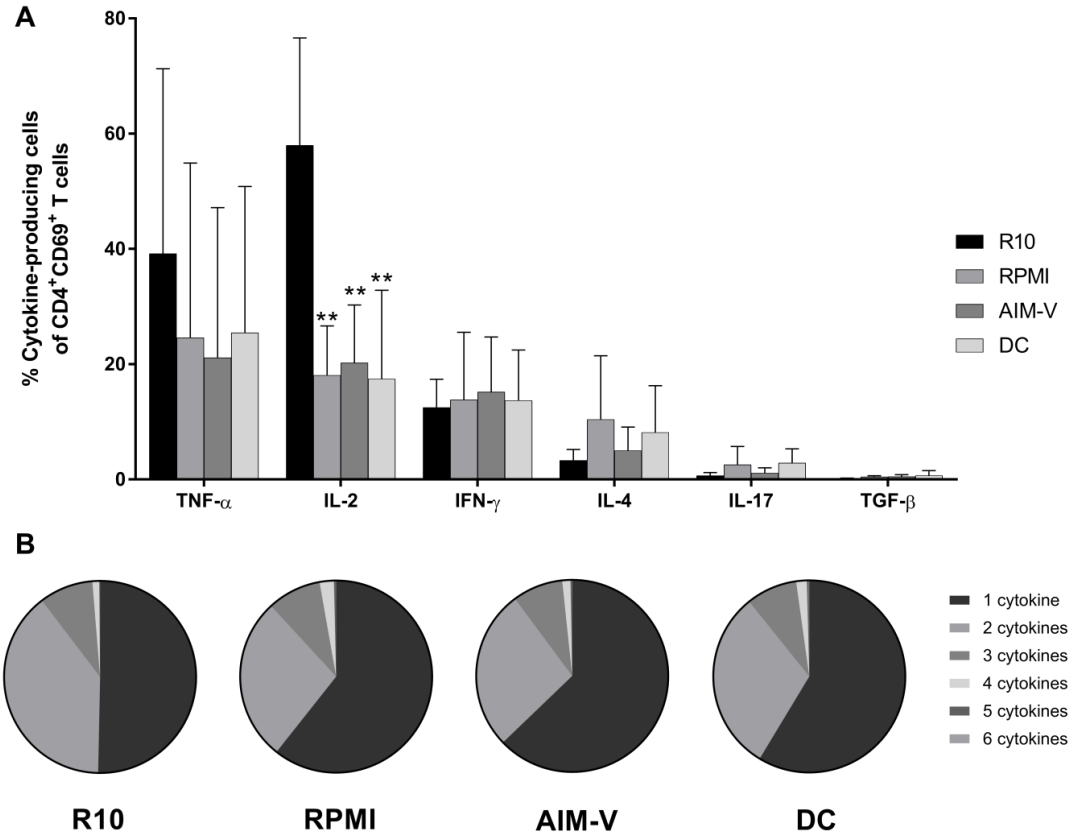


Fig. 7 Cytokine production profiles including TNF-α, IL-2, IFN-γ, IL-4, IL-17, and TGF-β of anti-CD3/28 expanded CD4+ T cells. (A) Percentages (mean ± SD) of different cytokine producing cells and (B) average proportions of polyfunctional cytokine producing cells were observed from different cultures in R10 (RPMI1640 with 10% FBS) and media containing 10% human serum including RPMI1640, AIM-V, and DC. (n = 3, ****p-value < 0.0001).

8. Bead-to-cell ratio comparison for anti-CD3/28 CD4+ T cell expansion

To achieve satisfied yields of the expanded cells, it is important to determine the optimum bead-to-cell ratio used for stimulation. In this study, three healthy volunteers were recruited for blood collection. Isolated CD4+ T cells were activated with anti-CD3/28 coated magnetic beads at different bead-to-cell ratios (i.e., 0.5:1, 1:1 and 2:1) and cultured in the absence of IL-2 for 21 days. Fold expansion, cell viability, growth kinetic and phenotypic characters were observed for proliferation efficiency of the expanded CD4+ T cells on days 0, 4, 7, 11, 14, 17 and 21.

There was no difference in fold expansion among 3 different bead-to-cell ratios during the first 14 days of culture, however, the fold expansion number of CD4+ T cells expanded with the 1:1 bead-to-cell ratio on day 17 showed remarkably higher than the others (Fig. 8A). On day 21 of culture, it was obvious that stimulation with the 1:1 bead-to-cell ratio provided the highest yield of the anti-CD3/28 expanded CD4+ T cells followed by the 2:1 and 0.5 bead-to-cell ratios (1,044 ± 259 –, 629 ± 457 –, and 301 ± 167 – fold, respectively). Cell viabilities of the expanded cells from the 3 different ratios were comparable with over 90% throughout the 3-week culture period (Fig. 8B). There were only slightly decreases in viable cells at the end of the culture for the 1:1 and 2:1 ratios (88 ± 7% and 83 ± 15%, respectively).

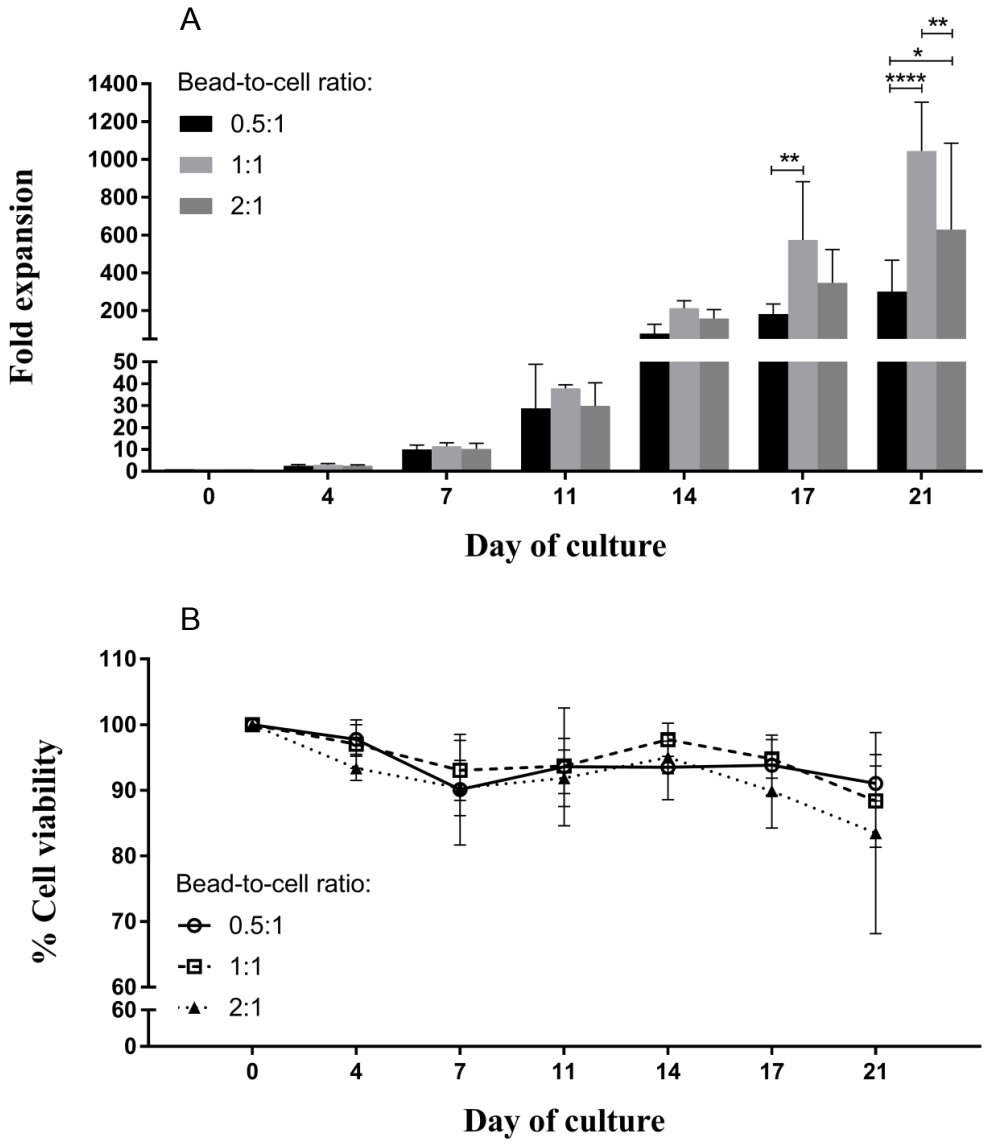


Fig. 8 Growth kinetics of anti-CD3/28 expanded CD4+ T cells when using different bead-to-cell ratios for stimulation. Fold expansion (a) and cell viability (b) of the expanded cells at different bead-to-cell ratios (i.e., 0.5:1, 1:1, and 2:1) were observed over the 21-day culture. All data are presented as mean ± SD (n = 3, *p-value < 0.05, **p-value < 0.01 and ****p-value < 0.0001).

Lymphocyte subset characters of the anti-CD3/28 stimulated CD4+ T cells were analyzed by a flow cytometer (Table 5). It was clearly demonstrated that the major population of the expanded cells was CD3+ T cells (> 99% of lymphocytes) with the dominant subset of CD3+CD4+CD8- for all bead-to-cell ratio groups over the culture period (> 98% of lymphocytes). The purity of the expanded cells was also confirmed with low frequencies of CD3+CD4-CD8+, CD3+CD4+CD8+, CD3-CD19+, CD3-CD16+CD56+, CD3+CD16+CD56+, and CD3+CD19+ (< 3.5% of expanded cells).

Cell population	**Day of culture**	**Bead-to-cell ratio**		
		0.5:1	**1:1**	**2:1**
$CD3^+$	0	99.3 ± 0.4	99.3 ± 0.4	98.8 ± 1.2
	14	99.8 ± 0.2	99.9 ± 0.1	99.9 ± 0.1
	21	99.6 ± 0.5	99.9 ± 0.1	99.9 ± 0.2
$CD3^+CD4^+CD8^-$	0	97.8 ± 1.3	98.1 ± 1.4	97.5 ± 1.7
	14	99.1 ± 0.6	99.8 ± 0.1	99.7 ± 0.1
	21	98.7 ± 1.5	99.5 ± 0.3	99.3 ± 0.4
$CD3^+CD4^-CD8^+$	0	0.0	0.0	0.0
	14	0.0 ± 0.1	0.0	0.0
	21	0.0	0.0	0.0
$CD3^+CD4^+CD8^+$	0	0.0	0.0	0.0
	14	0.0	0.0	0.0
	21	0.0 ± 0.1	0.0 ± 0.1	0.0
Others[a]	0	0.9 ± 0.5[b]	0.7 ± 0.4[b]	1.1 ± 0.9[b]
	14	1.7 ± 0.4[b]	3.5 ± 1.5[b]	2.4 ± 0.3[b]
	21	1.3 ± 0.7[b]	1.3 ± 0.4[b]	1.2 ± 0.4[b]

[a]Other cell populations include $CD3^-CD19^+$, $CD3^-CD16^+CD56^+$, $CD3^+CD16^+CD56^+$, and $CD3^+CD19^+$.
[b]A sum of average percentages of frequencies of the other cell populations.

Table 5 Characterization of anti-CD3/28 stimulated CD4+ T lymphocytes at different bead-to-cell ratios over the 21-day culture. Frequencies of all cell populations are represented as percentages of lymphocytes (mean ± SD, n = 3).

9. Effects of IL-2 supplementation on cell expansion

IL-2 supplementation has been generally used to promote cell proliferation in addition to the autocrine/paracrine IL-2 production by the activated T cells. High concentrations of IL-2 (100 and 300 IU/mL) have been reported to predominantly affect CD8+ T cell development. This study, therefore, used a low concentration of IL-2 at 20 IU/mL to support cell expansion and compared this expansion effect of IL-2 to the autocrine/paracrine IL-2 production (i.e., cell culture in the absence of

IL-2). Fold expansion, cell viability, growth kinetic and lymphocyte subset characters were observed for proliferation efficiency of the expanded CD4+ T cells on days 0, 4, 7, 11, 14, 17 and 21.

Data showed that fold expansion numbers between the culture without and with IL-2 supplementation were similar throughout the 21-day culture period (Fig. 9A). Only the expanded cells cultured in the absence of IL-2 supplementation on day 17 proliferated significantly higher than those in the presence of IL-2 supplement (582 ± 166 – and 455 ± 125 – fold, respectively). At the end of the culture, there was no significant difference in proliferation between the two culture groups. With respect to cell viability, the cultures without or with IL-2 supplementation maintained great numbers of viable cells with over 90% throughout the 3-week culture period (Fig. 9B).

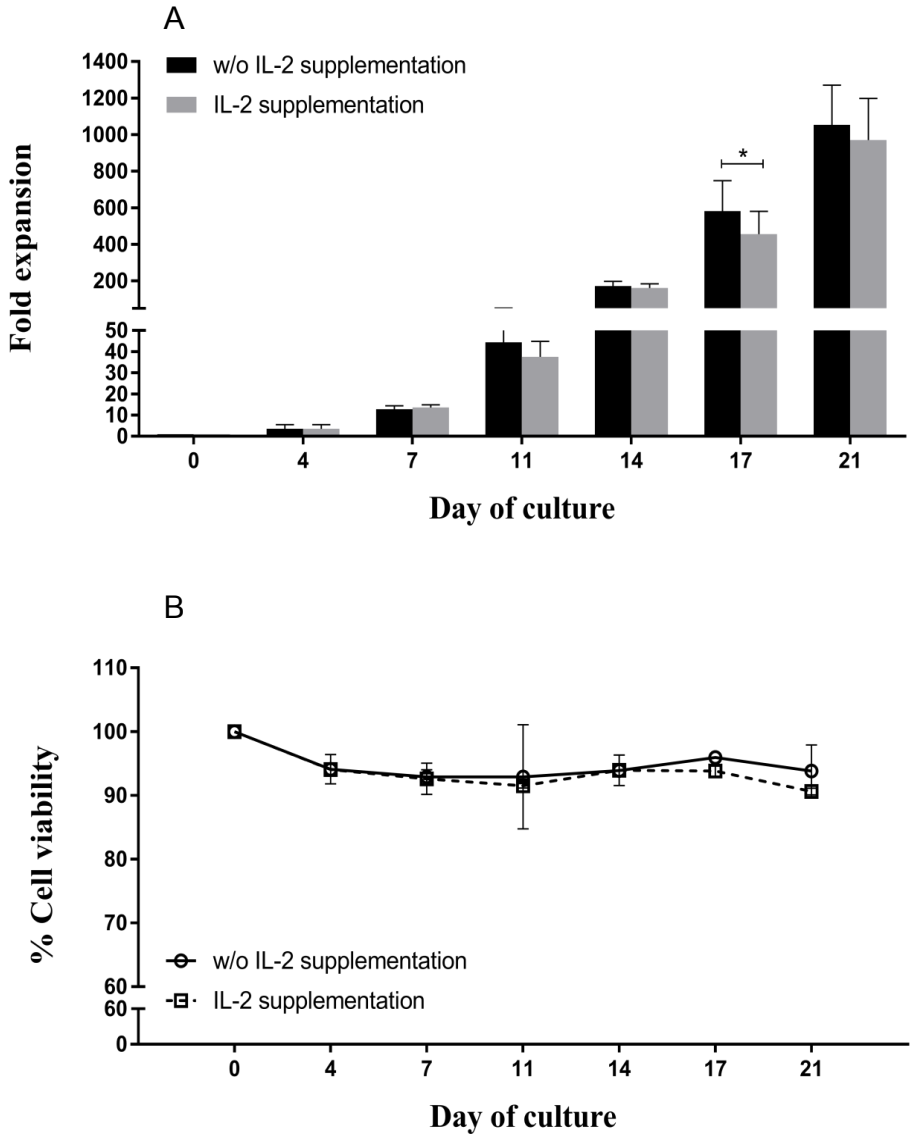


Fig. 9 Growth kinetics of anti-CD3/28 expanded CD4+ T cells when cultured without and with IL-2 supplementation. Fold expansion (a) and cell viability (b) of the expanded cells when cultured without (w/o) and with IL-2 supplementation at the concentration of 20 units/mL were observed over 21 days. All data are presented as mean ± SD (n = 3 and *p-value < 0.05).

Predominant phenotypes of the expanded cells from both culture groups were CD3+ T cells (> 97% of expanded cells) with the major CD3+CD4+CD8- subset (> 94% of expanded cells) as presented in Table 6. The minor cell populations including CD3+CD4-CD8+, CD3+CD4+CD8+, CD3-CD19+, CD3-CD16+CD56+, CD3+CD16+CD56+ and CD3+CD19+ were also found with very low frequencies (< 2% of expanded cells), suggesting a specific expansion of purified CD4+ T cells.

Cell population	**Day of culture**	**Supplementation**	
		w/o IL-2	**IL-2**
$CD3^+$	0	99.5 ± 0.2	99.2 ± 0.7
	14	96.7 ± 5.7	99.8 ± 0.1
	21	99.9 ± 0.1	99.9 ± 0.0
$CD3^+CD4^+CD8^-$	0	98.3 ± 0.6	97.9 ± 1.7
	14	94.4 ± 5.2	99.6 ± 0.1
	21	99.2 ± 0.1	99.0 ± 0.9
$CD3^+CD4^-CD8^+$	0	0.0	0.0
	14	0.0	0.0
	21	0.1 ± 0.1	0.0
$CD3^+CD4^+CD8^+$	0	0.0	0.0
	14	1.4 ± 2.4	0.1 ± 0.1
	21	0.0	0.0
Others[a]	0	0.7 ± 0.4[b]	0.8 ± 0.6[b]
	14	2.0 ± 1.0[b]	1.9 ± 0.6[b]
	21	1.3 ± 0.4[b]	0.6 ± 0.4[b]

[a]Other cell populations include $CD3^-CD19^+$, $CD3^-CD16^+CD56^+$, $CD3^+CD16^+CD56^+$, and $CD3^+CD19^+$.
[b]A sum of average percentages of frequencies of the other cell populations.

Table 6 Characterization of anti-CD3/28 stimulated CD4+ T lymphocytes when cultured without (w/o) and with IL-2 supplementation over 21 days. Frequencies of all cell populations are represented as percentages of lymphocytes (mean ± SD, n = 3).

10. Specific cell surface molecule expressions of anti-CD3/28 CD4+ T cells

As surface molecule expressions are related to cell maturation, activities and functions, this study thus explored several cell surface molecule expressions of anti-CD3/28 expanded CD4+ T cells which were divided into seven groups according to the molecules' functions and roles. The cell surface molecules included (i) chemokine receptors: CCR4, CCR5, CCR6, CCR7, CCR10, CXCR3, CXCR4 and CXCR5; (ii) adhesion molecules: CD11a, CD11b, CD11c, CD103 and $\alpha4\beta7$; (iii) co-stimulatory molecules: CD27, CD28, CD40, CD40L, CD134, PD-1 and ICOS; (iv) activation molecules: CD25, CD38, CD69, CD71 and HLA-DR; (v) maturation markers: CD45RO, CD45RA and CD62L; (vi) cytokine receptors: CD126 and CD127; and (vii) other molecules: CD57, CD95, CD95L,

and GITR. Frequencies of the expanded cells expressing these surface molecules in the cultures without and with IL-2 supplementation on day 21 were observed and compared to those of whole blood on day 0 as a baseline control.

For chemokine receptors, the expanded cells from cultures whether IL-2 supplementation or not exhibited significant lower expressions of CCR6, CCR7 and CXCR4 and dramatic higher expression of CXCR3 when compared to the unexpanded cells (i.e., whole blood) as seen in Fig. 10A. Other molecules, CCR4, CCR5, CCR10, CXCR5, remained similar after the expansion. The expanded cells also had marked increases in expressions of adhesion molecules, CD11b, CD11c and $\alpha 4\beta 7$ when compared to the unexpanded cells (Fig. 10B). There was also no change in expressions of CD11a and CD103 between the expanded and unexpanded cells.

With respect to co-stimulatory molecules, only frequencies of CD40L of the expanded cells from both expansion groups were significantly increased when compared to the unexpanded cells (Fig. 10C). All other molecules including CD27, CD28, CD40, CD134, PD-1 and ICOS remained unchanged after the expansion. Furthermore, expressions of all activation molecules, except CD69, on the expanded cells were significantly upregulated when compared to those of the unexpanded cells (Fig. 10D). The expanded cells also showed significant lower frequencies of CD45RO and higher frequencies of CD45RA, while their CD62L expression was similar to the unexpanded cells (Fig. 10E).

The expanded cells also exhibited notable downregulation of cytokine receptor, CD126, and upregulation in CD127 when compared to the unexpanded cells (Fig. 10F). Expressions of other molecules including CD57, CD95 and GITR were dramatically raised after the expansion, whereas there were slight increases in CD95L (Fig. 10G). Moreover, no significant difference in numbers of any chemokine receptors, adhesion molecules, co-stimulatory molecules, activation molecules, maturation markers, cytokine receptors and other molecules was found between the two culture groups with and without IL-2 supplementation (Figs. 10A-10G).

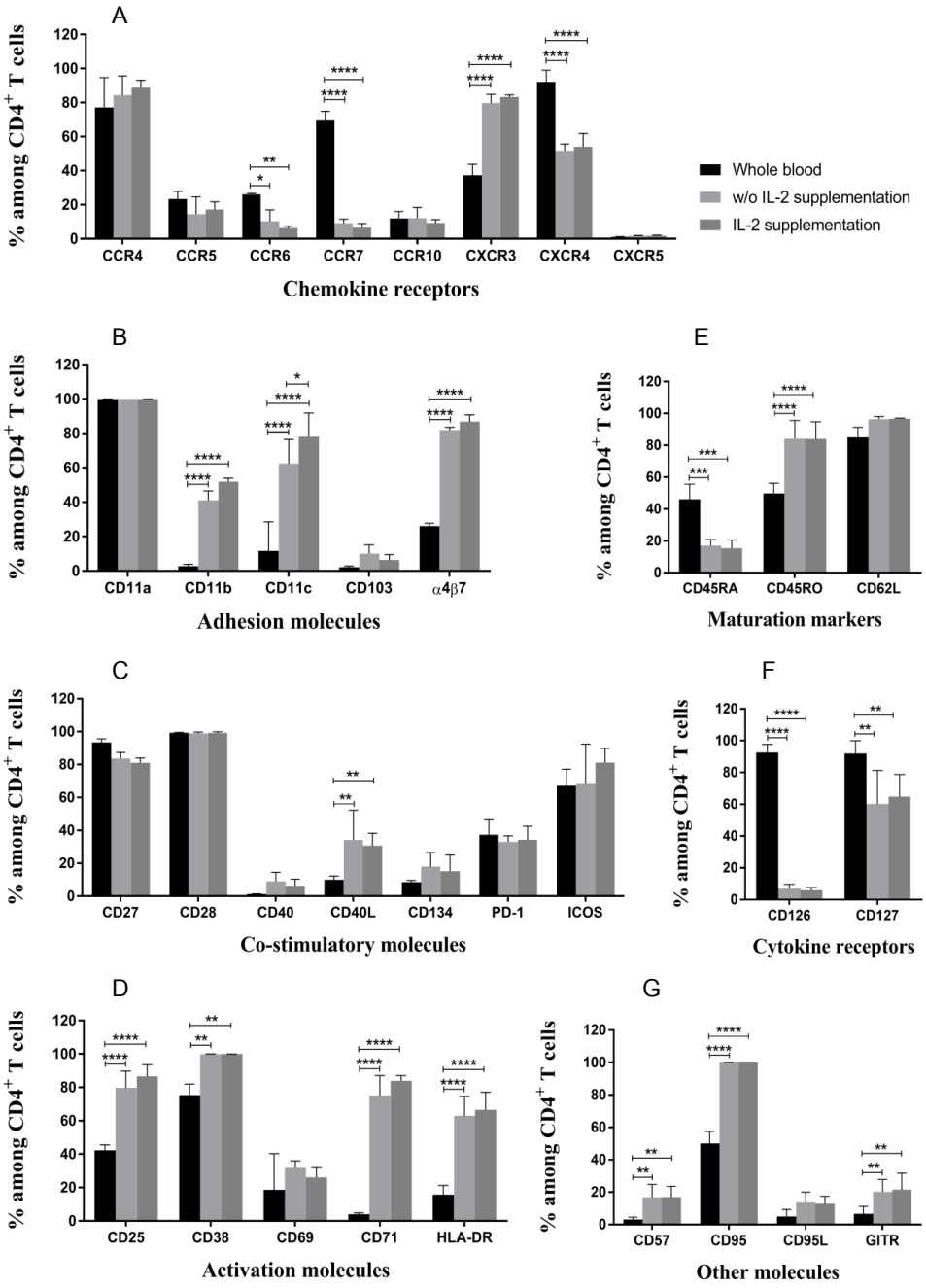


Fig. 10 Specific cell surface molecule expressions of anti-CD3/28 expanded CD4+ T cells. Expressions of a) chemokine receptors, b) adhesion molecules, c) co-stimulatory molecules, d) activation molecules, e) maturation markers, f) cytokine receptors and g) other molecules of the expanded cells when cultured without (w/o) and with IL-2 supplementation were observed on day 21 of the culture and compared to the expressions of whole blood observed on day 0. All data are presented as mean ± SD (n = 3, *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001 and ****p-value < 0.0001).

