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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