Vietnam Journal of Biotechnology **22**(1): 55-68, 2024. DOI: 10.15625/vjbt-19664

CONSTRUCTION AND EVALUATION OF THE DIAGNOSTIC VALUE OF THE RECOMBINANT ANTI-CD3ε ANTIBODY IN THE QUALIFICATION OF T CELLS BY FLOW CYTOMETRY

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Received: 15.12.2023
Accepted: 09.03.2024

ABSTRACT

Quantification of absolute and percentage values for T lymphocyte subsets is a common test for early detection, diagnosis, and treatment of immune-mediated diseases. By using monoclonal antibodies against CD3, CD4, and CD8 conjugated with fluorescent reagents, flow cytometry is a powerful technique to qualify CD3, CD4, and CD8⁻ positive T cells in the evaluation of primary (congenital) and secondary (acquired) immunodeficiencies such as HIV/AIDS, autoimmune diseases, leukemia, non-Hodgkin lymphoma, Hodgkin lymphoma, … However, almost all monoclonal antibodies used in hospitals around Vietnam are imported from foreign countries, increasing the financial burden on patients. Our study aims to create and evaluate the efficiency of a recombinant anti-CD3ε monoclonal antibody (mAb) obtained from the CHO-DG44 host cell system to identify antigen-specific T cells using flow cytometry. The monoclonal antibodies were purified from culture supernatant and have dimeric structures, a feature that is responsible for mAb activities. The flow cytometry results indicated that the recombinant anti-CD3εmAb was able to accurately qualify the cell-surface antigen *in vitro*. Furthermore, the mAb developed in this study specifically recognized T cells as opposed to other major human blood cell types such as B cells, NK cells, and monocytes in human peripheral blood. These data collectively suggested that anti-CD3εmAb expressed by CHO- DG44 cells has the potential for further development towards the application of $CD3^+$ T cell quantification in disease diagnosis by using flow cytometry.

Keywords: recombinant anti-CD3εmAb, CHO- DG44 cells, $CD3^-$ positive T cells quantification, flow cytometry.

INTRODUCTION

Monoclonal antibodies (mAbs) are molecules that bind to specific epitopes expressed on the targets. These antibodies are made from homogeneous hybrid cells (B cells) that are derived from a unique parent cell (Nelson *et al*., 2000; Steinitz, 2009).

Recombinant mAbs are antibodies produced by recombinant DNA technology from suitable host cell systems harboring the

corresponding genes. Various recombinant production systems are used to proceduce recombinant proteins, including prokaryotic *and* eukaryotic expression hosts such as bacteria, yeast, insect cells, phages, transgenic animals, and mammalian cells,. Approximately 70% of therapeutic recombinant proteins are collected from mammalian cell-cultures of Chinese hamster ovary (CHO) cell lines such as K1, DukX B11, and DG44 (Frenzel *et al.*, 2013; Kunert *et al.*, 2016; Li *et al.*, 2010).

Lymphocytes, a type of white blood cell, play a central role in adaptive immunity by producing antibodies and other substances that protect the body. In human ciculating blood, approximately 60 to 70% of lymphocytes are T cells. These cells are primarily responsible for eliminating infected or cancerous cells, activating other immune cells, producing cytokines, and regulating the immune response (Ho *et al.*, 2013). The number of T cells is reliable data for diagnosing, classifying, and monitoring the activity of the immune system. Abnormal T cell numbers have been linked to autoimmune diseases (such as leukemia, non-Hodgkin lymphoma, Hodgkin lymphoma), and immunosuppression caused by HIV. T-cell counts also provide a useful biosignature for monitoring transplant rejection *(*Berliner *et al.*, 2020*;* Holland *et al.*, 2020; McPherson *et al.*, 2022*)*. The quantity of T cells in the blood can aid doctors in diagnosis, staging, and therapy progress tracking of patients.

CD3 (cluster of differentiation 3) is a surface structure associated with the T-cell receptor (TCR) that plays a critical role in the differentiation, survival, and function of T cells. CD3 is a common T-lymphocyte antigen present on more than 95% of the surface of T-lymphocytes in normal human peripheral blood lymphocytes (Malviya *et al.,* 2009; Kleiveland et al., 2015).

The quantification of T lymphocyte subsets in human peripheral blood using flow cytometry is a standard procedure in many hospitals in Vietnam. T cell surface CD3 is labelled using monoclonal anti-CD3ε antibody- conjugated fluorescent agents. Currently, all monoclonal antibodies used in Vietnamese hospitals are imported, thereby increasing the financial burden on patients.