Discussions and conclusions

With respect to implementation of in vitro CD4+ T lymphocyte expansion for adoptive immunotherapy in human, there is a clinical study conducting adoptive transfer of up to 3x1010 autologous anti-CD3/28 activated CD4+T cells in HIV-infected patients and the transfusion was scheduled approximately $5x10^9$ cells at 8-week intervals [14, 15], indicating that large-scale expanded CD4+ T lymphocytes is required. A large-scale production of CD4+ T cells from HIV-1-infected donors using anti-CD3/28 coated beads, IL-2 supplementation, and gas-permeable bags was established and achieved 37-fold expansion after 14 days [21] which is not yet satisfied for CD4+ T cell numbers demanded for transfusion.

GMP-grade culture bags are in a concern to reduce contamination risks from microorganisms during a cell production aiming for treatment in clinics. GMP-grade Teflon cell culture (TCC) bags were used to compare a growth ability of normal human CD8+ T cells with conventional tissue culture flasks and plates [25]. Expanded CD8+ T cells in TCC bags yielded considerably higher than those in flasks and plates after 10 days (73-fold, 41-fold and 48-fold, respectively). Nevertheless, our study shows that anti-CD3/28 expanded CD4+ T cells from healthy donors in flasks proliferated better than those in commercially available GMP-grade culture bags (797-fold and 331-fold, respectively) after 21 days. Even so, fold expansion of CD4+ T cells from bag culture is still considerably high enough for cell production. High cell viability (75%) and high purity (97%) of expanded CD4+ T cells also support the use of bag for cell expansion.

Previous studies showed that expansion of both human and rhesus macaque CD4+ T cells with anti-CD3/28 coated beads provided a large number of homogenous CD4+ T cell population with Th1-like phenotype [16, 26]. Moreover, TNF-α production of anti-CD3/28 expanded CD4+ T cells played an important role in regulating synthesis of various β-chemokines [27] which support cellular resistance to R5-tropic HIV strain. Our results show that TNF-α producing cells were a major population presenting in the expanded cells which can play a protective role in resistance to infection. The presence of high frequency of IL-2 producing cells can also provide sufficient amount of IL-2 that supports in vitro expansion without exogenous IL-2 supplementation. Interestingly, although only one-third of anti-CD3/28 expanded CD4+ T cells showed Th1-like phenotype, other CD4+ T cell types including Th2, Th17 and Treg were almost diminished suggesting that anti-CD3/28 expansion promotes Th1 dominant population of the expanded cells. More importantly, simultaneous detection of cytokine production at a single cell level by polychromatic flow cytometry demonstrates that the majority of expanded cells simultaneously produced 2-3 cytokines (approximately 65%), suggesting a polyfunctional role of these cells.

To optimize the protocol for clinical adoptive T-cell therapy, other serum supplements besides FBS were considered in this study. HS was chosen for CD4+ T cell expansion development as it is commercially available and can be obtained as human AB serum. The culture media including RPMI1640, AIM-V and DC were then added with 10% HS for expansion comparison with RPMI1640 supplemented with 10% FBS. Culture media with 10% HS supplementation supported CD4+ T cell expansion similar to normal culture medium (i.e., culture medium with 10% FBS

supplementation or R10 in our study) in the first 2 weeks of culture. The growth rates of expanded cells from all culture media were 224-330 -fold and cell viability was approximately 70% after two weeks of cell activation and expansion. Fold expansions of all media, except R10, maintained at the same levels on day 17 before gradual declines on day 21. Unlike the others, fold expansion of R10 was considerably augmented on day 17 and then decreased on day 21. However, the expanded cells from the R10 culture on day 21 still proliferated ~2–fold higher than those on day 14. CD4+ T cell expansion in R10 provided the highest growth rate and the most consistency when compared to that in the others. Of all cultures using specialized media, RPMI1640 supplemented with 10% HS rendered the lowest value of fold expansion after a 3-week culture. It is then assumed that FBS supplementation render the higher expansion rates, and yields as well as better consistency than HS supplementation. Our findings are opposed to the previous study demonstrating that 10% FBS provided inconsistency in cell expansion when compared to HS supplementation [24]. Therefore, FBS supplementation can be a choice of interest for clinical uses to achieve the best expansion as long as its source is from an area free of bovine spongiform encephalopathy (BSE), such as Australia or New Zealand [28].

Furthermore, AIM-V and DC with 10% HS supplementation were able to provide suitable CD4+ T cell expansion with average 250-fold which is similar to the results achieved from AIM-V and DC with 5% HS supplementation from the previous study [24]. It is suggested that 5% HS supplementation is sufficient to support CD4+ T cell expansion and higher concentration of HS does not improve the proliferation rates and yields. Both AIM-V and DC are also good candidates as GMP-grade media used for cell expansion following requirement of clinical trials.

Besides FBS and HS supplementation, a xeno-free serum replacement (SR) was introduced as a novel platform strategy for clinical-grade cell manufacturing [29]. In this study, T-cell expansion was conducted by using a serum-free medium AIM-V supplemented with 5% SR and anti-CD3/28 coated bead stimulation. The expansion kinetic of T cells using SR was comparable and polyfunctional profiles were similar to that using FBS and HS [29]. SR supplementation, therefore, has become an alternative choice for FBS and HS substitution and for clinical-grade cell production since a potential risk of cross-species contamination can be avoided. Addition of exogenous cytokines, particularly IL-2, is worth considerable for improvement in cell expansion. Previous studies showed that growth rates of the expanded T cells cultured in media with HS and IL-2 supplementation were increased when compared to those without IL-2 supplementation [24, 29]. In this case, addition of HS and IL-2 together can synergistically promote the cell expansion.

Changes in specific cell surface molecule expressions of the anti-CD3/28 CD4+ T cells were also observed in this study. Several chemokine receptors have been identified as coreceptors for the HIV entry, such as CCR5, CXCR4, CCR4, CCR6 and CCR10. The in vitro expanded CD4+ T cells with anti-CD3/28 activation were proved to be resistant to HIV-1 infection via the reduction in frequencies and densities of CCR5 molecules [18,30]. Our expanded cells also rendered a low frequency in CCR5 (< 15% of CD4+ T cells) which presumably maintain at this low level because the recovery of CCR5 expression was low when activation with anti-CD3/28 coated beads compared

to stimulation with anti-CD3/28 immobilized on the surface of a tissue culture plate [30]. Our anti-CD3/28 activation protocol also rendered the expanded CD4+ T cells with twice as less CXCR4 expression than the unexpanded cells. Although a high number of the expanded cells still expressed CXCR4 (52% of CD4+ T cells), the chance of viral entry when switching coreceptor usage from CCR5 to CXCR4 [31] will feasibly diminished when compared to the unexpanded cells.

CCR4, CCR6 and CCR10 were also reported to be other HIV-1 coreceptors of primary HIV-1 isolates [32-34]. Our expanded cells showed that CCR4 was highly expressed throughout the expansion period (84% of CD4+ T cells), whereas a significant downregulation in CCR6 by 2.5 – fold and low frequency of CCR10 (12% of CD4+ T cells) were found. Although CCR4 expression was high, soluble viral protein gp120 had greater affinity to CCR5 than CCR4 [34]. CCR4+ T cells are also able to be defined as Th2 cells, while Th1 cells are classified by CXCR3+ [35]. In our study, the frequency of CXCR3+ T cells was 2-fold higher than that of the unexpanded cells (~83% of CD4+ T cells) which was almost equal to CCR4+ T cells. However, we cannot specify that our CXCR3+ T cells are absolutely purified Th1 cells as they need to be further characterized with expressions of CCR4-, CCR5+, and CXCR6+ [35]. These evidences clearly demonstrate that our expanded cells are suitable for reinfusion due to their highly feasible ability for HIV-1 resistance in vivo.

As the gut compartment is a major portal for HIV entry, the considerable depletion in mucosal CD4+ T cells was observed during acute infection [36]. Our study shows that the expanded cells had a pretentious increase in $\alpha 4\beta 7$ expression to 82% of CD4+ T cells. In contrary, the level of another gut mucosal specific adhesion molecule, CD103 [37], was low at the end of the culture (10% of CD4+ T cells) even though it was 5 – fold higher expressed in the expanded cells. It is then worth proposing that these cells feasibly migrate to the site of depletion and improve immune response at the gut-associated lymphoid tissues.

In conclusion, results from this study address that our developed closed-culture system for CD4+ T lymphocyte expansion using anti-CD3/28 coated beads and GMP-grade gas-permeable culture bags is practical, effective, and reproducible. Using our protocol, a large number of anti-CD3/28 expanded CD4+ T lymphocytes is achieved together with high viability and high purity within reasonable time (21 days). This method is also successful to expand both freshly isolated CD4+ T cells from healthy volunteers and cryopreserved CD4+ T cells from HIV-infected patients. It is then suggested that our refined procedure is useful for cell production used for adoptive transfer immunotherapy, although, there are still many aspects require further improved and investigated, such as effects of different culture media, impact of exogenous supplementation, and cell manufacturing processes under GMP standard. This study also addresses the expansion and polyfunctional profiles of expanded CD4+ T cells when cultured in commercially available GMP-grade media with human serum supplementation. We found that AIM-V and DC are good candidates for clinical-grade cell manufacturing and HS supplementation can well support cell expansion. Although animal-derived substances, mainly FBS, is not recommended to use in cell products due to their risk in cross-species contamination, FBS supplementation is still able to render the highest fold expansion with high viability and consistency when compared to HS. There is also no significant

difference in polyfunctional profiles between FBS and HS. To use FBS for cell production, its origin must be free of BSE. Therefore, our expansion protocol using either AIM-V or DC supplemented with HS provides satisfied yields of the expanded cells of which polyfunctions are similar, suggesting the suitable protocol for further uses in clinical studies.

Moreover, this study demonstrated that the 1:1 bead-to-cell ratio of anti-CD3/28 coated magnetic beads for CD4+ T cell expansion was the most optimum bead quantity to achieve the satisfied yield of the expanded cells. The autocrine cytokines, mainly IL-2, produced by the expanded cells themselves are also adequate for a 3-week proliferation without additional IL-2 supplementation. After the expansion, phenotypic profiles of the expanded cells were changed. The expanded cells likely become more resistant to HIV-1 via downregulation of dominant coreceptors for HIV entry, CCR5 and CXCR4, as well as migrate to the site of depletion and improve immune response at the gut-associated lymphoid tissues due to higher expressions of gut-homing molecules, $\alpha 4\beta 7$ integrin. Furthermore, other specific surface molecule expressions related to activation, proliferation, differentiation, homeostasis and apoptosis revealed certain functions of the expanded cells. It is thus worth suggesting that these expanded cells following our optimized protocol are suitable for CD4+ T cell immunotherapy used in HIV-infected patients, even though further investigation on CD4+ T cells from HIV-infected patients and a large-scale production are required.

Suggestion for further study

A study to develop cell based immunotherapy using autologous transferred of CD3/28 expanded CD4+ T cells with an aim at increasing CD4+ T cell level can be a promising therapeutic tool for immunological discordant patients to support a conventional antiretroviral drug treatment. Moreover, a successful effort to reduce antiretroviral drug cost in HIV infected patients with HAART is possible since CD4 transfusion will supplement with HAART interruption in successive HIV viral control patients by providing long-term anti-HIV viral control using patient's immune response. More importantly, when HAART interruption is needed to reduce drug toxicity and prevent a development of drug resistant HIV viral strain, HAART supplemented with CD4 transfusion before treatment interruption will ensure a successful control of HIV viral replication during treatment cessation.

With this developed protocol, clinical grade expansion procedure could be applied to regulatory T cell which can be used for the treatment of autoimmune disease. Furthermore, Tumor specific T cell from infiltrated T lymphocytes in a tissue from solid tumor can be isolated and expanded for being used in a treatment of certain type of cancer. The cell expansion protocol can also be used for the expansion of Chimeric Antigen Receptor (CAR) T cells to obtain a large number of CAR T cells for infusion in patients with specific cancer type.

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Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ

   1.1 Thitilertdecha P, Suwannachod P, Poungpairoj P, Tantithavorn V, Khowawisetsut L, Ammaranond P, Onlamoon N. A closed-culture system using a GMP-grade culture bag and anti-CD3/28 coated bead stimulation for CD4+ T cell expansion from healthy and HIV-infected donors. J Immunol Methods. 2018 Jun 9. pii: S0022-1759(17)30458-1. doi: 10.1016/j.jim.2018.06.004. [Epub ahead of print]

   1.2 Thitilertdecha P, Tantithavorn V, Poungpairoj P, Ammaranond P, Loharungsikul S, Onlamoon N. Comparison of serum supplementation and culture medium for CD4+ T cell expansion by using anti-CD3/28 coated beads. (Submitted to Biological procedures online)

   1.3 Thitilertdecha P, Poungpairoj P, Tantithavorn V, Ammaranond P, Onlamoon N. Differential expression of functional-associated cell surface molecules on anti-CD3/28 expanded CD4+ T cells. (Submitted to Human Immunology)

2. การนำผลงานวิจัยไปใช้ประโยชน์
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3. อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร) : ไม่มี

Appendix

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Research paper

A closed-culture system using a GMP-grade culture bag and anti-CD3/28 coated bead stimulation for CD4$^+$ T cell expansion from healthy and HIV-infected donors

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Adoptive immunotherapy

ABSTRACT

CD4 immunotherapy is potentially useful in immune reconstitution of CD4$^+$ T cells for HIV-infected patients. Transfusion of anti-CD3/28 expanded CD4$^+$ T cells is also proved to be safe and effective in both SIV-infected macaques and HIV-infected patients. However, there is no such standardized and practical protocol available for cell production in order to use in clinics. This study thus aimed to develop a closed-culture system for *in vitro* CD4$^+$ T lymphocyte expansion by using a commercially available GMP-grade culture bag and anti-CD3/28 activation. Freshly isolated CD4$^+$ T cells by immunorosette formation from healthy donors and cryopreserved CD4$^+$ T cells from HIV-infected patients with CD4 count over 500 cells/μL were stimulated with anti-CD3/28 coated beads. The activated cells were then expanded in conventional culture flasks and GMP-grade culture bags for three weeks. Fold expansion, cell viability, growth kinetic and phenotypic characters were observed. Results revealed that purified CD4$^+$ T cells from healthy individuals cultured in flasks showed better expansion than those cultured in bags (797-fold and 331-fold, respectively), whereas, their cell viability, growth kinetic and expanded CD4$^+$ T cell purity were almost similar. A large-scale production was also conducted and supported consistency of cell proliferation in the closed-culture system. Frozen CD4$^+$ T lymphocytes from the patients were able to remain their growth function and well expanded with a good yield of 415-fold, 85% viability and 96% purity of CD4$^+$ T cells at the end of a 3-week culture in bags. This developed closed-culture system using culture bags and anti-CD3/28 coated beads, therefore, can achieve a large number of expanded CD4$^+$ T lymphocytes with good reproducibility, suggesting a promising protocol required for adoptive immunotherapy.

1. Introduction

Highly active antiretroviral therapy (HAART) is greatly effective and safe for human immunodeficiency virus (HIV)-infected patients by lowering the HIV viral load into an undetectable level. However, this therapy does not eradicate latent reservoirs of virus (Finzi et al., 1997) and not completely restore immune system (Carcelain et al., 2001; Lange and Lederman, 2003; Valdez et al., 2002; Valdez et al., 2003). Adoptive transfer of autologous activated CD4$^+$ T cells then becomes an alternative approach for HIV treatment due to its tentative benefit for immune reconstitution. This approach is proved to be effective and safe by several *in vivo* studies in both simian immune deficiency virus (SIV)-infected rhesus macaques and HIV-infected patients (Onlamoon et al., 2007; Villinger et al., 2002; Onlamoon et al., 2006; Bernstein et al., 2004; Levine et al., 2002).

CD4$^+$ T cell expansion method was established with results showing that expanded CD4$^+$ T cells were intrinsic resistant to macrophage-tropic isolates of HIV-1 infection when using anti-CD3/28 coated magnetic beads for cell stimulation (Levine et al., 1996; Carroll et al., 1997; Riley et al., 1997). Anti-CD3/28 activated CD4$^+$ T cells also had

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lower expression of CCR5 as well as greater expression of RANTES, MIP-1α, and MIP-1β (Onlamoon et al., 2007; Onlamoon et al., 2006; Bernstein et al., 2004; Levine et al., 1996). Furthermore, cytokine secretion of these expanded $CD4^+$ T cells was associated with T helper cell type 1 function along with increases of telomerase activity and diversity of TCR Vβ repertoires (Onlamoon et al., 2007; Bernstein et al., 2004).

Although several *in vitro* culture methods are available for anti-CD3/28 activated $CD4^+$ T lymphocytes (Levine et al., 1997; Onlamoon et al., 2013; Garland et al., 1999; Levine et al., 1998), none of them is yet standardized and practical to be used in clinics. There are many concerns in cell production for adoptive immunotherapy, such as contamination risk, production scale, and source of starting cells. To reduce contamination risk of microorganisms, a closed-culture system is introduced by expanding the cells in gas-permeable culture bags instead of conventional flasks. No current protocols have yet reached satisfaction in cell yields for transfusion. According to the production scale for clinical uses, previous studies demonstrated that a large number of up to 3×10^{10} expanded $CD4^+$ T cells were demanded of reinfusion into HIV-infected patients (Bernstein et al., 2004; Levine et al., 2002), suggesting that a large number of purified $CD4^+$ T cells are required for *in vitro* expansion. Most HIV-infected patients, however, have low $CD4^+$ T cell counts and then becoming a limitation for sufficient cell number.

Our study then purposed to develop a closed-culture system for *in vitro* $CD4^+$ T lymphocyte expansion by using a commercially available GMP-grade culture bag and anti-CD3/28 activation as well as a large-scale production aiming for adoptive immunotherapy. This developed procedure was also utilized for purified $CD4^+$ T cells from HIV-infected patients.

2. Materials and methods

2.1. Samples

Five healthy volunteers were recruited and signed informed consents which were approved by the Institutional Review Board of the Faculty of Medicine Siriraj Hospital at Mahidol University. Sixteen to twenty-five milliliters of whole blood were collected into sodium heparin-containing vacutainer tubes and kept at room temperature prior to phenotypic determination of whole blood and $CD4^+$ T lymphocyte isolation.

Cryopreserved $CD4^+$ T lymphocytes were obtained from HIV-infected patients with $CD4^+$ T cell count over 500 cells/μL and stored in a liquid nitrogen tank at −196 °C for 1.5–2 years.

2.2. Antibodies

Monoclonal antibodies (mAbs) and their conjugated fluorochromes including anti-CD3 conjugated with fluorescein isothiocyanate (FITC), anti-CD4 conjugated with allophycocyanin (APC), anti-CD8 conjugated with phycoerythrin (PE), anti-CD19 conjugated with allophycocyanin and cyanine dye (APC-Cy7), anti-CD45 conjugated with peridinin chlorophyll protein (PerCP), and anti-CD56 conjugated with phycoerythrin and cyanine dye (PE-Cy7) were purchased from Becton Dickinson Bioscience (BDB, San Jose, CA). In addition, anti-CD3 conjugated with AlexaFluor® (A700), anti-CD4 conjugated with Brilliant Violet™ 605 (BV605), anti-CD8 conjugated with PE/Dazzle™ 594, anti-CD69 conjugated with PerCP/Cy5.5, anti-IL-2 conjugated with BV510, anti-IL-4 conjugated with FITC, anti-IL-17 conjugated with PE, anti-IFN-γ conjugated with APC, anti-TNF-α conjugated with BV650 and anti-TGF-β conjugated with BV421 were obtained from BioLegend (San Diego, CA).

2.3. $CD4^+$ T lymphocyte isolation

$CD4^+$ T lymphocytes were directly isolated from fresh whole blood through immunorosette formation by using RosetteSep® Human $CD4^+$ T cell enrichment cocktail (STEMCELL Technologies, Vancouver, BC, Canada) according to the manufacturer's instruction. Purified $CD4^+$ T lymphocytes were isolated by a standard Ficoll-Hypaque gradient centrifugation (Histopaque, Sigma-Aldrich,Co., St.Louis, MO, USA) and were ready for phenotypic characterization and cell expansion.

2.4. Cell stimulation and expansion for freshly isolated $CD4^+$ T cells

Freshly purified $CD4^+$ T cells of 1×10^6 cells were stimulated with anti-CD3/28 coated beads (Dynabeads® Human T-Activator CD3/CD28, Invitrogen Dynal, Oslo, Norway) at a bead-to-cell ratio of 1:1. The stimulated $CD4^+$ T cells were then expanded in complete media (RPMI1640 with 10% fetal bovine serum (FBS)), 50 μg/mL pencillin-streptomycin and 2 mM L-glutamine. The expanded cells were incubated at 37 °C and 5% CO_2 humidification and reactivated on day 7. The cells were expanded for a 3-week culture period. Cell numbers and viability were observed by using trypan blue exclusion and a TC10™ automated cell counter. Phenotypic characters were analyzed by flow cytometry on days 14 and 21.

For an expansion method of flask culture, the stimulated cells of 1×10^6 cells were placed in a 24-well plate (Costar® 24 well clear TC-treated multiple well plates, sterile, Corning Inc., Life Sciences, NY, USA) on day 0 and expanded at a concentration of 0.5×10^6 cells/mL before transferring to T25, T75 and T175 plastic tissue culture flasks (Corning® U-shaped canted neck cell culture flask with vent cap, Corning Inc., Life Sciences, NY, USA) on days 4, 7, and 11, respectively. Cell numbers and viability were observed on days 4, 7, 11, 14, 17 and 21 while the media were replenished with calculated amounts of fresh media on days 4, 7, 11, 14, and 17 to maintain the cell suspension concentration at 0.5×10^6 cells/mL.

With respect to bag culture, the expansion process was similar to flask culture during the first week of cell expansion. The expanded cells were re-stimulated in T25 flasks on day 7 and replenished with calculated amounts of fresh media at a concentration of 0.5×10^6 cells/mL before transferring to a GMP-grade culture bag (Vuelife® cell culture bags, CellGenix, Freiburg, Germany) with a size of 72c (maximum volume of 72 mL). The culture bag was clamped by half and placed on a steel grating culture stage prior to cell transfer. Fresh media were added to reach maximum volume of the bag (72 mL) on day 11. After that, the expanded cells were transferred to another culture bag with a size of 197c on day 14, filled with media up to 197 mL on day 17, and leaved for growth until day 21. Cell numbers and viability were observed on days 4, 7, 14, and 21.

2.5. Large-scale production of freshly isolated $CD4^+$ T cells in a closed-culture system

Freshly purified $CD4^+$ T cells of 8×10^6 cells from healthy donors were mixed with anti-CD3/28 coated beads (Dynabeads® Human T-Activator CD3/CD28, Invitrogen Dynal, Oslo, Norway) at a bead-to-cell ratio of 1:1 in a plastic tube (Falcon® high clarity polypropylene centrifuge tube, conical bottom, sterile, Corning Inc., Life Sciences, NY, USA) before injecting into a GMP-grade culture bag (Vuelife® cell culture bags, CellGenix, Freiburg, Germany) with a size of 32c. Complete media (RPMI1640 with 10% fetal bovine serum, 50 μg/mL pencillin-streptomycin and 2 mM L-glutamine) were added in order to achieve a concentration of 0.5×10^6 cells/mL. The expanded cells were incubated at 37 °C and 5% CO_2 humidification. Only 20×10^6 cells of anti-CD3/28 expanded $CD4^+$ T cells were reactivated on day 7. The expanded cells were consequently transferred to larger culture bags with sizes of 72c and 196c on days 7 and 14. Fresh culture media were added to reach suggested maximum volume of individual bag size on

days 4, 11 and 17. Cell numbers and viability were observed on days 7, 14 and 21 by using trypan blue exclusion and a TC10™ automated cell counter. Phenotypic characters were analyzed by flow cytometry on days 14 and 21.

2.6. Cell stimulation and expansion for cryopreserved CD4$^+$ T cells

Cryopreserved CD4$^+$ T lymphocytes from HIV-infected patients were removed from a liquid nitrogen tank at −196 °C and then thawed in a sterile water bath at 37 °C. Cryopreserved cells of 1×10^6 cells were activated and expanded following the developed bag culture protocol of freshly isolated CD4$^+$ T cells. In addition, fresh media with IL-2 supplementation at a concentration of 100 U/mL was used on day 7 onwards. Cell numbers and viability were observed on days 4, 7, 14 and 21 by using trypan blue exclusion and a TC10™ automated cell counter. Phenotypic characters were analyzed by flow cytometry on days 14 and 21.

2.7. Immunofluorescence staining and analysis

Samples of whole blood, purified and expanded CD4$^+$ T cells were stained with fluorochrome-conjugated mAbs for 15 min before adding FACS lysing solution (BDB, San Jose, CA) in order to lyse and fix the cells. The stained cells of at least 100,000 events were acquired for each analysis by a BD FACSVerse™ flow cytometer (BDB, San Jose, CA) and the data were analyzed by using FlowJo Software (Tree Star, San Carlos, CA). The percentages of lymphocyte subsets were determined by the expression of CD3, CD4, CD8, CD19 and CD56.

2.8. Cell stimulation, intracellular cytokine staining (ICS) and analysis

Expanded CD4$^+$ T cells from a bag culture of cryopreserved CD4$^+$ T cells from HIV-infected patients were used to determined different types of cytokine production. Expanded CD4$^+$ T cells at 1×10^6 cells/mL were stimulated with 25 ng phorbol 12-myristate 13-acetate (PMA) and 1 μg ionomycin (I) in the presence of brefeldin A (BFA) at 10 μg, whereas expanded CD4$^+$ T cells in the presence of BFA without stimulation were used as a control. The samples were then incubated at 37 °C/5% CO_2 for 4 h. After incubation, PMA/I stimulated and unstimulated samples were stained with Zombie NIR™ dye (BioLegend, San Diego, CA) at 4 °C for 15 min. A washing buffer (phosphate buffered saline (PBS) with 2% FBS) were added and the samples were washed by centrifugation at 450 *g* for 5 min. The samples were then surface stained with a combination of mAbs including anti-CD3 A700, anti-CD4 BV605, anti-CD8 PE/Dazzle™ 594 and anti-CD69 PerCP/Cy5.5 at 4 °C for 15 min and washed once. The stained samples were fixed and permeabilized in 0.5 mL of BD Cytofix/Cytoperm™ Fixation and Permeabilization Solution (BDB, San Jose, CA) at 4 °C for 20 min. After incubation, the samples were washed by adding 1× BD Perm/Wash™ Buffer (BDB, San Jose, CA) and centrifuged at 500 *g* for 5 min. ICS were performed by staining with a combination of mAbs including anti-IL-2 BV510, anti-IL-4 FITC, anti-IL-17 PE, anti-IFN-γ APC, anti-TNF-α BV650, anti-TGF-β BV421 at 4 °C for 30 min. After staining, the samples were washed with 1× BD Perm/Wash™ Buffer and resuspended in PBS. The stained cells of at least 100,000 events were acquired for each analysis by a BD Fortessa™ flow cytometer (BDB, San Jose, CA) and the data were analyzed by using FlowJo Software. The cytokine producing cell subsets were determined from activated populations expressing CD69 and the percentages of cytokine producing cell subsets were determined from a total cytokine producing cell population. A Boolean gating strategy was used for the analysis of polycytokine producing cell subsets in order to evaluate cytokine producing cell subsets with ability to simultaneously produce 1, 2, 3, 4, 5 or 6 cytokines.

2.9. Cell harvesting and quality control

Cell suspensions containing expanded CD4$^+$ T cells from a bag culture of cryopreserved CD4$^+$ T cells from HIV-infected patients were collected on day 21 of cell expansion. Aliquots of cell suspensions were transferred to 50-mL tubes and centrifuged at 450 *g* for 5 min. Culture supernatants were removed, collected in small aliquots and stored in a − 80 °C freezer prior to thaw for using in quality control. Cell pellets were re-suspended in 10 mL of complete media and transferred to 15-mL tubes.

To remove anti-CD3/28 coated beads, each 15-mL tube was placed in a DynaMag™-15 Magnet (Invitrogen Dynal) for 2 min and the cell suspension was transferred to another 15-mL tube. The same procedure was repeated once and aliquots of cell suspensions were pooled. The centrifugation process was repeated and media was removed. Cell pellets were re-suspended in complete media and pooled into a single tube at a volume of 20 mL. Cell numbers and viability were observed by using trypan blue exclusion and a TC10™ automated cell counter. Aliquots of the expanded CD4$^+$ T cells at 1×10^7 cells/mL were cryopreserved in RPMI-1640 media containing 20% FBS with 10% DMSO and stored at 180 °C prior to thaw for using in quality control and intracellular cytokine staining.

For quality control, frozen expanded CD4$^+$ T cells were thawed and re-suspended in PBS containing 5% human albumin. To detect residual bead contamination, samples containing 1×10^7 cells were mixed with sodium hypochlorite solution and centrifuged at 450 *g* for 5 min. Supernatants were removed and the pellets were re-suspended with PBS. Residual bead counts were determined on a hemacytometer and the total numbers of beads were calculated. For sterility testing, a 14-days United States Pharmacopoeia (USP) sterility testing on aliquots of thawed cells and culture supernatants was performed at Siriraj Hospital Microbiology Laboratory by using fluid thioglycollate medium (for the detection of anaerobic bacteria) and soybean-casein digest medium (for the detection of aerobic bacteria and fungi). Sterile results were identified as no growth of microorganism.

2.10. Data analysis

Fold expansion number was calculated by using the viable cell number at each indicated time point divided by the viable cell number at the beginning of cell expansion. For a large-scale production experiment, fold expansion numbers were calculated to get an approximated number if a total number of expanded cells on day 7 was expanded until day 21. Fold expansion, cell viability and frequencies of T lymphocytes and their subsets were shown as mean ± SD (standard deviation). Statistical differences of mean values of fold expansion and cell viability of expanded CD4$^+$ T lymphocytes were analyzed by paired *t*-tests. Statistical significance was considered when *p*-value was below 0.05. For cytokine producing cell subset analysis, the percentages of specific-cytokine producing cell subsets were shown as mean ± SD. The proportions of cytokine producing cell subsets were presented as pie-charts. All data were analyzed by using Prism software (GraphPad, La Jolla, CA).

3. Results

3.1. Expanded CD4$^+$ T lymphocytes in small-scale plastic flasks and GMP-grade culture bags after anti-CD3/28 coated bead stimulation

To develop a closed-culture system for CD4$^+$ T lymphocytes, GMP-grade culture bags were used for cell expansion and compared to conventional plastic flasks. Freshly isolated CD4$^+$ T cells were obtained from five healthy volunteers for the study. Fold expansion, cell viability, growth kinetic and phenotypic characters were observed for proliferation efficiency of expanded CD4$^+$ T cells in flasks and bags.

Fold expansion numbers of CD4$^+$ T lymphocytes expanded in both

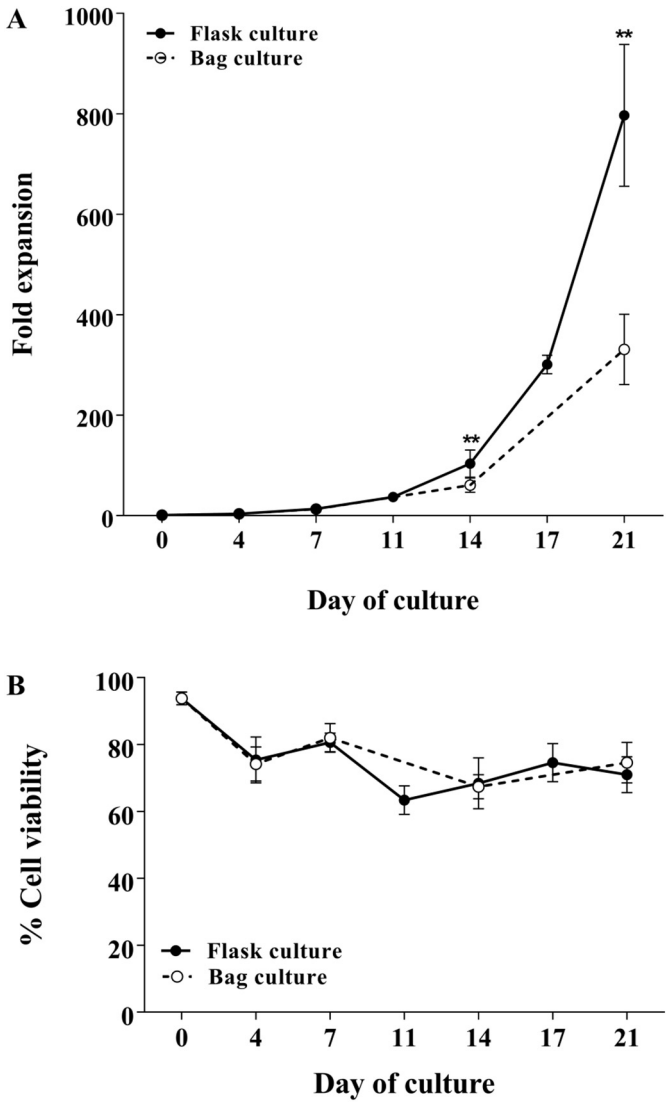

Fig. 1. Growth kinetics: (A) fold expansion, (B) cell viability of anti-CD3/28 expanded $CD4^+$ T cells of freshly isolated $CD4^+$ T cells from healthy volunteers expanded in flasks and bags ($n = 5$, ** p-value < 0.01).

flasks and bags were similar at the beginning and started dramatically increasing on day 14 as shown in Fig. 1A. A flask culture exhibited significantly higher fold expansion than a bag culture (103.8 ± 27.2 and 60.9 ± 14.4, respectively, p-value = 0.0075). At the end of the culture period, $CD4^+$ T lymphocytes expanded in flasks were proliferated better than those expanded in bags (796.7 ± 141.2 –fold and 330.9 ± 70.0 –fold, respectively, p-value = 0.0078).

Cell viability of anti-CD3/28 expanded $CD4^+$ T cells from both flask and bag cultures were similar throughout a 3-week expansion period (Fig. 1B). There were slightly decreases of viable cells from day 0 to day 21 (93.8 ± 1.9% to 71.0 ± 5.4% in flasks and 93.8 ± 1.9% to 74.6 ± 6.1% in bags) and no significant difference between the two different culture methods.

Phenotypes of whole blood, isolated $CD4^+$ T lymphocytes, and anti-CD3/28 expanded $CD4^+$ T lymphocytes were determined by a flow cytometer (Fig. 2). As shown in Table 1, phenotypic profiles showed that the immunorosette formation technique was effective and reproducible for $CD4^+$ T cell isolation from whole blood with high frequency of $CD3^+CD4^+CD8^-$ subset (91% of lymphocytes). After a 3-week culture, anti-CD3/28 expanded $CD4^+$ T cells ($CD3^+CD4^+CD8^-$) in either flasks or bags exhibited high frequencies (> 97% of lymphocytes) on days 14 and 21. The cell purity was also confirmed with low frequencies of $CD3^+CD4^-CD8^+$ and $CD3^+CD4^+CD8^+$ populations (< 0.3% and < 2%, respectively) throughout the expansion period.

3.2. Large-scale expansion of $CD4^+$ T lymphocytes in a closed-culture system

As a high number of purified $CD4^+$ T lymphocytes are demanded for cell transfusion, a large-scale production of freshly isolated $CD4^+$ T cells from five healthy donors in a closed-culture system was developed in this study. Fold expansion, cell viability, growth kinetic and phenotypic characters were observed for large-scale proliferation.

A large-scale production of $CD4^+$ T cells exhibited a gradual increase of fold expansion from day 0 to day 14 (1.0 ± 0.0 on day 0, 5.5 ± 1.0 on day 7, and 20.4 ± 3.4 on day 14) and a marked increase from day 14 to day 21 (109.1 ± 18.4 on day 21). Growth kinetic of this large-scale expansion in culture bags also had a similar pattern with that of a small-scale bag culture prescribed earlier. Cell viability of large-scale anti-CD3/28 expanded $CD4^+$ T cells was good with a slight decrease from 91.2 ± 2.6% on day 0 to 77.4 ± 6.3% on day 21.

Again, phenotypic characterization of whole blood and isolated $CD4^+$ T cells was performed for purity confirmation of isolated $CD4^+$ T cells before culture as presented in Table 2. Anti-CD3/28 expanded $CD4^+$ T lymphocytes from a large-scale production showed high frequency of $CD3^+CD4^+CD8^-$ population (> 98%) on days 14 and 21. Low numbers of $CD3^+CD4^-CD8^+$ and $CD3^+CD4^+CD8^+$ populations (< 0.8% and < 0.5%, respectively) were detected.

3.3. Bag culture of anti-CD3/28 expanded $CD4^+$ T cells from HIV-infected patients

Using freshly isolated $CD4^+$ T cells from HIV-infected patients for cell expansion may possibly be inconvenient; therefore, frozen cells become alternative source and more practical for expansion. Cryopreserved $CD4^+$ T lymphocytes from five healthy donors were able to expand in bags with a similar fold expansion when compared to freshly isolated $CD4^+$ T lymphocytes (data not shown). Cryopreserved $CD4^+$ T lymphocytes from HIV-infected patients were then expanded in the developed close-culture system.

Anti-CD3/28 expanded $CD4^+$ T cells from frozen cells showed the same trend and similar numbers of fold expansion as those from freshly isolated cells (1.0 ± 0.0 on day 0, 3.4 ± 0.4 on day 4, 13.6 ± 3.5 on day 7, 63.6 ± 16.2 on day 14 and 414.9 ± 67.6 on day 21). Cell viability of cryopreserved cells was quite high and remained stable until the end of expansion (76.7 ± 5.8% on day 0 to 85.0 ± 1.7% on day 21).

Purity of isolated $CD4^+$ T cells from cryopreservation was ensued by phenotypic characterization (Table 3). $CD3^+CD4^+CD8^-$ population was detected to be dominant for anti-CD3/28 expanded $CD4^+$ T cells throughout the expansion period (> 98%). This was confirmed by minor populations of $CD3^+CD4^-CD8^+$ and $CD3^+CD4^+CD8^+$ T cells (< 0.8% and < 0.5%, respectively).

3.4. Determination of cytokines produced by anti-CD3/28 expanded $CD4^+$ T cells

Although cell expansion by using anti-CD3/28 coated beads showed a homogenous population of expanded $CD4^+$ T lymphocytes, the obtained phenotypic character were not able to provide information on a variety of expanded cells in term of function. Since $CD4^+$ T lymphocytes can be divided into subpopulations including T helper 1 (Th1), T helper 2 (Th2), T helper 17 (Th17), and regulatory T cells (Treg) based on types of cytokine produced by these cells, anti-CD3/28 expanded $CD4^+$ T cells from a bag culture of cryopreserved $CD4^+$ T cells from HIV-infected patients were investigated for their T helper cell subset identity.

CD69 expression was used to determine the level of activated cells

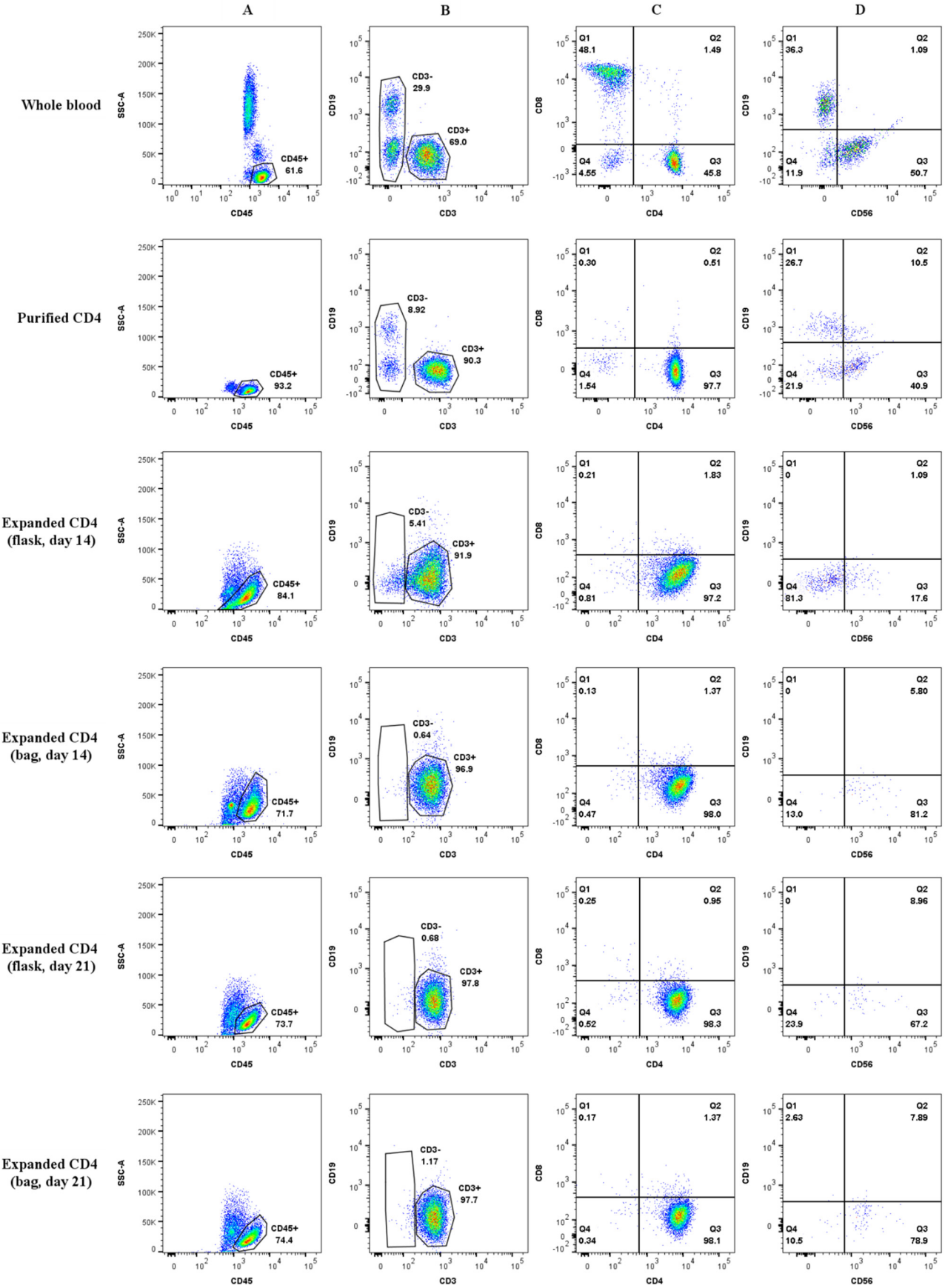

Fig. 2. Phenotype characterization of whole blood, purified $CD4^+$ T cells, expanded $CD4^+$ T cells on days 14 and 21 in flask and bag cultures. Gating identified (A) lymphocytes among leukocyte population, (B) $CD3^+$ and $CD3^-$ cells among lymphocyte population, (C) $CD4^+$ and $CD8^+$ T cells among $CD3^+$ cells, and (D) $CD19^+$ B cells and $CD56^+$ NK cells among $CD3^-$ cells.

Table 1
Phenotypes of whole blood, isolated $CD4^+$ T cells, and anti-CD3/28 expanded $CD4^+$ T cells from healthy volunteers (mean ± SD, $n = 5$).