This study aimed to produce and analyze the biological activity of recombinant anti-CD3ε Abs expressed by the CHO-DG44 system. This recombinant mAbs was engineered with sequences of a heavy chain (HC) and light chain (LC) variable region similar to the published sequence of Muromonab, an anti-CD3ε mouse mAb (Drug bBank, DB00075), and a mouse constant region of HC IgG1 and LC Kappa (pFUSE-CHIg-mG1, pFUSE2-CLIg-mK, Invivogen). The present study demonstrated that the recombinant anti-CD3 mAbs are efficacious in qualifying CD3ε-positive T cells by flow cytometry, thereby developing a potential bioproduct that offers additional diagnosis options at a lower cost.

MATERIALS AND METHODS

Cells, agents

CHO- DG44, SUP T1, and RajiB cells were obtained from ATCC and cultured in MEM (-) medium (Biological Industries, Kibbutz Beit-Haemek, Israel), RPMI or DMEM high glucose (Thermo Fisher, CA), respectively, supplemented with 10% fetal bovine serum (Thermo Fisher) at 37 °C, 5% CO_2. The cells were confirmed to be negative for mycoplasma contamination.

Construction of plasmids encoding CD3 mAbs expressed by CHO- DG44 cells

HC and LC variable regions of Muromonab, a monoclonal anti-CD3εmAb bound specifically with CD3ε on human T cells, were obtained from publicly available source (https://www.drugbank.ca/drugs/DB00075). Artificial gDNA fragments encoding HC and LC of Muromonab were synthesized by Integrated DNA Technologies, Inc. (IDT, Korea). These DNAs were amplified by PCR using a pair of primers: F1 (5'-CGTGTCCTGT CCCAAGTGCAGCTGCA GCA-3'), R1 (5'-TTTATTAGCGCTGCTG CTCACGGTC-3') for HC region and primers F2 (5'-CTTCAGTCATAATGTCC AGAGGACAGATCGTGCTGACACAGA GCCC-3') and R2 (5'-GTTTTTGCAGCAT CTGCGTTGATCTCCAG-3') for LC (All primers in this study were synthesized by IDT Inc.). Approximately 25 ng of DNA templates were used for each reaction. The final concentration of each primer was 100 nM. Cycling conditions were as follows: 95 °C for 5 minutes, followed by 35 cycles of 95 °C for 30 seconds, 60 °C for 1 minute 30 seconds and 72 °C for 30 seconds. A final, 10-minute elongation step was performed at 72 °C.

The PCR products of 702 bp for the LC and 1398 bp for the HC were cloned into pOptiVEC-TOPO and pcDNA3.3 TOPO vectors, respectively, which were supplied in the pOptiVEC™-TOPO™ TA Cloning™ Kit or pcDNA™ 3.3-TOPO™ TA Cloning™ Kit (Thermo Fisher), as described in Thermo's user guide. Four constructs containing anti-CD3εmAb heavy and light chain genes downstream of the CMV promoter were generated (Table 1). The DNA constructs were transformed into chemically competent DH5α *E. coli* cells using the heat shock method (Froger A., et al, 2007). Positive colonies were selected by PCR using a pair of primers CMV-F (5'-CGCAAATGGGCGGTAGGCGTG-3'), R1 (5'-TTTATTAGCGCTGCTGCTCACGGT C-3') for the HC region, and primers CMV-F and R2 (5'-GTTTTTGCAGCATCTGC GTTGATCTCCAG-3') for LC. The final concentration of each primer was 100 nM. Cycling conditions were as follows: 95 °C for 5 minutes, followed by 35 cycles of 95 °C for 30 seconds, 60 °C for 1 minute 30 seconds, and 72 °C for 30 seconds. A final, 10-minute elongation step was performed at 72 °C.

The plasmids were extracted from the positive colonies using GenJET plasmid miniprep kit (Thermo Fisher) and confirmed again by DNA sequencing analysis.

Table 1. Constructions of plasmids encoding CD3εmAbs.

	Vector	Gene	Construct name
1	pOptiVEC-TOPO	*CD3ε*-HC	pOp-CD3ε-HC
2	pOptiVEC-TOPO	*CD3ε*-LC	pOp-CD3ε-LC
3	pcDNA3.3-TOPO	*CD3ε*-HC	pc-CD3ε-HC
4	pcDNA3.3-TOPO	*CD3ε*-LC	pc-CD3ε-LC

Transfection of CHO-DG44 and generation of stable cell lines

One day before transfection, the cells were plated at a density of 1 × 10^6 cells/mL. On the day of transfection, cells were transformed according to the manufacturer's instructions (FreeStyle CHO-DG44 Cells, Invitrogen, USA). Transfection efficiency was assessed by fluorescence microscopy of cells with the pEGFP plasmids.