Phenotypes	Whole blood	Isolated $CD4^+$ T cells	Anti-CD3/28 expanded $CD4^+$ T cells[a]			
	Day 0	Day 0	Day 14[b]		Day 21	
			Flasks	Bags	Flasks	Bags
% of lymphocytes						
$CD3^+CD4^-CD8^-$	4.4 ± 3.2	2.2 ± 1.3	0.8 ± 0.5	0.7 ± 0.6	0.8 ± 0.9	0.7 ± 0.4
$CD3^+CD4^+CD8^-$	32.6 ± 4.3	91.0 ± 4.8	98.2 ± 0.4	97.8 ± 1.3	98.3 ± 1.2	97.4 ± 1.5
$CD3^+CD4^-CD8^+$	36.0 ± 2.0	0.1 ± 0.0	0.2 ± 0.1	0.1 ± 0.1	0.3 ± 0.3	0.2 ± 0.1
$CD3^+CD4^+CD8^+$	0.5 ± 0.4	2.2 ± 1.3	0.8 ± 0.5	1.4 ± 1.4	0.6 ± 0.5	1.0 ± 0.4
$CD3^-CD19^+CD56^-$	10.9 ± 3.5	1.4 ± 1.6	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^+$	13.5 ± 2.5	0.8 ± 0.8	N/A	N/A	N/A	N/A
$CD3^-CD19^+CD56^+$	0.2 ± 0.3	0.1 ± 0.0	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^-$	2.0 ± 1.4	4.2 ± 3.8	N/A	N/A	N/A	N/A
% of $CD3^+$ T cells						
$CD3^+CD4^-CD8^-$	5.9 ± 4.1	2.3 ± 1.4	N/A	N/A	N/A	N/A
$CD3^+CD4^+CD8^-$	43.9 ± 4.1	97.3 ± 1.3	N/A	N/A	N/A	N/A
$CD3^+CD4^-CD8^+$	49.5 ± 4.1	0.1 ± 0.0	N/A	N/A	N/A	N/A
$CD3^+CD4^+CD8^+$	0.7 ± 0.7	0.3 ± 0.2	N/A	N/A	N/A	N/A
% of $CD3^-$ T cells						
$CD3^-CD19^+CD56^-$	42.3 ± 7.8	24.0 ± 25.9	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^+$	51.1 ± 7.3	10.2 ± 8.5	N/A	N/A	N/A	N/A
$CD3^-CD19^+CD56^+$	0.8 ± 1.2	0.8 ± 0.4	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^-$	7.2 ± 4.9	65.0 ± 27.1	N/A	N/A	N/A	N/A

N/A = Not available; [b] = 4 subjects (data from one donor was ruled out due to machine error); [a] = gating of $CD45^+$ lymphocytes was 100% of $CD45^+CD3^+$ T cells.

Table 2
Phenotypes of whole blood, isolated $CD4^+$ T cells, and anti-CD3/28 expanded $CD4^+$ T cells from healthy volunteers expanded in culture bags for large-scale production (mean ± SD, $n = 5$).

Phenotypes	Whole blood	Isolated $CD4^+$ T cells	Anti-CD3/28 expanded $CD4^+$ T cells*	
	Day 0	Day 0	Day 14	Day 21
% of lymphocytes				
$CD3^+CD4^-CD8^-$	4.7 ± 1.5	1.3 ± 0.4	0.3 ± 0.1	0.8 ± 0.3
$CD3^+CD4^+CD8^-$	37.5 ± 7.2	97.5 ± 1.2	99.1 ± 0.3	98.0 ± 0.6
$CD3^+CD4^-CD8^+$	28.7 ± 6.7	0.0 ± 0.0	0.1 ± 0.1	0.8 ± 0.4
$CD3^+CD4^+CD8^+$	0.9 ± 0.3	0.1 ± 0.1	0.5 ± 0.3	0.3 ± 0.5
$CD3^-CD19^+CD56^-$	14.4 ± 5.9	0.2 ± 0.2	N/A	N/A
$CD3^-CD19^-CD56^+$	11.4 ± 3.5	0.2 ± 0.1	N/A	N/A
$CD3^-CD19^+CD56^+$	0.3 ± 0.2	0.0 ± 0.0	N/A	N/A
$CD3^-CD19^-CD56^-$	2.0 ± 1.1	0.7 ± 0.7	N/A	N/A
% of $CD3^+$ T cells				
$CD3^+CD4^-CD8^-$	6.8 ± 2.1	1.3 ± 0.4	N/A	N/A
$CD3^+CD4^+CD8^-$	52.1 ± 9.4	98.6 ± 0.5	N/A	N/A
$CD3^+CD4^-CD8^+$	39.9 ± 8.7	0.0 ± 0.0	N/A	N/A
$CD3^+CD4^+CD8^+$	1.2 ± 0.6	0.1 ± 0.1	N/A	N/A
% of $CD3^-$ T cells				
$CD3^-CD19^+CD56^-$	41.0 ± 11.1	20.6 ± 17.6	N/A	N/A
$CD3^-CD19^-CD56^+$	51.2 ± 10.8	17.0 ± 12.7	N/A	N/A
$CD3^-CD19^+CD56^+$	1.0 ± 0.5	1.3 ± 1.9	N/A	N/A
$CD3^-CD19^-CD56^-$	6.8 ± 2.0	61.1 ± 18.8	N/A	N/A

N/A = Not available; * = gating of $CD45^+$ lymphocytes was 100% of $CD45^+CD3^+$ T cells.

Table 3
Phenotypes of cryopreserved $CD4^+$ T cells, and anti-CD3/28 expanded $CD4^+$ T cells from cryopreserved HIV-infected samples (CD4 counts ≥ 500 cells/μL) expanded in bags with IL-2 supplementation (mean ± SD, $n = 3$).

Phenotypes	Cryopreserved $CD4^+$ T cells	Anti-CD3/28 expanded $CD4^+$ T cells*	
	Day 0	Day 14	Day 21
% of lymphocytes			
$CD3^+CD4^-CD8^-$	2.2 ± 1.1	4.7 ± 2.5	2.5 ± 2.3
$CD3^+CD4^+CD8^-$	93.6 ± 6.6	94.9 ± 2.3	95.9 ± 2.8
$CD3^+CD4^-CD8^+$	0.0 ± 0.0	0.1 ± 0.0	0.5 ± 0.5
$CD3^+CD4^+CD8^+$	0.0 ± 0.0	0.3 ± 0.2	1.1 ± 0.2
$CD3^-CD19^+CD56^-$	0.4 ± 0.5	N/A	N/A
$CD3^-CD19^-CD56^+$	0.5 ± 0.9	N/A	N/A
$CD3^-CD19^+CD56^+$	0.0 ± 0.0	N/A	N/A
$CD3^-CD19^-CD56^-$	3.1 ± 4.3	N/A	N/A

N/A = Not available; * = gating of $CD45^+$ lymphocytes was 100% of $CD45^+CD3^+$ T cells.

after stimulation with PMA/I which is a polyclonal activator. The average percentage of CD69 expressing cells among anti-CD3/28 expanded $CD4^+$ T cells was 95.4 ± 1.4%. Of these activated cells, the average percentage of 76.2 ± 5.0% was cytokine producing cells. Different types of cytokine producing cells including TNF-α, IL-2, IFN-γ, IL-4, IL-17 and TGF-β are shown in Fig. 3A. The major population among total cytokine producing cells was TNF-α producing cells with the average frequency of 81.4 ± 2.5%, followed by IL-2 producing cells (67.3 ± 15.1%). Determination of Th1 cells based on IFN-γ production showed the average percentage of 29.7 ± 11.9%. Low levels of Th2 and Th17 cells were detected based on the production of IL-4 and IL-17 (1.5 ± 0.9% and 1.2 ± 1.2%, respectively). On the contrary, the frequency of Treg cells based on TGF-β production was almost undetectable. When cytokine producing cell populations showing simultaneous production were determined, up to 99% of cytokine producing cells belongs to 3 major populations including single, double and triple cytokine producing cells with the average percentages of 34.3 ± 5.8%, 50.6 ± 3.9% and 14.5 ± 7.4%, respectively (Fig. 3B).

Proportions of specific-cytokine producing cell subsets determined among single, double and triple cytokine producing cells are presented in Fig. 4. For single cytokine producing cells, the majority was TNF-α producing cell with the average percentage of 51.1 ± 12.6%, followed by IL-2 and IFN-γ producing cells (32.8 ± 13.8% and 15.2 ± 4.3%, respectively). With respect of double cytokine producing cells, the highest frequency was observed for $TNF\text{-}\alpha^+$ $IL\text{-}2^+$ producing cells with the average percentage of 77.7 ± 14.7%, followed by the other 2 main populations including $TNF\text{-}\alpha^+$ $IFN\text{-}\gamma^+$ (16.8 ± 14.4%) and $IFN\text{-}\gamma^+$ $IL\text{-}2^+$ (4.1 ± 3.0%). For triple cytokine producing cells, $TNF\text{-}\alpha^+$ $IL\text{-}2^+$ $IFN\text{-}\gamma^+$ producing cells showed the highest frequency with the average percentage of 89.2 ± 7.4%, followed by $TNF\text{-}\alpha^+$ $IL\text{-}2^+$ $IL\text{-}4^+$ (4.6 ± 3.7%), and $TNF\text{-}\alpha^+$ $IL\text{-}2^+$ $IL\text{-}17^+$ (2.4 ± 1.8%).

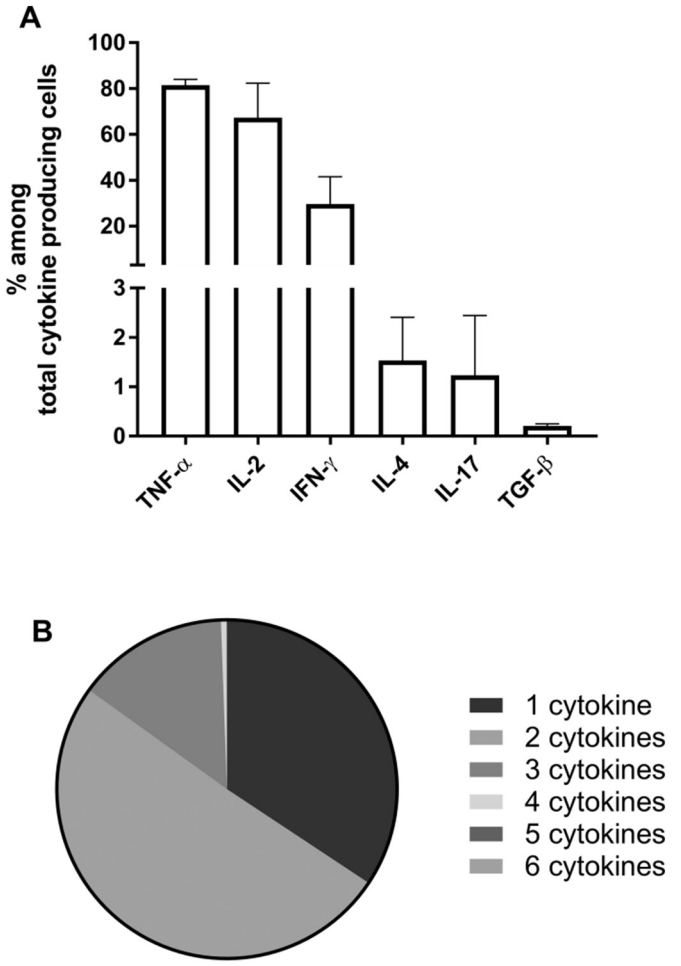

Fig. 3. Cytokine production profiles including TNF-α, IL-2, IFN-γ, IL-4, IL-17, and TGF-β of anti-CD3/28 expanded $CD4^+$ T cells: (A) percentages (mean ± SD) of different cytokine producing cells; (B) average proportions of polyfunctional cytokine producing cells ($n = 3$).

3.5. Residual bead detection and sterility testing

To ensure safety and sterility of the final products due to magnetic beads and microorganism contamination, a small-scale bead removal process was performed together with sterility testing on the final cell culture supernatants (day 21) and harvested expanded $CD4^+$ T cells. Since a large volume of culture medium containing expanded $CD4^+$ T cells was harvested, the sample was concentrated by centrifugation before bead removal. By using a small scale depletion method, the average number of 5.5×10^4 residual beads per 10^7 expanded cell products was measured. Since the average number of 414.9×10^6 cells was harvested on day 21, the average number of 2.3×10^6 residual beads may be obtained in the final products. While the average number of beads before harvesting was 13.6×10^6 beads (average cell number for reactivation on day 7), the result showed 83% depletion of beads. For sterility testing, a 14-day incubation period in specialized media was performed according to USP. The sterile results were presented as the culture supernatant colleting on the final day of cell expansion and expanded cells from cryopreservation were free from any bacterial and fungal contaminations.

4. Discussion

This report introduces a promising closed-culture system for anti-CD3/28 expanded $CD4^+$ T lymphocytes by using GMP-grade culture bags. Immunorosette formation was chosen for $CD4^+$ T cell purification in our study as this method showed the best expansion (1000-fold) with high viability (90%) and high purity of $CD4^+$ T cells (> 95%) after a 3-week culture with anti-CD3/28 activation in flasks when compared to other methods (*i.e.*, negative selection and CD8 depletion) (Onlamoon et al., 2013). Our phenotypic profiles for all freshly isolated $CD4^+$ T cells confirm effectiveness and reproducibility of the immunorosette formation technique with high purity of $CD3^+CD4^+CD8^-$ populations (> 91% of lymphocytes).

Levine et al. (1997) conducted a long-term *in vitro* proliferation of polyclonal adult $CD4^+$ T cells using immobilized anti-CD3/28 stimulation in flasks, showing an exponential growth of $CD4^+$ T cells for over 60 days (Levine et al., 1997). The culture condition with IL-2 supplement provided significant higher expansion than that without IL-2 supplementation (10^9- to 10^{11}-fold and 10^5-fold, respectively). However, Onlamoon et al. (2013) presented that autocrine cytokines were sufficient to support proliferation of anti-CD3/28 expanded $CD4^+$ T cells in absence of IL-2 throughout a 3-week culture (1000-fold expansion) (Onlamoon et al., 2013). Our enriched $CD4^+$ T cells expanded in flasks thus underpin good proliferation of approximately 797-fold after 21 days without IL-2 supplementation.

GMP-grade culture bags are in a concern to reduce contamination risks from microorganisms during a cell production aiming for treatment in clinics. GMP-grade Teflon cell culture (TCC) bags were used to compare a growth ability of normal human $CD8^+$ T cells with conventional tissue culture flasks and plates (Garland et al., 1999). Expanded $CD8^+$ T cells in TCC bags yielded considerably higher than those in flasks and plates after 10 days (73-fold, 41-fold and 48-fold, respectively). Nevertheless, our study shows that anti-CD3/28 expanded $CD4^+$ T cells from healthy donors in flasks proliferated better than those in commercially available GMP-grade culture bags (797-fold and 331-fold, respectively) after 21 days. Even so, fold expansion of

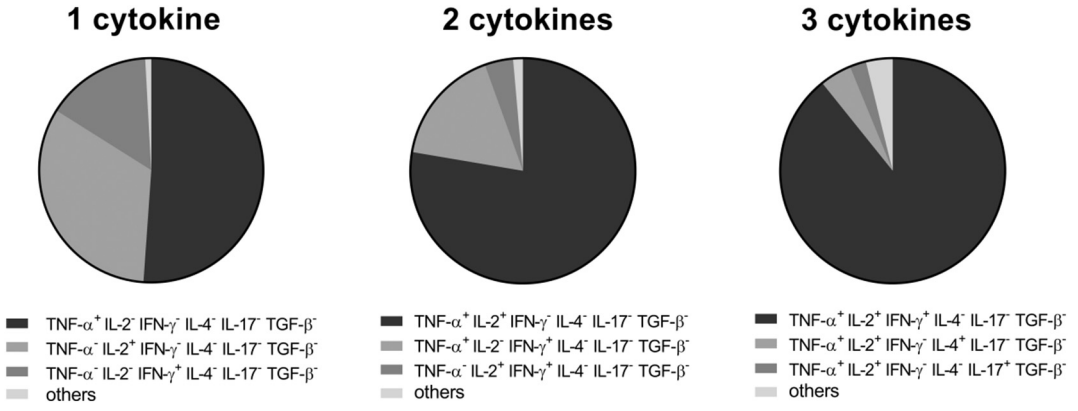

Fig. 4. Average proportions of polyfunctional cytokine producing cell subsets in single, double and triple cytokine producing cells ($n = 3$).

$CD4^+$ T cells from bag culture is still considerably high enough for cell production. High cell viability (75%) and high purity (97%) of expanded $CD4^+$ T cells also support the use of bag for cell expansion.

With respect to implementation of *in vitro* $CD4^+$ T lymphocyte expansion for adoptive immunotherapy in human, there is a clinical study conducting adoptive transfer of up to 3×10^{10} autologous anti-CD3/28 activated $CD4^+$ T cells in HIV-infected patients and the transfusion was scheduled approximately 5×10^9 cells at 8-week intervals (Bernstein et al., 2004; Levine et al., 2002), indicating that large-scale expanded $CD4^+$ T lymphocytes is required. A large-scale production of $CD4^+$ T cells from HIV-1-infected donors using anti-CD3/28 coated beads, IL-2 supplementation, and gas-permeable bags was established and achieved 37-fold expansion after 14 days (Levine et al., 1998) which is not yet satisfied for $CD4^+$ T cell numbers demanded for transfusion. Therefore, our study provides an improved expansion method under the closed-culture system for large-scale anti-CD3/28 expanded $CD4^+$ T cells from healthy volunteers with 109-fold increase after 21 days.

For expansion of anti-CD3/28 activated $CD4^+$ T cells from HIV-infected patients in our developed closed-culture system, $CD4^+$ T cells from patients with CD4 count > 500 cells/μL were selected for the study. Anti-CD3/28 expanded $CD4^+$ T cells from HIV-infected patients with CD4 count > 500 cells/μL were found to have better proliferation ability than patients with CD4 counts 200–500 and < 200 cells/μL (Onlamoon et al., 2015). They also reached 1000-fold expansion when supplemented with IL-2 which was quite similar level as seen in healthy volunteers (Onlamoon et al., 2013). Nevertheless, freshly isolated $CD4^+$ T cells from the patients may be not usually accessible and cryopreserved $CD4^+$ T cells become substitute. Frozen $CD4^+$ T cells from healthy donors were successfully expanded (data not shown) before we carried out the expansion of those from the patients. Our results show that anti-CD3/28 activated $CD4^+$ T cells from cryopreserved cells of HIV-infected patients still remained their growth ability even after a long storage (1.5–2 years). The proliferation was great with 415-fold when IL-2 in presence for growth enhancement, although it was only half of expanded cells from flask culture system of freshly isolated $CD4^+$ T cells from HIV-infected patients reported previously (Onlamoon et al., 2013). This achievement may be resulted from ability of IL-2 for restoration of $CD4^+$ T cell proliferation by either prevention or reversion of T cell unresponsiveness. Although using IL-2 supplementation was considered to tentatively promote apoptosis of expanded cells which rapidly disappeared after transfusion (Tan et al., 1999), our protocol still provides high cell viability (85%) at the beginning and indeed requires further investigation for a relation between anti-CD3/28 stimulation with IL-2 supplementation and induction of apoptotic signaling molecules (*e.g.*, Bcl-2, Bcl-xL and capase-3).

Previous studies showed that expansion of both human and rhesus macaque $CD4^+$ T cells with anti-CD3/28 coated beads provided a large number of homogenous $CD4^+$ T cell population with Th1-like phenotype (Levine et al., 1996; Brice et al., 1998). Moreover, TNF-α production of anti-CD3/28 expanded $CD4^+$ T cells played an important role in regulating synthesis of various β-chemokines (Brice et al., 2000) which support cellular resistance to R5-tropic HIV strain. Our results show that TNF-α producing cells were a major population presenting in the expanded cells which can play a protective role in resistance to infection. The presence of high frequency of IL-2 producing cells can also provide sufficient amount of IL-2 that supports *in vitro* expansion without exogenous IL-2 supplementation. Interestingly, although only one-third of anti-CD3/28 expanded $CD4^+$ T cells showed Th1-like phenotype, other $CD4^+$ T cell types including Th2, Th17 and Treg were almost diminished suggesting that anti-CD3/28 expansion promotes Th1 dominant population of the expanded cells. More importantly, simultaneous detection of cytokine production at a single cell level by polychromatic flow cytometry demonstrates that the majority of expanded cells simultaneously produced 2–3 cytokines (approximately 65%), suggesting a polyfunctional role of these cells.

With respect of safety concern, before releasing the expanded cell product for cell transfusion, anti-CD3/28 coated beads have to be removed prior to preparing the final product due to the potential toxicity hazard associated with the administration of microspheres. Interestingly, the previous study showed the absence of an adverse toxicity in a rat model receiving an intravenous administration of sheep anti-mouse immunoglobulin G coated paramagnetic-polystyrene beads at a high level of bead exposure (White et al., 1995). However, a removal of beads from cell therapy products has become a general practice for the manufacturing of *in vitro* expanded T cell products (Hollyman et al., 2009; Tumaini et al., 2013). In these studies, a large-scale depletion of anti-CD3/28 coated beads was achieved by using the Dynal ClinExVIVO magnetic particle concentrator magnet which consists of a large primary magnet allowing the major removal of beads and a smaller secondary magnet that traps residual beads. The effectiveness of bead depletion was assessed in a final product for the presence of residual beads by lysing 10^7 cells of the final product in chloride bleach. The lysed sample was centrifuged and supernatant was removed. The pellet was then re-suspended in a buffer and placed on a hemacytometer for residual bead counting and calculation of residual bead quantity. While approximately 80% bead depletion was achieved in this study, a higher depletion level may be obtained by using a large-scale depletion method.

More importantly, sterility testing must be performed on cell therapy products in order to ensure that the products are free of bacterial and fungal contamination. Sterility testing can be conducted in a hospital microbiology laboratory where Gram staining and a 14-day culture in culture medium detecting aerobic and anaerobic microorganisms are tested. In this study, all cell culture supernatants and expanded cells of the final products had no contamination of bacteria and fungi when testing with standard USP methods. Alternatively, automated culture methods such as Bact/ALERT (bioMérieux) and Bactec (Becton Dickinson) have already been validated and used for sterility testing of cell therapy products (Khuu et al., 2006; Hocquet et al., 2014). The routine evaluation for sterility testing of cell therapy products also showed the effectiveness in decreasing rate of contamination of cell harvest, suggesting the significances of routine sterility testing (Golay et al., 2018).

5. Conclusions

Results from this study address that our developed closed-culture system for $CD4^+$ T lymphocyte expansion using anti-CD3/28 coated beads and GMP-grade gas-permeable culture bags is practical, effective, and reproducible. Using our protocol, a large number of anti-CD3/28 expanded $CD4^+$ T lymphocytes is achieved together with high viability and high purity within reasonable time (21 days). This method is also successful to expand both freshly isolated $CD4^+$ T cells from healthy volunteers and cryopreserved $CD4^+$ T cells from HIV-infected patients. It is then suggested that our refined procedure is useful for cell production used for adoptive transfer immunotherapy, although, there are still many aspects require further improved and investigated, such as effects of different culture media, impact of exogenous supplementation, and cell manufacturing processes under GMP standard.

Conflicts of interest

None.

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Biological Procedures Online

Comparison of serum supplementation and culture medium for CD4+ T cell expansion by using anti-CD3/28 coated beads

--Manuscript Draft--

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Abstract:	Background: Although several in vitro expansion methods for CD4+ T cells have been established for adoptive immunotherapy, the information about effects of commercially available GMP-grade media with serum supplementation in cell expansion and functions is limited. Results: Isolated CD4+ T cells from healthy donors were stimulated with anti-CD3/28 coated beads and expanded in different media supplemented with either human serum (HS) or fetal bovine serum (FBS) for 3 weeks. Fold expansion, cell viability, growth kinetics and phenotypic characters were periodically observed throughout the culture period. Cytokine production of IL-2, IL-4, IL-17, IFN-γ, TNF-α and TGF-β of the expanded cells was detected by intracellular cytokine staining. Results showed that the expanded cells from the cultures using AIM-V and DC media with 10% HS were well expanded (~255-fold) and had similar polyfunctional patterns, however, they showed poorer proliferation rates than those from the cultures using RMPI1640 with 10% FBS (R10; ~637-fold). Moreover, cell viability of the expanded cells from R10 was also significantly higher than the others after a 3-week culture. High purity with CD3+CD4+CD8- phenotype of the expanded cells from all cultures was also observed. The expanded cells from all conditions were found predominantly producing TNF-α, IL-2 and IFN-γ and frequency of IL-2 producing cells in R10 was the highest. Conclusions: Our expansion protocol using GMP-grade media, AIM-V and DC with HS supplementation rendered satisfied proliferation rates and yields without differences in polyfunctional patterns, indicating the promising method for further uses in clinical trials. Addition of FBS is also suggested for expansion improvement but under the condition that its source must be free from bovine spongiform encephalopathy.
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Additional Information:	
Question	**Response**
Is this study a clinical trial?<hr>A clinical trial is defined by the Word Health Organisation as 'any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes'.	No

Comparison of serum supplementation and culture medium for CD4$^+$ T cell expansion by using anti-CD3/28 coated beads

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ABSTRACT

Background: Although several *in vitro* expansion methods for $CD4^+$ T cells have been established for adoptive immunotherapy, the information about effects of commercially available GMP-grade media with serum supplementation in cell expansion and functions is limited.

Results: Isolated $CD4^+$ T cells from healthy donors were stimulated with anti-CD3/28 coated beads and expanded in different media supplemented with either human serum (HS) or fetal bovine serum (FBS) for 3 weeks. Fold expansion, cell viability, growth kinetics and phenotypic characters were periodically observed throughout the culture period. Cytokine production of IL-2, IL-4, IL-17, IFN-γ, TNF-α and TGF-β of the expanded cells was detected by intracellular cytokine staining. Results showed that the expanded cells from the cultures using AIM-V and DC media with 10% HS were well expanded (~255-fold) and had similar polyfunctional patterns, however, they showed poorer proliferation rates than those from the cultures using RMPI1640 with 10% FBS (R10; ~637-fold). Moreover, cell viability of the expanded cells from R10 was also significantly higher than the others after a 3-week culture. High purity with $CD3^+CD4^+CD8^-$ phenotype of the expanded cells from all cultures was also observed. The expanded cells from all conditions were found predominantly producing TNF-α, IL-2 and IFN-γ and frequency of IL-2 producing cells in R10 was the highest.

Conclusions: Our expansion protocol using GMP-grade media, AIM-V and DC with HS supplementation rendered satisfied proliferation rates and yields without differences in polyfunctional patterns, indicating the promising method for further uses in clinical trials. Addition of FBS is also suggested for expansion improvement but under the condition that its source must be free from bovine spongiform encephalopathy.

Keywords: $CD4^+$ T lymphocytes; anti-CD3/28 coated beads; *in vitro* cell expansion; culture medium; serum supplementation.

BACKGROUND

Anti-CD3/28 co-stimulation is an effective method to induce *in vitro* expansion of $CD4^+$ T cells. A major advantage of this method is that anti-CD3/28 expanded $CD4^+$ T cells became resistant to the infection of M-tropic isolates of human immunodeficiency virus (HIV)-1 [1]. Some experiments in non-human primates showed that expanded autologous $CD4^+$ T cells from pre- and post- simian immunodeficiency virus (SIV) infection promoted the immune responses for viral suppression and plasma viremia control by using in combination with a transient initial antiretroviral treatment in SIV-infected models [2,3]. An adoptive transfer of the expanded autologous $CD4^+$ T cells also exhibited an increased level of $CD4^+$ T cells in antiretroviral-treated HIV-infected patients [4]. Therefore, the optimized protocol of a large-scale $CD4^+$ T cell expansion is warranted for clinical uses. This expanded $CD4^+$ T cell reinfusion can be beneficial for improvement in immune restoration and become an alternative treatment for HIV-infected patients with immunological discordance.

Several studies demonstrated many parameters affecting T cell proliferation, such as cell isolation, disease progression, and culture media. Onlamoon *et al.* (2013) determined the effect of cell isolation on cell expansion rates and cell phenotypes. Of three different $CD4^+$ T cell isolation methods including immunorosette formation, negative selection and CD8 depletion, enriched $CD4^+$ T lymphocytes from normal whole blood by immunorosette formation exhibited the highest yield of over 1,000-fold expansion with good viability (90%) and high purity of $CD4^+$ T lymphocytes (> 95%) within three weeks of culture. $CD4^+$ T lymphocyte enrichment through immunorosette formation thus provided the optimal isolation method for a large-scale expansion of anti-CD3/28 expanded $CD4^+$ T lymphocytes [5].

Following the same protocol, the expansion of $CD4^+$ T cells from the HIV-infected patients was conducted and found different growth kinetics among the patients with CD4 counts < 200, 200-500, and > 500 cells/μL [6]. Isolated $CD4^+$ T cells from the patients still retained proliferation ability and their yields depended on disease progression. Even so, their fold expansion numbers were still lower than those of healthy volunteers. IL-2 supplementation at the concentration of 100 U/mL was also found to significantly help increasing fold expansion of $CD4^+$ T cells from HIV-infected patients with both low and high CD4 counts (< 200 and > 500 cells/μL, respectively). However, $CD4^+$ T cells isolated from the patients with more progressive disease (CD4 count < 200 cell/μL) showed lower rates in cell survival and

proliferative responses to anti-CD3/CD28 co-stimulation with IL-2 supplementation when compared to those from HIV-infected patients with high CD4 counts [6].

For media supplementation, basal culture media are generally supplemented with animal serum (e.g., fetal bovine serum (FBS)) which is essential for cell growth, metabolism and proliferation. The functions of serum in culture media are (i) to provide hormonal factors stimulating cell growth, proliferation and differentiation, (ii) to transport proteins carrying hormones, minerals, trace elements and lipids, (iii) to attach and spread factors, acting as germination points for cell attachment and (iv) to stabilize and detoxify factors needed for maintaining pH as well as to inhibit proteases and other toxic molecules. Nevertheless, the use of animal serum, particularly FBS, has been debated in some serious scientific and ethical concerns in terms of serum production and cell harvesting. Development of serum-free or animal/human protein-free media is then important for safety improvement in biological products for cell therapy and vaccination [7].

FBS can be substituted with defined chemical components in serum-free cell culture. Trickett *et al.* (2002) demonstrated that the expansion of functional T lymphocytes from HIV-infected patients was good when stimulated with anti-CD3/28 coated microspheres and propagated in serum-free media. However, the greater T cell proliferation was observed when supplemented with FBS in the initial period of cell expansion, whereas, human albumin (AB) serum supplementation failed to increase T cell numbers. Plasma supplementation also provided a low level of $CD4^+$ T cells which was resulted from phenotypic switching of $CD8^+$ T lymphocytes [8]. Carlens *et al.* (2000) studied expansion rates of anti-CD3 stimulated T lymphocytes in three different serum-free media (i.e., X-VIVO 15, AIM-V and Cellgro SCGM) compared to standard RPMI1640 media with 5% human serum (HS) and 10% FBS. A 3-week activation in serum-free media resulted in a small increase in expansion rates, whereas the culture with serum supplementation rendered better consistency and effectiveness in cell expansion. The additional low level of HS thus supported the T-cell expansion in all culture media types. On the other hands, supplementation with 10% FBS showed inconsistency in cell expansion when compared to that with 5% HS. Increased levels of IFN-γ secretion were detected for all media combinations when compared with serum-free culture. No IL-4 and IL-10 production was found [9].

Although the *in vitro* cultures with HS supplementation supported better $CD4^+$ T cell expansion than those with serum-free media and more consistency than those with FBS

supplementation, the fold expansion numbers (< 267-fold) [9] was not high when compared to our previous method [5]. While GMP-grade culture media are required for cell production, the information about the effect of serum supplementation in cell functions is limited. Our study, therefore, aimed to assess the expansion rates and yields of expanded $CD4^+$ T cells by using commercially available GMP-grade culture media with HS supplementation in comparison with the established protocol. Their cytokine production was also investigated to observe the potential diversity of cell functions.

RESULTS

Expanded $CD4^+$ T lymphocytes in different culture conditions after anti-CD3/28 coated bead stimulation

To assess an optimal cell culture condition for *in vitro* expansion of $CD4^+$ T lymphocytes by using anti-CD3/28 coated beads, different culture conditions based on serum supplementation and culture media usages were compared. Fold expansion, cell viability, growth kinetic and phenotypic characters from different culture conditions were observed for cell expansion efficiency.

Fold expansion numbers of $CD4^+$ T lymphocytes expanded in all culture conditions were similar during the first 2-week period. After that, the expanded cells cultured in RPMI1640 with 10% FBS (R10) were dramatically increased on day 17 (755.7 ± 337.1-fold), whereas the other cultures maintained similar expansion rates (Fig. 1A). At the end of the culture period (day 21), slight decreases were found in every culture media. The culture in R10 showed the highest fold expansion (637.1 ± 265.3 –fold) which was approximately 2.5-fold higher than the other culture conditions with 10% HS (< 255-fold). No significant difference was observed among 10% HS supplemented media including GMP-grade media (i.e., AIM-V and DC) and standard media (i.e., RPMI1640).

Gradual declines in viable cells were observed throughout the 21-day culture period (Fig. 1B). All culture conditions exhibited a similar pattern in cell viability at over 70% during the first two weeks. Only the culture in R10 still maintained high numbers of viable cells on day 17 (75.9 ± 11.0%) before markedly decreasing on day 21 (54.8 ± 5.8%). On the other hand, the other culture media including RPMI1640, AIM-V and DC supplemented with 10% HS showed notable decreases since day 17 (47.1 ± 7.7%, 56.0 ± 6.4% and 52.6 ± 5.5%, respectively) and lowered to less than 36% on day 21.

Immunophenotypes of whole blood, isolated $CD4^+$ T lymphocytes, and anti-CD3/28 expanded $CD4^+$ T lymphocytes were determined by a flow cytometer (Table 1). Phenotypic profiles revealed that purification of $CD4^+$ T cells from whole blood by using the immunorosette formation technique provided high frequency of $CD3^+CD4^+CD8^-$ subset (> 95% of lymphocytes). After a 3-week culture, anti-CD3/28 expanded $CD4^+$ T cells ($CD3^+CD4^+CD8^-$) from R10 and DC with 10% HS exhibited high frequencies (> 90% of lymphocytes), whereas slightly lower frequencies were observed for RPMI1640 and AIM-V with 10% HS. The cell purity was also confirmed with low frequencies of $CD3^+CD4^-CD8^+$ and $CD3^+CD4^+CD8^+$ populations at the end of the expansion period.

Table 1 Lymphocyte subset analyses of whole blood, isolated $CD4^+$ T cells, and anti-CD3/28 expanded $CD4^+$ T cells (mean ± SD, n = 5).

Phenotypes	**Whole blood**	**Isolated $CD4^+$ T cells**	**Anti-CD3/28 expanded $CD4^+$ T cells**			
			10% FBS	**10% HS**		
			RPMI1640*	**RPMI1640**	**AIM-V**	**DC**
% of lymphocytes						
$CD3^+CD4^-CD8^-$	6.1 ± 1.9	1.6 ± 0.6	5.7 ± 7.9	7.4 ± 9.3	10.0 ± 6.4	3.5 ± 2.1
$CD3^+CD4^+CD8^-$	34.7 ± 6.2	96.0 ± 2.6	91.9 ± 8.1	89.0 ± 11.3	84.8 ± 7.5	94.1 ± 3.9
$CD3^+CD4^-CD8^+$	30.3 ± 7.2	0.0 ± 0.0	1.0 ± 0.8	2.5 ± 2.2	3.6 ± 1.8	1.2 ± 1.2
$CD3^+CD4^+CD8^+$	0.5 ± 0.3	0.1 ± 0.1	1.4 ± 1.0	0.9 ± 0.5	1.5 ± 1.3	1.0 ± 0.9
$CD3^-CD19^+CD56^-$	11.2 ± 5.1	0.5 ± 0.8	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^+$	15.8 ± 7.4	0.6 ± 0.7	N/A	N/A	N/A	N/A
$CD3^-CD19^+CD56^+$	0.3 ± 0.3	0.0 ± 0.1	N/A	N/A	N/A	N/A
$CD3^-CD19^-CD56^-$	1.1 ± 0.2	1.0 ± 1.2	N/A	N/A	N/A	N/A

N/A = Not available as gated population of $CD45^+$ lymphocytes was 100% of $CD45^+CD3^+$ T cells.
*RPMI1640 with 10% FBS is R10

Determination of cytokines produced by anti-CD3/28 expanded $CD4^+$ T cells

Although a homogenous population of expanded $CD4^+$ T lymphocytes was observed in all culture conditions with different degree of expansion ability, it remains unclear whether there are any functional changes induced by different culture conditions. Anti-CD3/28 expanded $CD4^+$ T cells from various culture conditions were then investigated in this study for their functional variances based on types of cytokines produced by these cells.

To determine the levels of activated cells among anti-CD3/28 expanded $CD4^+$ T cells after stimulation with PMA/I, the CD69 expression levels were assessed. There was no significant difference in frequencies of CD69 expressing cells among different culture conditions (Fig. 2A). These CD69 expressing cell populations were also consequently determined for cytokine producing cells in order to ensure the presence of cytokine production in the activated cells. While total cytokine producing cells (i.e., a combination of all types of cytokine producing cells) from all culture conditions were not significantly different, the expanded cells from R10 exhibited higher percentages of total cytokine producing cells than the others (Fig. 2B).

When different cytokine-producing cell populations including TNF-α, IL-2, IFN-γ, IL-4, IL-17 and TGF-β were determined, the major population among total cytokine-producing cells was TNF-α producing cells (Fig. 3A). No difference in all types of cytokine-producing cells was observed among different culture conditions, except IL-2 producing cells with a significant high percentage (58.0 ± 18.6 % of $CD4^+CD69^+$ T cells) from the R10 culture. Ability in simultaneous production of cytokines was also determined. Results demonstrated that up to 99% of cytokine-producing cells from all culture conditions belong to 3 main populations including single, double and triple cytokine-producing cells in which a single cytokine-producing cell population showed the highest percentages of over 50% of total cytokine-producing cells (Fig. 3B).

The 3 main populations of single, double and triple cytokine-producing cells were then evaluated for specific-cytokine producing cell subsets. IL-2 producing cells were predominantly found in the single cytokine-producing cells from all culture conditions. For the double cytokine-producing cells, TNF-α^+ IL-2^+ producing cells showed the highest frequency for all culture conditions, except the DC culture presenting the highest proportion of IFN-γ^+ IL-2^+ producing cells instead. The triple cytokine-producing cells in all media supplemented with 10% HS exhibited TNF-α^+ IL-2^+ IFN-γ^+ producing cells as a majority, whereas TNF-α^+ IL-2^+ IL-4^+ producing cells were found predominant in the R10 culture (data not shown).

DISCUSSION

Over the last decade, manufacturing processes for T-cell therapy have been extensively refined to improve the quality of effector cells and to increase the speed of production [4,10]. Adoptive transfer of anti-CD3/28 expanded $CD4^+$ T lymphocytes was already achieved in HIV-infected patients and SIV-infected nonhuman primates for effectiveness in their immune responses [2-4,11], suggesting a promising treatment strategy for HIV-infected patients. According to our previous studies, the isolation and *ex vivo* expansion protocols of $CD4^+$ T cells from healthy and HIV-infected individuals by using anti-CD3/28 coated bead stimulation were optimized [5,6]. Even so, the platform of clinical cell transfusion for patient safety is still demanded for further investigation and was thus proposed in this study.

To optimize the protocol for clinical adoptive T-cell therapy, other serum supplements besides FBS were considered in this study. HS was chosen for $CD4^+$ T cell expansion development as it is commercially available and can be obtained as human AB serum. The culture media including RPMI1640, AIM-V and DC were then added with 10% HS for expansion comparison with RPMI1640 supplemented with 10% FBS. Culture media with 10% HS supplementation supported $CD4^+$ T cell expansion similar to normal culture medium (i.e., culture medium with 10% FBS supplementation or R10 in our study) in the first 2 weeks of culture. The growth rates of expanded cells from all culture media were 224-330 -fold and cell viability was approximately 70% after two weeks of cell activation and expansion. Fold expansions of all media, except R10, maintained at the same levels on day 17 before gradual declines on day 21. Unlike the others, fold expansion of R10 was considerably augmented on day 17 and then decreased on day 21. However, the expanded cells from the R10 culture on day 21 still proliferated ~2–fold higher than those on day 14. $CD4^+$ T cell expansion in R10 provided the highest growth rate and the most consistency when compared to that in the others. Of all cultures using specialized media, RPMI1640 supplemented with 10% HS rendered the lowest value of fold expansion after a 3-week culture. It is then assumed that FBS supplementation render the higher expansion rates, and yields as well as better consistency than HS supplementation. Our findings are opposed to the previous study demonstrating that 10% FBS provided inconsistency in cell expansion when compared to HS supplementation [9]. Therefore, FBS supplementation can be a choice of interest for clinical uses to achieve the best expansion as long as its source is from an area free of bovine spongiform encephalopathy (BSE), such as Australia or New Zealand [12].

Furthermore, AIM-V and DC with 10% HS supplementation were able to provide suitable $CD4^+$ T cell expansion with average 250-fold which is similar to the results achieved from AIM-V and DC with 5% HS supplementation from the previous study [9]. It is suggested that 5% HS supplementation is sufficient to support $CD4^+$ T cell expansion and higher concentration of HS does not improve the proliferation rates and yields. Both AIM-V and DC are also good candidates as GMP-grade media used for cell expansion following requirement of clinical trials.

Besides FBS and HS supplementation, a xeno-free serum replacement (SR) was introduced as a novel platform strategy for clinical-grade cell manufacturing [13]. In this study, T-cell expansion was conducted by using a serum-free medium AIM-V supplemented with 5% SR and anti-CD3/28 coated bead stimulation. The expansion kinetic of T cells using SR was comparable and polyfunctional profiles were similar to that using FBS and HS [13]. SR supplementation, therefore, has become an alternative choice for FBS and HS substitution and for clinical-grade cell production since a potential risk of cross-species contamination can be avoided. Addition of exogenous cytokines, particularly IL-2, is worth considerable for improvement in cell expansion. Previous studies showed that growth rates of the expanded T cells cultured in media with HS and IL-2 supplementation were increased when compared to those without IL-2 supplementation [9,13]. In this case, addition of HS and IL-2 together can synergistically promote the cell expansion.

Regarding to functions of the expanded $CD4^+$ T cells, previous studies revealed that $CD4^+$ T cell population with type 1 T helper cell (Th1)-like phenotype was expanded when *in vitro* expansion of both human and rhesus macaque $CD4^+$ T cells was performed by using anti-CD3/28 coated beads [14,15]. We found that anti-CD3/28 expanded $CD4^+$ T cells produced 3 major cytokines including TNF-α, IL-2 and IFN-γ. Interestingly, a frequency of IL-2 producing cells in the culture condition using FBS was the highest which was correlated to its superior fold expansion when compared to the other conditions. This can presume that a higher level of IL-2 released from these cells under FBS supplementation may self-support such high *in vitro* expansion. A simultaneous detection of cytokine production at a single cell level also confirmed the presence of IL-2 producing cells in the largest proportion when compared to other cytokines, highlighting its significant role in promoting cell expansion.

CONCLUSIONS

This study addresses the expansion and polyfunctional profiles of expanded CD4+ T cells when cultured in commercially available GMP-grade media with human serum supplementation. We found that AIM-V and DC are good candidates for clinical-grade cell manufacturing and HS supplementation can well support cell expansion. Although animal-derived substances, mainly FBS, is not recommended to use in cell products due to their risk in cross-species contamination, FBS supplementation is still able to render the highest fold expansion with high viability and consistency when compared to HS. There is also no significant difference in polyfunctional profiles between FBS and HS. To use FBS for cell production, its origin must be free of BSE. Therefore, our expansion protocol using either AIM-V or DC supplemented with HS provides satisfied yields of the expanded cells of which polyfunctions are similar, suggesting the suitable protocol for further uses in clinical studies.

MATERIALS AND METHODS

Samples

Five healthy volunteers were recruited and signed informed consents which were approved by the Institutional Review Board of the Faculty of Medicine Siriraj Hospital at Mahidol University. Sixteen to twenty-five milliliters of whole blood were collected into sodium heparin-containing vacutainer tubes and kept at room temperature prior to phenotypic determination of whole blood and $CD4^+$ T lymphocyte isolation.

Antibodies

Monoclonal antibodies (mAbs) and their conjugated fluorochromes including anti-CD3 conjugated with fluorescein isothiocyanate (FITC), anti-CD4 conjugated with allophycocyanin (APC), anti-CD8 conjugated with phycoerythrin (PE), anti-CD19 conjugated with allophycocyanin and cyanine dye (APC-Cy7), anti-CD45 conjugated with peridinin chlorophyll protein (PerCP), and anti-CD56 conjugated with phycoerythrin and cyanine dye (PE-Cy7) were purchased from Becton Dickinson Bioscience (BDB, San Jose, CA). In addition, anti-CD3 conjugated with AlexaFluor® (A700), anti-CD4 conjugated with Brilliant Violet™ 605 (BV605), anti-CD8 conjugated with PE/Dazzle™ 594, anti-CD69 conjugated with PerCP/Cy5.5, anti-IL-2 conjugated with BV510, anti-IL-4 conjugated with

FITC, anti-IL-17 conjugated with PE, anti-IFN-γ conjugated with APC, anti-TNF-α conjugated with BV650 and anti-TGF-β conjugated with BV421 were obtained from BioLegend (San Diego, CA).

$CD4^+$ T lymphocyte isolation

$CD4^+$ T lymphocytes were freshly isolated from whole blood through immunorosette formation by using RosetteSep® Human $CD4^+$ T cell enrichment cocktail (STEMCELL Technologies, Vancouver, BC, Canada) following the manufacturer's instruction. Purified $CD4^+$ T lymphocytes were isolated by a standard Ficoll-Hypaque gradient centrifugation (Histopaque, Sigma-Aldrich,Co., St.Louis, MO, USA) prior to phenotypic characterization and cell expansion.

Cell stimulation and expansion of purified $CD4^+$ T cells

Freshly purified $CD4^+$ T cells ($1x10^6$ cells) were stimulated with anti-CD3/28 coated beads (Dynabeads® Human T-Activator CD3/CD28, Invitrogen Dynal, Oslo, Norway) at a bead-to-cell ratio of 1:1 in a 24-well plate (Costar® 24 well clear TC-treated multiple well plates, sterile, Corning Inc., Life Sciences, NY). The stimulated $CD4^+$ T cells were then expanded in complete media composing RPMI1640 supplemented with 10% heat inactivated FBS, 50 µg/mL pencillin-streptomycin, and 2 mM L-glutamine (Gibco, Paisley, UK) at a concentration of $0.5x10^6$ cells/mL. The expanded cells were incubated at 37 °C and 5% CO_2 humidification before reactivation on day 7 at a similar bead-to-cell ratio. Cell numbers and viability were observed by using trypan blue exclusion and a TC10™ automated cell counter on days 4, 7, 11, 14, 17 and the calculated amounts of fresh media were replenished to maintain the cell suspension concentration at $0.5x10^6$ cells/mL. The cell suspensions were also transferred to T25, T75 and T175 plastic tissue culture flasks (Corning® U-shaped canted neck cell culture flask with vent cap, Corning Inc., Life Sciences, NY) on days 4, 7, 11 and maintained in T175 until the end of a 3-week culture period. Final cell numbers and viability were determined on day 21. The beads were removed and the expanded cells were re-suspended in a cryopreservation media and stored in a liquid nitrogen tank at -196 °C for further analyses.

For comparison between culture medium and serum supplementation, $1x10^6$ cells of purified $CD4^+$ T cells were expanded in 3 different culture conditions including (i) RPMI1640 supplemented with 10% heat inactivated HS (Gibco, Paisley, UK), (ii) AIM-V® medium

CTS™ (Therapeutic grade, Gibco, Paisley, UK) supplemented with 10% heat inactivated HS and (iii) CellGro® GMP serum-free dendritic cell medium (CellGro® DC medium, CellGenix GMBH, Freiburg, Germany) supplemented with 10% heat inactivated HS. Cell expansions were conducted by using the same protocol described above.

Immunofluorescence staining and analysis

For phenotypic characterization, samples of whole blood, purified and expanded $CD4^+$ T cells were stained with fluorochrome-conjugated mAbs for 15 min before adding FACS lysing solution (BDB, San Jose, CA) for cell lysis and fixation. The stained cells of at least 100,000 events were acquired for each analysis by a BD FACSVerse™ flow cytometer (BDB, San Jose, CA) and the data was analyzed by using FlowJo Software (Tree Star, San Carlos, CA). Percentages of lymphocyte subsets were determined by expressions of CD3, CD4, CD8, CD19 and CD56.

Intracellular cytokine staining (ICS) and analysis

Cryopreserved expanded $CD4^+$ T cells from different culture conditions were used to determine different types of cytokine production. Expanded $CD4^+$ T cells at $1x10^6$ cells/mL were stimulated with 25 ng phorbol 12-myristate 13-acetate (PMA) and 1 µg ionomycin (I) in the presence of brefeldin A (BFA) at 10 µg, whereas the expanded $CD4^+$ T cells in the presence of BFA without stimulation were used as a control. The samples were then incubated at 37 °C and 5% CO_2 for 4 h. After the incubation, PMA/I stimulated and unstimulated samples were stained with Zombie NIR™ dye (BioLegend, San Diego, CA) at 4 °C for 15 min. A washing buffer (phosphate buffered saline (PBS) with 2% FBS) were added and the samples were washed by centrifugation at 450g for 5 min. The samples were then surface stained with a combination of mAbs including anti-CD3 A700, anti-CD4 BV605, anti-CD8 PE/Dazzle™ 594 and anti-CD69 PerCP/Cy5.5 at 4 °C for 15 min and washed once. The stained samples were fixed and permeabilized in 0.5 mL of BD Cytofix/Cytoperm™ fixation and permeabilization solution (BDB, San Jose, CA) at 4 °C for 20 min. After the incubation, the samples were washed by adding 1X BD Perm/Wash™ buffer (BDB, San Jose, CA) and centrifuged at 500g for 5 min. ICS was performed by staining with a combination of mAbs including anti-IL-2 BV510, anti-IL-4 FITC, anti-IL-17 PE, anti-IFN-γ APC, anti-TNF-α BV650, anti-TGF-β BV421 at 4 °C for 30 min. After staining, the samples were washed with 1X BD Perm/Wash™ buffer (BDB, San Jose, CA) and re-suspended in PBS.

The stained cells of at least 100,000 events were acquired for each analysis by a BD Fortessa™ flow cytometer (BDB, San Jose, CA) and the data was analyzed by using FlowJo Software (Tree Star, San Carlos, CA). Cytokine producing cell subsets were determined from activated populations expressing CD69 and percentages of cytokine producing cell subsets were determined from a total cytokine producing cell population. A Boolean gating strategy was used for the analysis of polycytokine producing cell subsets in order to evaluate cytokine producing cell subsets with ability to simultaneously produce 1, 2, 3, 4, 5 or 6 cytokines.

Data analysis

Fold expansion numbers were calculated by using the viable cell numbers at each indicated time point divided by viable cell numbers at the beginning of cell expansion. All statistical analyses were performed using GraphPad Prism® version 7.02 (GraphPad Software Inc., CA, USA). Datasets were expressed as mean ± standard deviation (S.D.) and compared for statistical significance at p-value ≤ 0.05 with 2-way ANOVA followed by Bonferroni's multiple comparisons test. Proportions of cytokine producing cell subsets were presented as pie-charts.

LIST OF ABBREVIATIONS

A700	AlexaFluor®
AB	Albumin
APC	Allophycocyanin
APC-Cy7	Allophycocyanin and cyanine dye
BFA	Brefeldin A
BSE	Bovine spongiform encephalopathy
BV605	Brilliant Violet™ 605
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
HIV	Human immunodeficiency virus
HS	Human serum
I	Ionomycin
IFN	Interferon

IL	Interleukin
mAbs	Monoclonal antibodies
OKT3	Muromonab-CD3
PBS	Phosphate buffered saline
PE	Phycoerythrin
PE-Cy7	Phycoerythrin and cyanine dye
PerCP	Peridinin chlorophyll protein
PMA	Phorbol 12-myristate 13-acetate
RPMI	Roswell Park Memorial Institute
SIV	Simian immunodeficiency virus
SR	Serum replacement
TGF	Transforming growth factor
Th1	Type 1 T helper T cells
TNF	Tumor necrosis factor

DECLARATIONS

Ethical approval and consent to participate:

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable standards. The study was approved by the Institutional Review Board of the Faculty of Medicine Siriraj Hospital at Mahidol University. Informed consent was obtained from all individual participants recruited in the study.

Consent for publication:

Written informed consent was obtained from the patients and participants for publication of their individual details and accompanying images in this manuscript. The consent form is held by the authors and is available for review by the Editor-in-Chief.

Availability of data and material:

All data generated or analyzed during this study are included in this published article.

Competing interests:

The authors declare that they have no conflict of interest.

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Authors' contributions:

PT: performed experiments, data analysis and manuscript writing; VT, PP and PA: performed experiments and data collection; NO: research idea formation, research monitoring, and manuscript editing. All authors read and approved the final manuscript.

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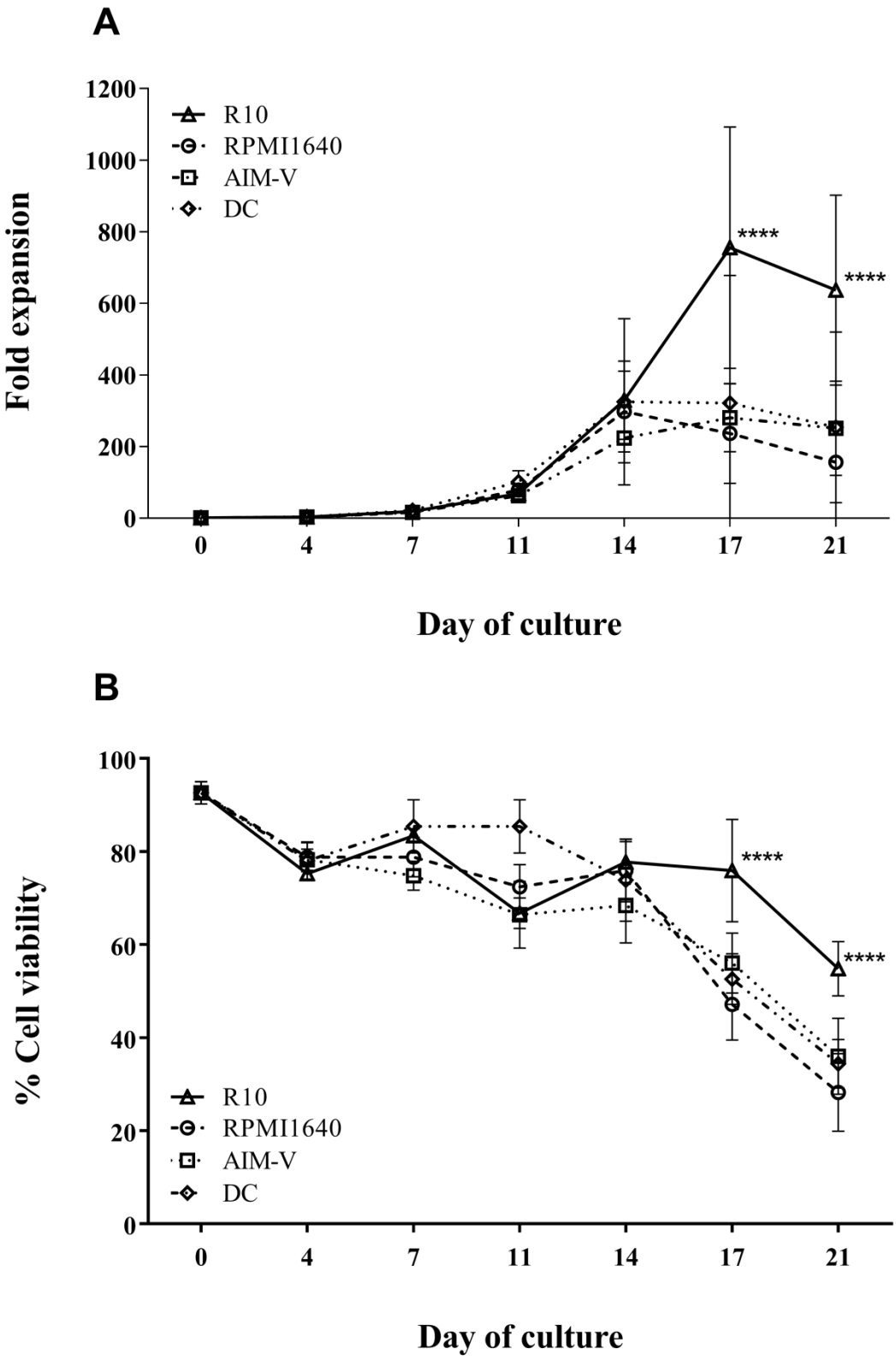

Fig. 1 Growth kinetics of anti-CD3/28 expanded $CD4^+$ T cells from different culture conditions. (A) Fold expansion and (B) cell viability of the expanded cells were observed from different cultures in R10 (RPMI1640 with 10% FBS) and media containing 10% human serum including RPMI1640, AIM-V, and DC. All data are presented as mean ± SD (n = 5, ****p-value < 0.0001).

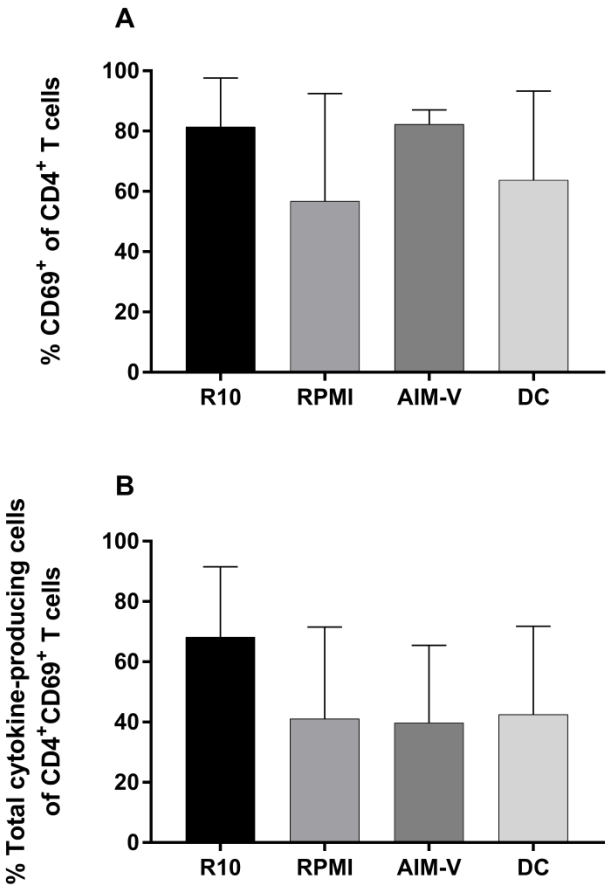

Fig. 2 Activation and cytokine production of anti-CD3/28 expanded $CD4^+$ T cells from different culture conditions. (A) Percentages of CD69 expressing cells and (B) percentages of cytokine producing cells were observed from different cultures in R10 (RPMI1640 with 10% FBS) and media containing 10% human serum including RPMI1640, AIM-V, and DC. All data are presented as mean ± SD (n = 3).

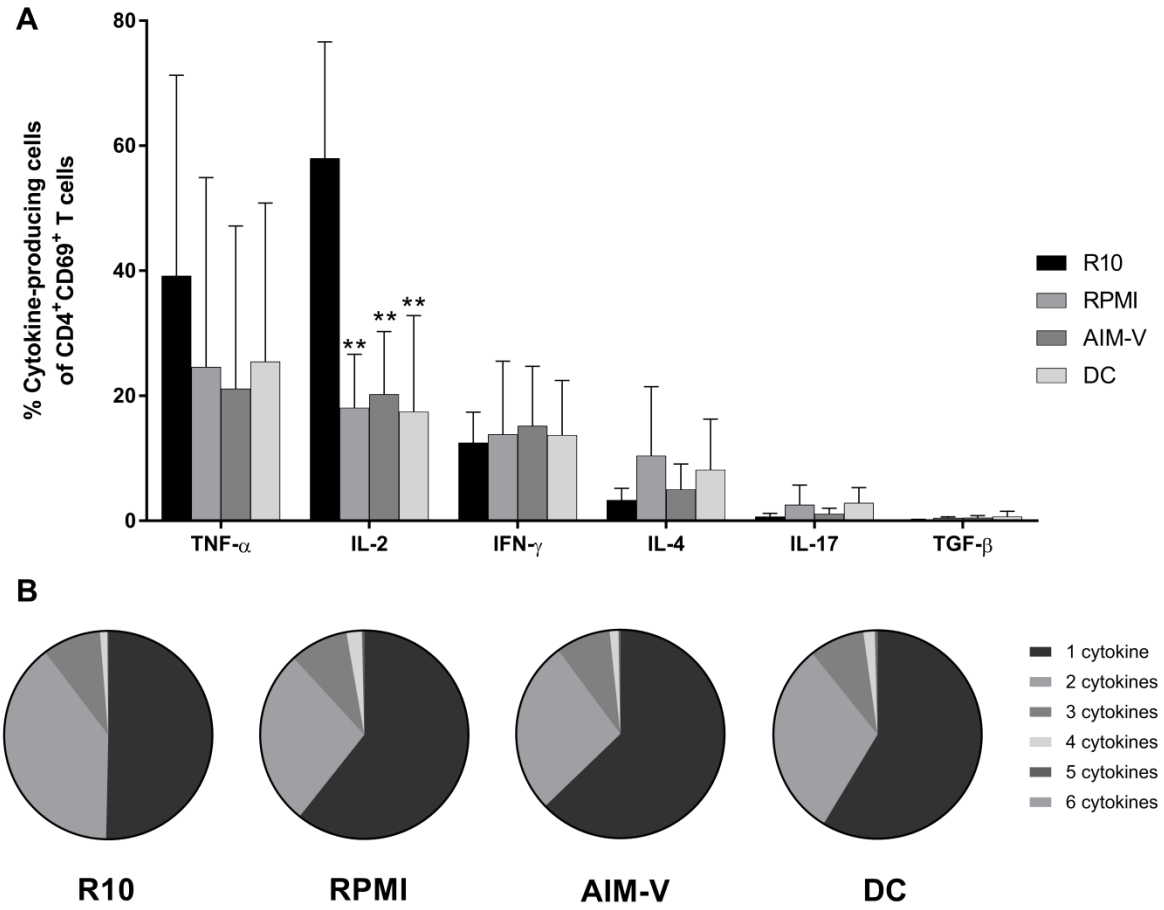

Fig. 3 Cytokine production profiles including TNF-α, IL-2, IFN-γ, IL-4, IL-17, and TGF-β of anti-CD3/28 expanded $CD4^+$ T cells. (A) Percentages (mean ± SD) of different cytokine producing cells and (B) average proportions of polyfunctional cytokine producing cells were observed from different cultures in R10 (RPMI1640 with 10% FBS) and media containing 10% human serum including RPMI1640, AIM-V, and DC. (n = 3, ****p-value < 0.0001).

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Abstract

CD4+ T cell immunotherapy has potential for treatment in HIV-infected patients. A large number of expanded CD4+ T cells and confirmation of functional-related phenotypes are required for ensuring the successful outcomes of treatment. Freshly isolated CD4+ T cells were activated with anti-CD3/28 coated magnetic beads at different bead-to-cell ratios and cultured in the absence and presence of IL-2 supplementation for three weeks. Fold expansion, cell viability, growth kinetic and lymphocyte subset identities were determined. Data demonstrated that a 1:1 bead-to-cell ratio rendered the highest expansion of 1,044 –fold with 88% viability and 99.5% purity followed by the 2:1 and 0.5:1 ratios. No significant difference in proliferation and phenotypes was found between non-IL-2 and IL-2 supplementation groups. Several specific surface molecule expressions of the expanded cells including chemokine receptors, adhesion molecules, co-stimulatory molecules, activation molecules, maturation markers, cytokine receptors and other molecules were altered when compared to the unexpanded cells. This optimized expansion protocol using the 1:1 bead-to-cell ratio of anti-CD3/28 coated magnetic beads and culture condition without IL-2 supplementation provided the satisfied yield with good reproducibility. Specific surface molecule expressions of the expanded cells presented potential roles in proliferation, differentiation, homeostasis, apoptosis and organ-homing.

Keywords	CD4+ T lymphocytes, cell surface molecules, immunotherapy, anti-CD3/28 coated beads, HIV.

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Dear Editor-in-Chief,

My colleagues and I would like to submit a manuscript for consideration as an original article in the Human Immunology entitled "Differential expression of functional-associated cell surface molecules on anti-CD3/28 expanded $CD4^+$ T cells" by authors, Premrutai Thitilertdecha, Poonsin Poungpairoj, Varangkana Tantithavorn, Palanee Ammaranond, and Nattawat Onlamoon.

This manuscript highlights optimization of the expansion protocol using anti-CD3/28 coated magnetic beads in terms of bead quantity and medium supplementation for $CD4^+$ expansion in order to achieve the satisfied yield for clinical uses. This study also firstly provide the whole range of specific cell surface molecule expressions of the expanded $CD4^+$ T cells including chemokine receptors, adhesion molecules, co-stimulatory molecules, activation molecules, maturation markers, cytokine receptors and other molecules related to apoptosis and cell exhaustion. Results show that a 1:1 bead-to-cell ratio of anti-CD3/28 coated beads and autocrine cytokines produced by the expanded cells were sufficient to maintain the culture and provided the good yield. Phenotypic profiles of the expanded cells also suggested that the expression of markers for activation, proliferation, differentiation, homeostasis and apoptosis revealed certain functions of the expanded cells. Interestingly, the expanded cells presented advanced capabilities in HIV resistance and migration to gut-associated lymphoid tissues. Therefore, the expanded cells from our optimized culture method are promising to use in adoptive immunotherapy for HIV-infected patients.

This manuscript has been seen and approved by all authors and has not been submitted elsewhere.

Yours sincerely,

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Differential expression of functional-associated cell surface molecules on anti-CD3/28 expanded $CD4^+$ T cells

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ABSTRACT

$CD4^+$ T cell immunotherapy has potential for treatment in HIV-infected patients. A large number of expanded $CD4^+$ T cells and confirmation of functional-related phenotypes are required for ensuring the successful outcomes of treatment. Freshly isolated $CD4^+$ T cells were activated with anti-CD3/28 coated magnetic beads at different bead-to-cell ratios and cultured in the absence and presence of IL-2 supplementation for three weeks. Fold expansion, cell viability, growth kinetic and lymphocyte subset identities were determined. Data demonstrated that a 1:1 bead-to-cell ratio rendered the highest expansion of 1,044 –fold with 88% viability and 99.5% purity followed by the 2:1 and 0.5:1 ratios. No significant difference in proliferation and phenotypes was found between non-IL-2 and IL-2 supplementation groups. Several specific surface molecule expressions of the expanded cells including chemokine receptors, adhesion molecules, co-stimulatory molecules, activation molecules, maturation markers, cytokine receptors and other molecules were altered when compared to the unexpanded cells. This optimized expansion protocol using the 1:1 bead-to-cell ratio of anti-CD3/28 coated magnetic beads and culture condition without IL-2 supplementation provided the satisfied yield with good reproducibility. Specific surface molecule expressions of the expanded cells presented potential roles in proliferation, differentiation, homeostasis, apoptosis and organ-homing.

Keywords: $CD4^+$ T lymphocytes, cell surface molecules, immunotherapy, anti-CD3/28 coated beads, HIV.

1 INTRODUCTION

Human immunodeficiency virus (HIV) infection causes a progressive decrease of $CD4^+$ T lymphocytes and an increase of HIV viral load (or HIV RNA level), leading to higher susceptibility to opportunistic infections which can further develop to acquired immune deficiency syndrome (AIDS) [1]. HIV enters target cells through the binding of viral envelope glycoproteins to CD4 receptors along with CCR5 and CXCR4 co-receptors markedly expressed on the target $CD4^+$ T lymphocytes [2-4]. Although highly active antiretroviral therapy (HAART) succeeds to control the HIV viral load into an undetectable level and recovers the CD4 counts in HIV-infected patients, the latent reservoir of virus still exists [5] and the immune restoration is incomplete [6-9]. A life-long treatment of HAART has also feasible consequences in cumulative drug toxicities, emergent drug-resistant viruses and unaffordable costs due to more complicated regimens. Moreover, some patients who have discordant immune responses (DIR) to HAART fail to achieve target CD4 count levels despite accomplished virological control, suggesting a higher risk in mortality [10].

An alternative approach, such as adoptive transfer of autologous activated $CD4^+$ T lymphocytes, has been proposed to be a potential treatment for the benefit of both virological control and direct immune reconstitution. Its effectiveness and safety have been confirmed by *in vivo* studies in both simian deficiency virus (SIV)-infected rhesus macaques and HIV-infected patients [11-15]. To expand $CD4^+$ T cells *in vitro*, anti-CD3/28 coated magnetic beads are widely used for stimulation. The anti-CD3/28 activated $CD4^+$ T cells showed intrinsic resistance to macrophage (M)-tropic isolates of HIV-1 infection [16-18] and promoted expression of RANTES, MIP-1α and MIP-1β as well as reduced expression of CCR5 [11,13,14,16]. Furthermore, the expanded $CD4^+$ T cells induced interferon (IFN)-γ production which is associated to type 1 T helper (Th1) cell function and increased the density of variable beta (Vβ) chain T cell receptor (TCR) repertoires [14] together with telomerase activity, resulting in a longer survival of the cells [11].

With respect to the clinical uses, a large number of $CD4^+$ T cells expanded *in vitro* was required for reinfusion in HIV-infected patients [14,15]; therefore, optimization of expansion protocols is warranted. There have been established *in vitro* culture methods for anti-CD3/28 stimulated $CD4^+$ T lymphocytes providing different yields [19-21] which can be related to different cell isolation methods, bead-to-cell ratios used for stimulation, and medium supplementation. More importantly, functional-associated phenotypic characters of the

expanded cells are essential which are not only related to cell characterization but also maturation and activation stages as well as cell migration. Even so, there is limited information concerning specific cell surface molecule expressions of the expanded $CD4^+$ T cells, such as chemokine receptors and maturation markers.

This study thus purposed to investigate the optimum bead-to-cell ratios and supplementation used for $CD4^+$ T cell expansion as well as to explore the whole series of surface molecule expressions of the expanded cells including chemokine receptors, adhesion molecules, co-stimulatory molecules, activation molecules, maturation markers, cytokine receptors, and other functional-specific molecules.

2 MATERIALS AND METHODS

2.1 Sample collection

Three healthy volunteers aged 26-30 years were enrolled in this study. The protocol was approved by the Institutional Review Board of the Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand. Written informed consent was obtained from each subject prior to sample collection.

2.2 Characterization of lymphocyte subsets and specific surface molecule expressions

Monoclonal antibodies (mAbs) and their conjugated fluorochromes were obtained from Becton Dickinson Biosciences (BDB: San Jose, CA) and used at the concentrations recommended by the manufacturer. The fluorescent-labeled mAbs used for phenotypic characterization of the cells were 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. The fluorescent-labeled mAbs used for identification of specific surface molecule expression were anti-CD4 PerCP, anti-CD3 FITC, anti-CD45RO FITC, anti-CD45RA FITC, anti-CD57 FITC, anti-CD27 FITC, anti-CCR7 PE, anti-CD62L PE, anti-CD11a PE, anti-CD11b PE, anti-CD11c PE, anti-CD126 PE, anti-CD127 PE, anti-CD95 PE, anti-CD95L PE, anti-CD154 (CD40L) PE, anti-CD40 PE, anti-CD134 (OX40) PE, anti-CD278 (ICOS) PE, anti-CD71 PE, anti-HLA-DR PE, anti-GITR PE, anti-CD28 PE, anti-CD103 PE, anti-CD38 PE, anti-CD69 PE, anti-CD25 PE, anti-CD184 (CXCR4) PE, anti-CD183 (CXCR3) PE, anti-CCR10 PE, anti-CD195 (CCR5) PE, anti-PD-1 PE, anti-CXCR5 PE, anti-CCR6 PE, anti-CCR4 PE and anti-$\alpha 4\beta 7$ PE.

2.3 *CD4$^+$ T lymphocyte isolation using immunorosettes formation method*

CD4$^+$ T lymphocytes can be directly isolated from whole blood by an immunorosettes formation method using RosetteSep® human CD4$^+$ T cell enrichment cocktail (STEMCELL Technologies, Vancouver, BC, Canada). Briefly, CD4$^+$ T lymphocytes were isolated from 5 mL of whole blood by adding 250 µL of RosetteSep® human CD4$^+$ T cell enrichment cocktail. After that, the samples were thoroughly mixed and incubated at room temperature for 20 minutes. The samples were then diluted with an equal volume of phosphate buffered saline (PBS) containing 2% fetal bovine serum (FBS) and gently mixed. The diluted blood samples were carefully layered on top of LSM® lymphocyte separation medium and centrifuged at 1200g with no break at room temperature for 20 minutes. After centrifugal separation, the samples were divided into four layers including plasma, enriched CD4$^+$ T cells, LSM® lymphocyte separation medium and red blood cells (from top to bottom). Pasteur pipettes were used to remove the plasma layer and collect enriched CD4$^+$ T cells from the layer interface. The collected CD4$^+$ T cells were then washed with 10 mL of PBS containing 2% FBS and centrifuged twice at 1400 rpm at room temperature for 5 minutes. The cell pellets were collected and re-suspended with a complete medium (RPMI1640 with 10% FBS, 50 µg/mL pencillin-streptomycin and 2 mM L-glutamine). Cell number and viability of the enriched CD4$^+$ T cells were determined by trypan blue exclusion using a hemacytometer.

2.4 *Expansion of isolated CD4$^+$ T lymphocytes using anti-CD3/28-coated beads*

The isolated CD4$^+$ T cells from the immunorosettes formation method were stimulated with anti-CD3/28 coated beads. Dynabeads® human T–activators (Invitrogen Dynal, Oslo, Norway) were used in this study. The expansion procedures of CD4$^+$ T lymphocytes were divided into two steps including bead washing for elimination of preservatives and cell activation. The bead number was calculated for 0.5:1, 1:1, and 2:1 bead-to-cell ratios to use for the expansion. Anti-CD3/CD28 coated beads with calculated amounts were then transferred into the tube and washed with 2 mL PBS to an original volume of beads. After that, the tube was placed on the magnet for 1 minute in order to remove the washing solvent. The washed beads remaining in the tube were re-suspended in the complete medium with an equal volume to the initial volume of beads.

For bead-to-cell ratio comparison, 1x10^6 enriched CD4$^+$ T cells were stimulated with anti-CD3/CD28 coated beads in the absence of exogenous interleukin (IL)-2. The stimulated CD4$^+$ T cells were then expanded in the complete medium at a concentration of 0.5x10^6

cells/mL and incubated at 37 °C and 5% CO_2 humidification before reactivation on day 7. The cells were expanded for a total of 3-week culture period and the medium was replenished with calculated amounts of fresh media on days 4, 7, 11, 14, and 17 to maintain the cell suspension concentration at $0.5x10^6$ cells/mL before transferring to appropriate culture vessels. Cell numbers and viability were observed on days 4, 7, 11, 14, 17 and 21 by using trypan blue exclusion and a hematocytometer. Lymphocyte subset characters were analyzed by a flow cytometer on days 0, 14 and 21.

With respect to IL-2 supplementation comparison, the similar activation and culture protocols were conducted by using only the 1:1 bead-to-cell ratio and cultures in the absence and in the presence of exogenous interleukin (IL)-2 at the low concentration of 20 units/mL (Prospec, Ness-Ziona, Israel). Cell numbers and viability were observed on days 4, 7, 11, 14, 17 and 21 by using trypan blue exclusion and a hematocytometer. Lymphocyte subset characters were analyzed by a flow cytometer on days 0, 14 and 21.

2.5 Immunofluorescent staining

Whole blood, purified and expanded $CD4^+$ T cells were stained with fluorochrome-conjugated mAbs and incubated for 15 minutes before adding 1X lysing solution for red blood cell lysis. The stained cells were then washed with PBS containing 2% FBS prior to centrifugation at 1,400 rpm at 25 °C for 5 minutes. Subsequently, the stained cells were re-suspended in PBS containing 1% paraformaldehyde. The stained cells were finally acquired by a BD FACSCalibur flow cytometer (BDB, San Jose, CA) and the data were analyzed by using FlowJo Software (Tree Star, San Carlos, CA).

2.6 Flow cytometric analysis

Six-parameter analysis including forward scatter (FSC), side scatter (SSC), FITC, PE, PerCP, and APC was performed using FlowJo Software (Tree Star, San Carlos, CA). The stained cells were gated using lymphogate (FSC/SSC) to determine a viable lymphocyte population. After that, lymphocyte subsets were defined using two-dimensional dot plots between CD45/SSC, CD45/CD3 and CD4/CD8 or CD19/CD16+CD56. Therefore, the lymphocyte subsets were detected into $CD4^+CD8^-$, $CD4^-CD8^+$, $CD4^+CD8^+$, $CD19^-CD16^+CD56^+$ and $CD19^+CD16^-CD56^-$ populations.

2.7 Statistical analysis

All statistical analyses were performed using GraphPad Prism® version 7.02 (GraphPad Software Inc., CA, USA). Datasets were expressed as mean ± standard deviation (S.D.) and compared for statistical significance at p-value ≤ 0.05 with 2-way ANOVA followed by Bonferroni's multiple comparisons test.

3 RESULTS

3.1 Bead-to-cell ratio comparison for anti-CD3/28 $CD4^+$ T cell expansion

To achieve satisfied yields of the expanded cells, it is important to determine the optimum bead-to-cell ratio used for stimulation. In this study, three healthy volunteers were recruited for blood collection. Isolated $CD4^+$ T cells were activated with anti-CD3/28 coated magnetic beads at different bead-to-cell ratios (i.e., 0.5:1, 1:1 and 2:1) and cultured in the absence of IL-2 for 21 days. Fold expansion, cell viability, growth kinetic and phenotypic characters were observed for proliferation efficiency of the expanded $CD4^+$ T cells on days 0, 4, 7, 11, 14, 17 and 21.

There was no difference in fold expansion among 3 different bead-to-cell ratios during the first 14 days of culture, however, the fold expansion number of $CD4^+$ T cells expanded with the 1:1 bead-to-cell ratio on day 17 showed remarkably higher than the others (Fig. 1a). On day 21 of culture, it was obvious that stimulation with the 1:1 bead-to-cell ratio provided the highest yield of the anti-CD3/28 expanded $CD4^+$ T cells followed by the 2:1 and 0.5 bead-to-cell ratios (1,044 ± 259 –, 629 ± 457 –, and 301 ± 167 – fold, respectively). Cell viabilities of the expanded cells from the 3 different ratios were comparable with over 90% throughout the 3-week culture period (Fig. 1b). There were only slightly decreases in viable cells at the end of the culture for the 1:1 and 2:1 ratios (88 ± 7% and 83 ± 15%, respectively).

Lymphocyte subset characters of the anti-CD3/28 stimulated $CD4^+$ T cells were analyzed by a flow cytometer (Table 1). It was clearly demonstrated that the major population of the expanded cells was $CD3^+$ T cells (> 99% of lymphocytes) with the dominant subset of $CD3^+CD4^+CD8^-$ for all bead-to-cell ratio groups over the culture period (> 98% of lymphocytes). The purity of the expanded cells was also confirmed with low frequencies of $CD3^+CD4^-CD8^+$, $CD3^+CD4^+CD8^+$, $CD3^-CD19^+$, $CD3^-CD16^+CD56^+$, $CD3^+CD16^+CD56^+$, and $CD3^+CD19^+$ (< 3.5% of expanded cells).

3.2 Effects of IL-2 supplementation on cell expansion

IL-2 supplementation has been generally used to promote cell proliferation in addition to the autocrine/paracrine IL-2 production by the activated T cells. High concentrations of IL-2 (100 and 300 IU/mL) have been reported to predominantly affect $CD8^+$ T cell development [22]. This study, therefore, used a low concentration of IL-2 at 20 IU/mL to support cell expansion and compared this expansion effect of IL-2 to the autocrine/paracrine IL-2 production (i.e., cell culture in the absence of IL-2). Fold expansion, cell viability, growth kinetic and lymphocyte subset characters were observed for proliferation efficiency of the expanded $CD4^+$ T cells on days 0, 4, 7, 11, 14, 17 and 21.

Data showed that fold expansion numbers between the culture without and with IL-2 supplementation were similar throughout the 21-day culture period (Fig. 2a). Only the expanded cells cultured in the absence of IL-2 supplementation on day 17 proliferated significantly higher than those in the presence of IL-2 supplement (582 ± 166 – and 455 ± 125 – fold, respectively). At the end of the culture, there was no significant difference in proliferation between the two culture groups. With respect to cell viability, the cultures without or with IL-2 supplementation maintained great numbers of viable cells with over 90% throughout the 3-week culture period (Fig. 2b).

Predominant phenotypes of the expanded cells from both culture groups were $CD3^+$ T cells (> 97% of expanded cells) with the major $CD3^+CD4^+CD8^-$ subset (> 94% of expanded cells) as presented in Table 2. The minor cell populations including $CD3^+CD4^-CD8^+$, $CD3^+CD4^+CD8^+$, $CD3^-CD19^+$, $CD3^-CD16^+CD56^+$, $CD3^+CD16^+CD56^+$ and $CD3^+CD19^+$ were also found with very low frequencies (< 2% of expanded cells), suggesting a specific expansion of purified $CD4^+$ T cells.

3.3 Specific cell surface molecule expressions of anti-CD3/28 $CD4^+$ T cells

As surface molecule expressions are related to cell maturation, activities and functions, this study thus explored several cell surface molecule expressions of anti-CD3/28 expanded $CD4^+$ T cells which were divided into seven groups according to the molecules' functions and roles. The cell surface molecules included (i) chemokine receptors: CCR4, CCR5, CCR6, CCR7, CCR10, CXCR3, CXCR4 and CXCR5; (ii) adhesion molecules: CD11a, CD11b, CD11c, CD103 and $\alpha4\beta7$; (iii) co-stimulatory molecules: CD27, CD28, CD40, CD40L, CD134, PD-1 and ICOS; (iv) activation molecules: CD25, CD38, CD69, CD71 and HLA-DR; (v)

maturation markers: CD45RO, CD45RA and CD62L; (vi) cytokine receptors: CD126 and CD127; and (vii) other molecules: CD57, CD95, CD95L, and GITR. Frequencies of the expanded cells expressing these surface molecules in the cultures without and with IL-2 supplementation on day 21 were observed and compared to those of whole blood on day 0 as a baseline control.

For chemokine receptors, the expanded cells from cultures whether IL-2 supplementation or not exhibited significant lower expressions of CCR6, CCR7 and CXCR4 and dramatic higher expression of CXCR3 when compared to the unexpanded cells (i.e., whole blood) as seen in Fig. 3a. Other molecules, CCR4, CCR5, CCR10, CXCR5, remained similar after the expansion. The expanded cells also had marked increases in expressions of adhesion molecules, CD11b, CD11c and $\alpha 4\beta 7$ when compared to the unexpanded cells (Fig. 2b). There was also no change in expressions of CD11a and CD103 between the expanded and unexpanded cells.

With respect to co-stimulatory molecules, only frequencies of CD40L of the expanded cells from both expansion groups were significantly increased when compared to the unexpanded cells (Fig. 3c). All other molecules including CD27, CD28, CD40, CD134, PD-1 and ICOS remained unchanged after the expansion. Furthermore, expressions of all activation molecules, except CD69, on the expanded cells were significantly upregulated when compared to those of the unexpanded cells (Fig. 3d). The expanded cells also showed significant lower frequencies of CD45RO and higher frequencies of CD45RA, while their CD62L expression was similar to the unexpanded cells (Fig. 3e).

The expanded cells also exhibited notable downregulation of cytokine receptor, CD126, and upregulation in CD127 when compared to the unexpanded cells (Fig. 3f). Expressions of other molecules including CD57, CD95 and GITR were dramatically raised after the expansion, whereas there were slight increases in CD95L (Fig. 3g). Moreover, no significant difference in numbers of any chemokine receptors, adhesion molecules, co-stimulatory molecules, activation molecules, maturation markers, cytokine receptors and other molecules was found between the two culture groups with and without IL-2 supplementation (Figs. 3a-3g).

4 DISCUSSION

In this report, we have described effects of bead-to-cell ratio of anti-CD3/28 coated magnetic beads for stimulation and IL-2 supplementation on the growth of expanded $CD4^+$ T cells as well as their specific surface molecule expression changes after the expansion. Ones of important concerns are quantity of anti-CD3/28 coated magnetic beads used for expansion and medium supplementation. Levine *et al.* (1997) used immobilized anti-CD3/28 coated beads at 1 - 3 beads/cell for a long-term proliferation of polyclonal $CD4^+$ T cells from normal donors. The expanded cells were able to maintain the exponential growth of 2.3×10^5 – fold over 40 days of culture without exogenous cytokines or feeder cells added to the culture. However, the fold expansion was dramatically increased to 10^9 – to 10^{11} – fold when supplemented with IL-2 at the late culture (~ days 28 – 106) [20]. Levine *et al.* (1998) also implemented the previous method for a large-scale production of $CD4^+$ T cells from HIV-infected patients by using anti-CD3/28 coated beads at 3 beads/cell for stimulation and the culture method without IL-2 supplementation. The cell numbers were increased by 37 – fold after a 14-day culture [21]. Later, our previous study showed that $CD4^+$ T cell stimulation with reduced numbers of anti-CD3/28 coated magnetic beads to a 1:1 bead-to-cell ratio and culture in the absence of IL-2 were able to prolong the cell expansion throughout a 3-week culture with the good yield of 1,000 – fold [19]. During the cell harvest, the higher number of beads used for expansion, the higher number of beads remaining in the final products which leads to the bead accumulation in the body after the cell reinfusion tentatively causing toxicity. It is then warranted to compromise between the bead quantity for the highest activation efficiency and the treatment safety for patients.

We then compared different bead-to-cell ratios (i.e., 0.5:1, 1:1, and 2:1) to minimize the number of beads used for expansion without sacrificing their promotion for the suitable yield. Our data showed that the 1:1 bead-to-cell ratio provided the highest fold expansion (1,044 ± 259 – fold) which was 1.7 and 3.5 times greater than the 2:1 and 0.5:1 ratios, respectively. Hence, cell stimulation at the 1:1 bead-to-cell ratio was sufficient to achieve the satisfied yield with good viability (88 ± 7%) and high purity (> 98% of lymphocytes) within the reasonable time (i.e., a 3-week culture). Autocrine cytokines produced from the expanded cells themselves were also adequate to supply the proliferation throughout the culture period. This was confirmed by our comparison between the cultures without and with IL-2 supplementation. No significant difference in fold expansion and cell viability was found between the two groups.

Changes in specific cell surface molecule expressions of the anti-CD3/28 $CD4^+$ T cells were also observed in this study. Several chemokine receptors have been identified as coreceptors for the HIV entry, such as CCR5, CXCR4, CCR4, CCR6 and CCR10. The *in vitro* expanded $CD4^+$ T cells with anti-CD3/28 activation were proved to be resistant to HIV-1 infection via the reduction in frequencies and densities of CCR5 molecules [18,23]. Our expanded cells also rendered a low frequency in CCR5 (< 15% of $CD4^+$ T cells) which presumably maintain at this low level because the recovery of CCR5 expression was low when activation with anti-CD3/28 coated beads compared to stimulation with anti-CD3/28 immobilized on the surface of a tissue culture plate [23]. Our anti-CD3/28 activation protocol also rendered the expanded $CD4^+$ T cells with twice as less CXCR4 expression than the unexpanded cells. Although a high number of the expanded cells still expressed CXCR4 (52% of $CD4^+$ T cells), the chance of viral entry when switching coreceptor usage from CCR5 to CXCR4 [24] will feasibly diminished when compared to the unexpanded cells.

CCR4, CCR6 and CCR10 were also reported to be other HIV-1 coreceptors of primary HIV-1 isolates [25-27]. Our expanded cells showed that CCR4 was highly expressed throughout the expansion period (84% of $CD4^+$ T cells), whereas a significant downregulation in CCR6 by 2.5 – fold and low frequency of CCR10 (12% of $CD4^+$ T cells) were found. Although CCR4 expression was high, soluble viral protein gp120 had greater affinity to CCR5 than CCR4 [27]. $CCR4^+$ T cells are also able to be defined as Th2 cells, while Th1 cells are classified by $CXCR3^+$ [28]. In our study, the frequency of $CXCR3^+$ T cells was 2-fold higher than that of the unexpanded cells (~83% of $CD4^+$ T cells) which was almost equal to $CCR4^+$ T cells. However, we cannot specify that our $CXCR3^+$ T cells are absolutely purified Th1 cells as they need to be further characterized with expressions of $CCR4^-$, $CCR5^+$, and $CXCR6^+$ [28]. These evidences clearly demonstrate that our expanded cells are suitable for reinfusion due to their highly feasible ability for HIV-1 resistance *in vivo*.

As the gut compartment is a major portal for HIV entry, the considerable depletion in mucosal $CD4^+$ T cells was observed during acute infection [29]. Our study shows that the expanded cells had a pretentious increase in $\alpha4\beta7$ expression to 82% of $CD4^+$ T cells. In contrary, the level of another gut mucosal specific adhesion molecule, CD103 [30], was low at the end of the culture (10% of $CD4^+$ T cells) even though it was 5 – fold higher expressed in the expanded cells. It is then worth proposing that these cells feasibly migrate to the site of depletion and improve immune response at the gut-associated lymphoid tissues.

Other surface adhesion molecules on T lymphocytes are β2 integrins (CD11/CD18 family) including CD11a, CD11b, and CD11c. These molecules facilitate the T cell engagement with target cells and endothelial cells. CD11a is generally highly expressed in $CD4^+$ T cells, whereas CD11b and CD11c are more prevalent in $CD8^+$ T cells. [31]. Our results affirm this evidence as the unexpanded $CD4^+$ T cells exhibited high expression of CD11a (100% of $CD4^+$ T cells) and low expressions of CD11b and CD11c (3% and 12% of $CD4^+$ T cells, respectively). Interestingly, after a-3 week culture, the expression of CD11a on the expanded cells remained high. Results also showed significant upregulation in CD11b (14.6 – fold) and CD11c (5.3 – fold). These expanded cells with the notable high CD11a expression are suitable for survival due to ability for T cell localization to areas rich in cytokines for promoting their homeostasis [32].

With respect to co-stimulatory molecules, a high frequency of CD28, in our study remained unchanged during the expansion (99% of $CD4^+$ T cells). This is in agreement with the previous report showing the constitutive expression of CD28 on $CD4^+$ T cells [33], suggesting that the function for an effective antigen-specific immune response is still active. However, our results are in contrary to another study reporting that its expression was transiently down-regulated following T-cell activation and progressively declined due to *in vitro* senescence [34]. For CD40 expression, it is not surprisingly to find its low level on the expanded $CD4^+$ T cells (9% of $CD4^+$ T cells) as they are commonly identified and functionally characterized on B cells [35]. On the other hand, CD40L is only expressed on activated $CD4^+$ T cells [35] which is in accordance with our results presenting a significantly increased CD40L expression by 3.4 – fold, proposing that the expanded $CD4^+$ T cells can probably promote isotype switching, maturation and survival of B cells [36].

We also found that the expression of CD134 (OX40) was slightly increased after the expansion (18% of $CD4^+$ T cells), possibly promoting survival of T cell numbers and accumulation of developed memory $CD4^+$ T cells over time [37]. For ICOS, it was highly expressed and comparable to that of the unexpanded cells (68% of $CD4^+$ T cells), even though this ICOS has to be *de novo* induced on the T-cell surface [33] and augmented by CD28 co-stimulation together with TCR engagement [38]. It was also reported to be matching CD28 in potency and enhancing all basic T-cell responses [33] as well as T-cell-dependent B-cell help [39]. Overall, all co-stimulatory molecules of the anti-CD3/28 $CD4^+$ T cells were endurable over the expansion period. Only CD40L was markedly higher expressed in the expanded cells. It is then suggested that these *in vitro* expanded cells with consistent co-

stimulatory molecules will be able to have normal proliferation and differentiation *in vivo* when reinfusion to HIV-infected patients in which some co-stimulatory molecules are dysregulated following the disease progression.

Expression kinetics of the CD25, CD69, CD38, CD71 and HLA-DR were previously described [40,41]. The results showed that while CD25 and CD69 expression on $CD4^+$ T cells reached over 90% at 24 hours after soluble anti-CD3/28 stimulation, whereas CD38 and CD71 reached their maximum levels at 72 hours [41]. Our activation molecules including CD25, CD38, CD71 and HLA-DR were also dramatically increased (80%, 100%, 75%, and 63% of $CD4^+$ T cells, respectively), while CD69 was slightly increased to 32% of $CD4^+$ T cells. It is then indicating that our expanded cells expressed full ranges of activation molecules in both early- (i.e., CD25 and CD69) and late-activation markers (i.e., CD38, CD71 and HLA-DR). Interestingly, the previous study also demonstrated that CD25 expression maintained at high level, whereas CD69 was significantly dropped to ~50% after 72 hours [41]. The low level of CD69 expression on our expanded cells revealed similar expression kinetics since the data was observed 2 weeks after re-stimulation.

Maturation stage of $CD4^+$ T cells subsets including naïve, effector, effector memory, and central memory cells is generally based on expression of CD45RA with CD62L or CD45RA with CCR7 [42]. We found that the unexpanded cells had equal numbers of naïve ($CD4^+CD45RA^+$) and memory ($CD4^+CD45RO^+$) subsets with ~50% of $CD4^+$ T cells. After a 3-week culture, $CD45RA^+$ cells were notably declined by ~2.7 – fold, whereas $CD45RO^+$ cells were 2-fold increased (84% of $CD4^+$ T cells). Furthermore, there were contradict expressions between CD62L and CCR7 which were supposed to be correlated each other. CD62L remained highly expressed (97% of $CD4^+$ T cells), whereas CCR7 was prominently decreased to 9% of $CD4^+$ T cells. However, this study had a limitation in T cell subset characterization due to those molecules were not determined simultaneously. Therefore, we cannot precisely specify T cell subsets. Even so, our results can assume that the expanded cells were probably in the transition towards memory cells either central memory ($CD45RA^-CCR62L^+$ or $CD45RA^-CCR7^+$) or effector memory ($CD45RA^-CCR62L^-$ or $CD45RA^-CCR7^-$) cells. A great number of $CD27^+$ T cells (84% of $CD4^+$ T cells) were also found, indicating that the cells have not been differentiated into effector cells which finally lose this CD27 expression [43].

Additionally, the expression of CXCR5 on CD4+ T cells indicates a distinct memory T cell subset with B cell helper function (i.e., follicular B helper T cells (T_{FH})) [44]. Our data exhibited a very low frequency of $CXCR5^+$ T cells (1.7% of $CD4^+$ T cells), revealing that our expanded cells are not T_{FH}. A low frequency of $GITR^+$ cells designated as regulatory T (T_{reg}) cells was also significantly increased but still less than 20% of $CD4^+$ T cells. This small augmented population of T_{reg} may help preventing an aberrant HIV-induced chronic T-cell hyperactivation, leading to retardation of disease progression [45]. Therefore, it is then suggested that our expanded cells may pose a regulatory function.

According to cytokine receptors, CD126 expression was markedly low in our expanded $CD4^+$ T cells compared to the unexpanded cells (7% vs 93% of $CD4^+$ T cells). This downregulation might be resulted from TCR cross-linking *in vitro*, indicating a non-naïve stage of T-cell differentiation [46]. This supports our cell maturation stage discussed previously. Our study also shows that CD127 was markedly decreased over the expansion by 1.5 – fold (from 92% to 60% of $CD4^+$ T cells). The reduced expression possibly cause from prolonged CD127 suppression via TCR stimulation [47].

During the expansion, activation-induced cell death of $CD4^+$ T lymphocytes can occur via Fas-dependent apoptosis by triggering Fas (CD95) with its ligand (FasL or CD95L) [48]. We found that the expanded cells expressing $CD95^+$ were significantly increased to 100% of $CD4^+$ T cells, whereas $CD95L^+$ cells remained at low level (14% of $CD4^+$ T cells). It is then assumed that the apoptosis following cell-cell contact between $CD95^+$ and $CD95L^+$ $CD4^+$ T cells will be limited. We also investigated the expression of CD57 which is associated to functions in termination of cell differentiation and submission to apoptosis [49]. A significant increase in $CD57^+$ cells was found but the level was low at 17% of $CD4^+$ T cells, indicating some of the expanded cells may be in the exhaustion stage. However, the expression of PD-1 which is also a critical mediator for T-cell exhaustion [50] was not changed during the expansion (33% of $CD4^+$ T cells). Our expanded cells thus possibly retain normal function in proliferation.

Moreover, a low concentration of IL-2 supplementation did not affect surface molecule expressions of any chemokine receptors, adhesion molecules, co-stimulatory molecules, activation molecules, maturation markers, cytokine receptors and other molecules of the expanded cells. These findings then support the previous experiments from Onlamoon *et al.* (2013).

This study demonstrated that the 1:1 bead-to-cell ratio of anti-CD3/28 coated magnetic beads for $CD4^+$ T cell expansion was the most optimum bead quantity to achieve the satisfied yield of the expanded cells. The autocrine cytokines, mainly IL-2, produced by the expanded cells themselves are also adequate for a 3-week proliferation without additional IL-2 supplementation. After the expansion, phenotypic profiles of the expanded cells were changed. The expanded cells likely become more resistant to HIV-1 via downregulation of dominant coreceptors for HIV entry, CCR5 and CXCR4, as well as migrate to the site of depletion and improve immune response at the gut-associated lymphoid tissues due to higher expressions of gut-homing molecules, $\alpha 4\beta 7$ integrin. Furthermore, other specific surface molecule expressions related to activation, proliferation, differentiation, homeostasis and apoptosis revealed certain functions of the expanded cells. It is thus worth suggesting that these expanded cells following our optimized protocol are suitable for $CD4^+$ T cell immunotherapy used in HIV-infected patients, even though further investigation on $CD4^+$ T cells from HIV-infected patients and a large-scale production are required.

5 LIST OF ABBREVIATIONS

ACT	Actin
AIDS	Acquired immune deficiency syndrome
APC	Allophycocyanin
CCR	Chemokine receptor
CXCR	CXC chemokine receptor
DIR	Discordance immune response
FBS	Fetal bovine serum
FSC	Forward scatter
FITC	Fluorescein isothiocyanate
GITR	Glucocorticoid-induced TNFR (tumor necrosis factor receptor) family related gene
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HLA-DR	Human leukocyte antigen – antigen D related
ICOS	Inducible costimulatory molecule
IFN	Interferon

IL	Interleukin
M	Macrophage
mAbs	Monoclonal antibodies
MIP	Macrophage inflammatory protein
PBS	Phosphate buffered saline
PD-1	Programmed cell death protein 1
PE	Phycoerythrin
PerCP	Peridinin chlorophyll protein
RANTES	Regulated on activation, normal T cell expressed and secreted
RNA	Ribonucleic acid
SIV	Simian deficiency virus
SSC	Side scatter
STI	Structured treatment interruption
TCR	T cell receptor
T_{FH}	Follicular B helper T cells
Th1	Type 1 T helper cells
T_{reg}	Regulatory T cells
Vβ	Variable beta

6 DECLARATIONS

6.1 Ethical approval and consent to participate:

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable standards. The study was approved by the Institutional Review Board of the Faculty of Medicine Siriraj Hospital at Mahidol University. Informed consent was obtained from all individual participants recruited in the study.

6.2 Consent for publication:

Written informed consent was obtained from the patients and participants for publication of their individual details and accompanying images in this manuscript. The consent form is held by the authors and is available for review by the Editor-in-Chief.

6.3 Availability of data and material:

All data generated or analyzed during this study are included in this published article.

6.4 Competing interests:

The authors declare that they have no conflict of interest.

6.5 Funding:

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6.6 Authors' contributions:

PT: performed the experiment, data analysis and manuscript writing; PP, VT, PA, and SL: performed the experiment and data analysis; NO: research idea formation, research monitoring, and manuscript editing. All authors read and approved the final manuscript.

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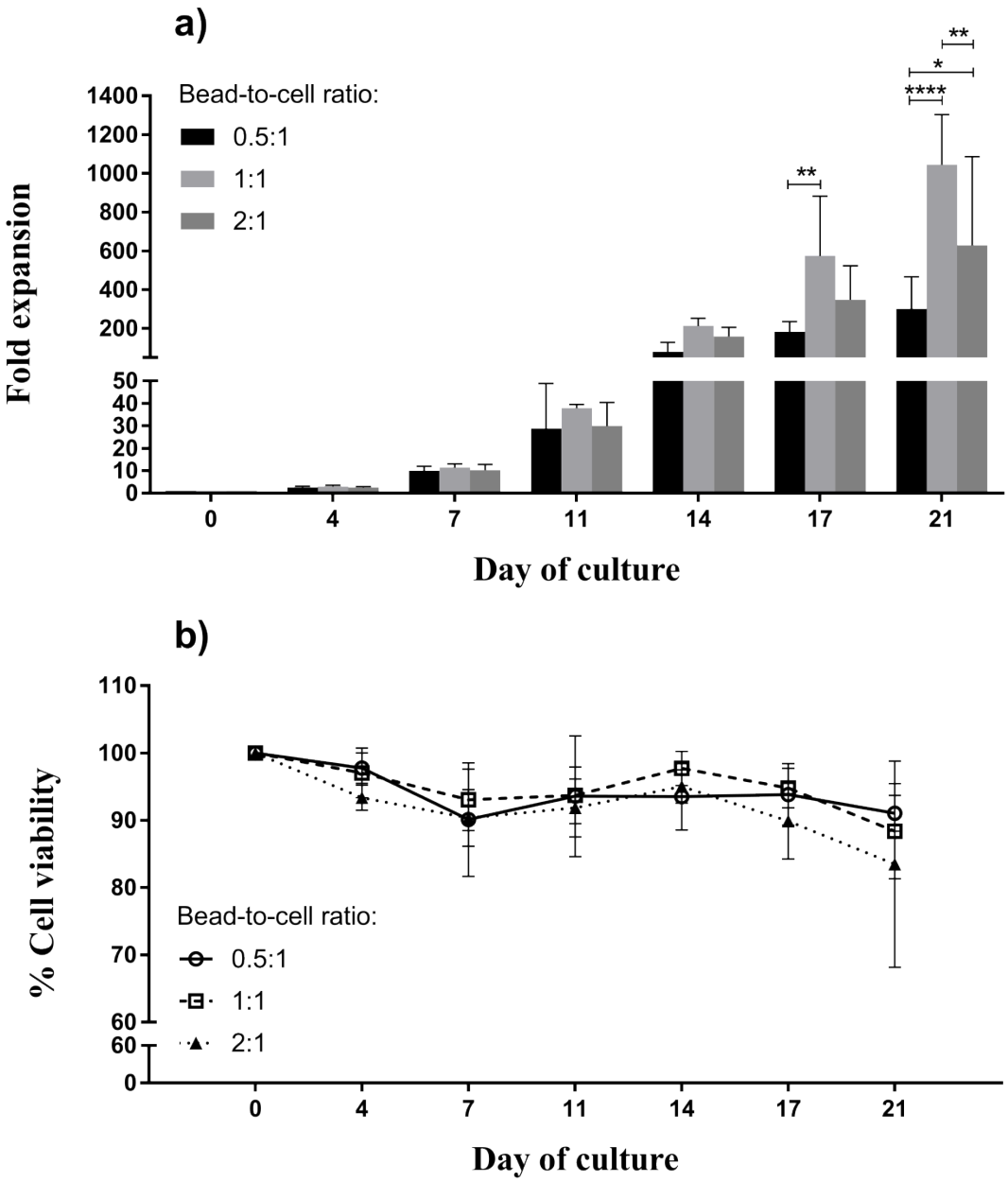

Fig. 1 Growth kinetics of anti-CD3/28 expanded $CD4^+$ T cells when using different bead-to-cell ratios for stimulation. Fold expansion (a) and cell viability (b) of the expanded cells at different bead-to-cell ratios (i.e., 0.5:1, 1:1, and 2:1) were observed over the 21-day culture. All data are presented as mean ± SD (n = 3, *p-value < 0.05, **p-value < 0.01 and ****p-value < 0.0001).

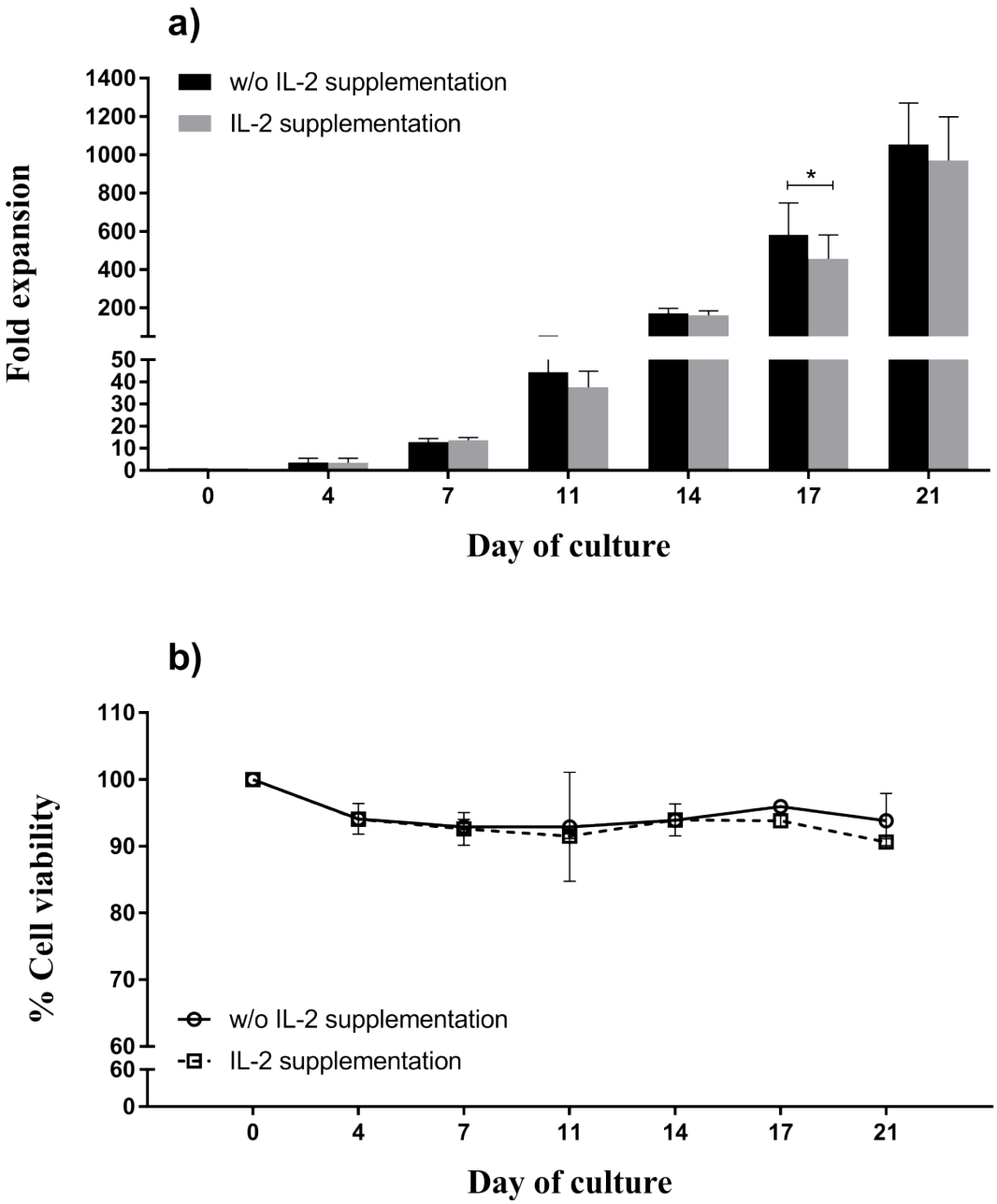

Fig. 2 Growth kinetics of anti-CD3/28 expanded $CD4^+$ T cells when cultured without and with IL-2 supplementation. Fold expansion (a) and cell viability (b) of the expanded cells when cultured without (w/o) and with IL-2 supplementation at the concentration of 20 units/mL were observed over 21 days. All data are presented as mean ± SD (n = 3 and *p-value < 0.05).

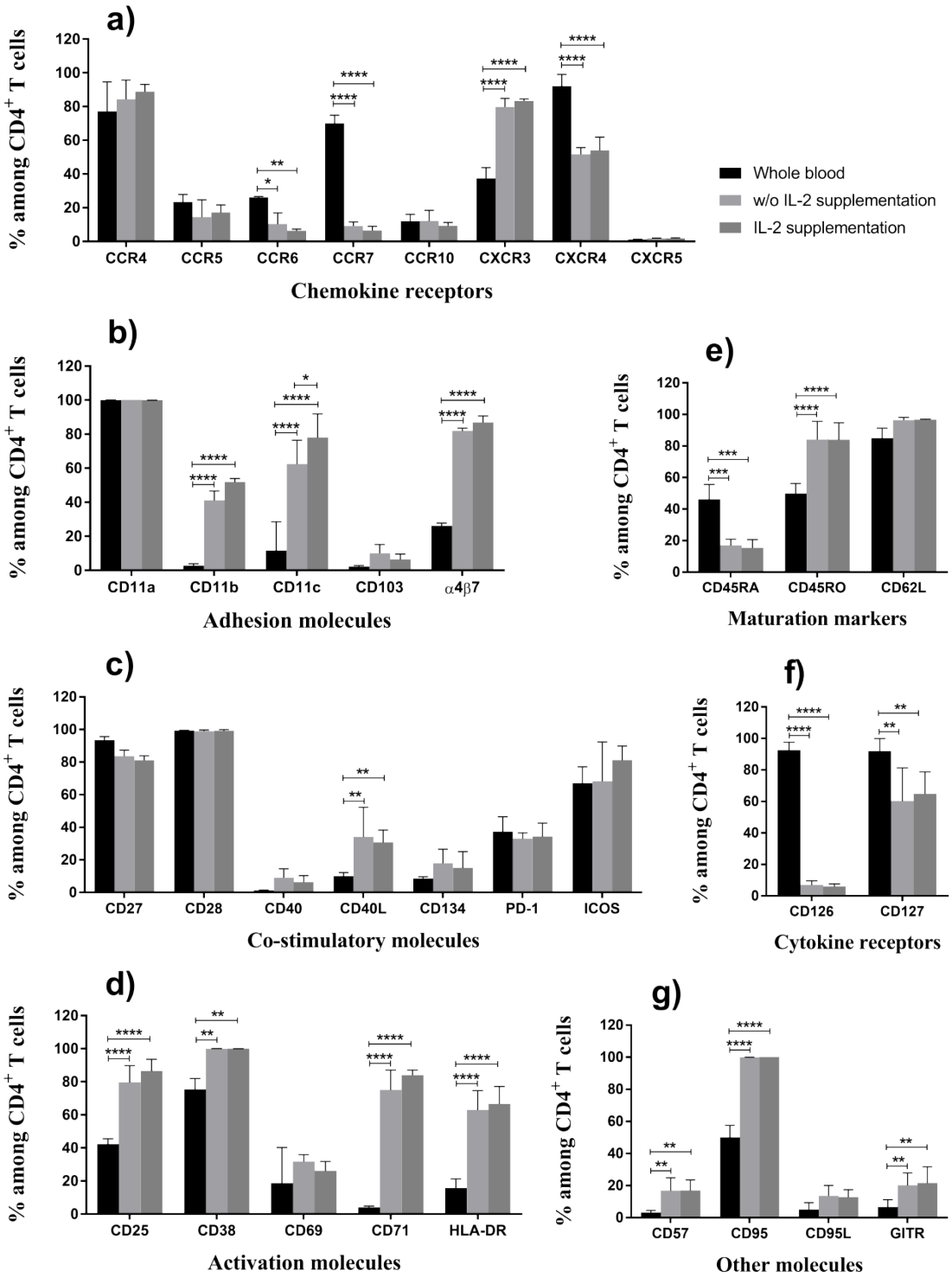

Fig. 3 Specific cell surface molecule expressions of anti-CD3/28 expanded CD4+ T cells. Expressions of a) chemokine receptors, b) adhesion molecules, c) co-stimulatory molecules, d) activation molecules, e) maturation markers, f) cytokine receptors and g) other molecules of the expanded cells when cultured without (w/o) and with IL-2 supplementation were observed on day 21 of the culture and compared to the expressions of whole blood observed on day 0. All data are presented as mean ± SD (n = 3, *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001 and ****p-value < 0.0001).

Table 1 Characterization of anti-CD3/28 stimulated $CD4^+$ T lymphocytes at different bead-to-cell ratios over the 21-day culture. Frequencies of all cell populations are represented as percentages of lymphocytes (mean ± SD, n = 3).

Cell population	Day of culture	Bead-to-cell ratio		
		0.5:1	**1:1**	**2:1**
$CD3^+$	0	99.3 ± 0.4	99.3 ± 0.4	98.8 ± 1.2
	14	99.8 ± 0.2	99.9 ± 0.1	99.9 ± 0.1
	21	99.6 ± 0.5	99.9 ± 0.1	99.9 ± 0.2
$CD3^+CD4^+CD8^-$	0	97.8 ± 1.3	98.1 ± 1.4	97.5 ± 1.7
	14	99.1 ± 0.6	99.8 ± 0.1	99.7 ± 0.1
	21	98.7 ± 1.5	99.5 ± 0.3	99.3 ± 0.4
$CD3^+CD4^-CD8^+$	0	0.0	0.0	0.0
	14	0.0 ± 0.1	0.0	0.0
	21	0.0	0.0	0.0
$CD3^+CD4^+CD8^+$	0	0.0	0.0	0.0
	14	0.0	0.0	0.0
	21	0.0 ± 0.1	0.0 ± 0.1	0.0
Others[a]	0	0.9 ± 0.5[b]	0.7 ± 0.4[b]	1.1 ± 0.9[b]
	14	1.7 ± 0.4[b]	3.5 ± 1.5[b]	2.4 ± 0.3[b]
	21	1.3 ± 0.7[b]	1.3 ± 0.4[b]	1.2 ± 0.4[b]

[a]Other cell populations include $CD3^-CD19^+$, $CD3^-CD16^+CD56^+$, $CD3^+CD16^+CD56^+$, and $CD3^+CD19^+$.

[b]A sum of average percentages of frequencies of the other cell populations.

Table 2 Characterization of anti-CD3/28 stimulated $CD4^+$ T lymphocytes when cultured without (w/o) and with IL-2 supplementation over 21 days. Frequencies of all cell populations are represented as percentages of lymphocytes (mean ± SD, n = 3).

Cell population	Day of culture	Supplementation	
		w/o IL-2	IL-2
$CD3^+$	0	99.5 ± 0.2	99.2 ± 0.7
	14	96.7 ± 5.7	99.8 ± 0.1
	21	99.9 ± 0.1	99.9 ± 0.0
$CD3^+CD4^+CD8^-$	0	98.3 ± 0.6	97.9 ± 1.7
	14	94.4 ± 5.2	99.6 ± 0.1
	21	99.2 ± 0.1	99.0 ± 0.9
$CD3^+CD4^-CD8^+$	0	0.0	0.0
	14	0.0	0.0
	21	0.1 ± 0.1	0.0
$CD3^+CD4^+CD8^+$	0	0.0	0.0
	14	1.4 ± 2.4	0.1 ± 0.1
	21	0.0	0.0
Others[a]	0	0.7 ± 0.4[b]	0.8 ± 0.6[b]
	14	2.0 ± 1.0[b]	1.9 ± 0.6[b]
	21	1.3 ± 0.4[b]	0.6 ± 0.4[b]

[a]Other cell populations include $CD3^-CD19^+$, $CD3^-CD16^+CD56^+$, $CD3^+CD16^+CD56^+$, and $CD3^+CD19^+$.

[b]A sum of average percentages of frequencies of the other cell populations.