The selection of stable cell populations was carried out following the standard protocol. After transfection for 24 hours, the cells were collected and suspended in MEM (-) containing a 500 µg/mL solution of G418 (Life Science, USA) and 10 nM MTX at a density of 1 cell/100 µL/well of a 96-well plate. The plates were cultured in a CO_2 incubator at 5% CO_2 at 37 °C and 95% humidity until reaching 80–100% confluence. The mAb-containing supernatant was analyzed by ELISA using an anti-human IgG1 (hIgG1) Fc antibody (Invitrogen). The selected positive pools with the highest productivity were subcultured into 6-well plates, re-selected for cell density and productivity by ELISA, and then transferred into T-75 flasks.

Western blot analysis

The expressed proteins were subjected to electrophoresis, transferred onto a nylon membrane, and hybridized with antibody (Ab) against human IgG1 Fc (Invitrogen, US).

Purification and analysis of structure of the recombinant antibodies from the culture supernatant

The recombinant anti-CD3ε antibodies in culture supernatants were purified using protein A affinity chromatography (GE, USA) at 20 °C. The purified mAb was re-buffered into phosphate-buffered saline, sterile-filtered, aliquoted, and frozen at –80 °C. SDS-PAGE with Laemmli buffer (Sambrook *et al.*, 1989) in the presence or absence of β-mercaptoethanol, was performed to analyse dimeric forms of mAb.

Peripheral blood mononuclear cells (PBMCs) isolation

PBMCs isolation from whole blood relies on density-gradient centrifugation with Ficoll-Paque (Merk, US) according to the manufacturer's protocol. Blood samples (5 mL) were diluted to a 1:1 volume ratio with PBS (Sigma, US). The diluted blood was gently layered on top of 5 mL of Ficoll-Paque and centrifuged at 800 × *g* for 30 minutes with the brake off. The upper layer was removed, following the harvest of the mononuclear cells at the interface. The PBMCs were then washed twice in PBS before being used in further experiments.

FACS analysis

Unstained and stained PBMCs, using mouse isotype IgG (Thermo, US), were utilized as negative controls. PBMCs were stained by the recombinant anti-CD3ε antibody (2 µg/test), anti-human CD3ε (BD, US), anti-human CD45 (BD, US), anti-human CD20 (BD, US), anti-human CD56 (BD, US), or anti-human CD14 (BD, US) Ab (at a ratio of 1:200 – 1:500 according to the manufacturer's recommendation) in dark for 1 hour at room temperature. The stained cells were washed with PBS and re-suspend in 200 µl FACS buffer (BD, US). Subsequently, the cells were analyzed using FACS AriaIII (BD, US).

Statistical analysis

The data are represented as the means of at least three independent experiments. Statistical analysis was performed using the ANOVA test with GraphPad Prism 6 software (GraphPad, USA). A p-value of less than 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

Construction of the recombinant anti-CD3ε antibody heavy and light chain plasmids

The anti-CD3ε mAb expression utilizes two separate plasmids, one encoding the HC and one encoding the LC. They were transformed into the dihydrofolate reductase (DHFR)-deficient Chinese hamster ovary (CHO) cell line DG44 - the most common mammalian host cell line for recombinant protein manufacturing (Kunert *et al*., 2016; Li *et al*., 2010; Frenzel *et al*., 2013). In this study, pOptiVEC-TOPO (Invitrogen) and pcDNA3.1 vectors were used as the backbone for generating populations of stably transfected cells in the selection medium. The DHFR protein-coding sequence from the pOptiVEC-TOPO vector is essential for CHO-DG44 cultures in a nutrient-deficient basal medium. Additionally, the antibiotic G418 resistance genes in the pcDNA3.3 vector facilitate the culture of CHO-DG44 cells during transient gene expression. Various combinations of plasmid constructs carrying HC and LC mAb genes are employed to establish stable cell lines.

Colony PCR was used to check the positive *E. coli* cells harboring recombinant vectors. All *E. coli* were amplified with forward primer paired with the pOptiVEC-TOPO vector and reverse primers paired directly with the LC constant regions, resulting in a fragment of *880 bp*. The PCR product size of the pOp-CD3ε-HC plasmid DNA from the selected single colonies was 1576 bp. The obtained results (Figure 1A) showed that all colonies of bacterial cells *carry* the recombinant plasmid pOp-CD3ε- LC.

Recombinant plasmids pc-CD3ε- LC and pc-CD3ε-HC were constructed by inserting DNA-*encoding antibodies* against human CD3ε mAb downstream of the CMV promoter located in the pcDNA3.3 *vector* backbone. Using primers flanking the CMV and reverse primers (only found in constant regions of mAb), we selected colonies with an expected molecular weight of 880 bp for the LC, and 1576 bp for the HC (including 702 bp for the LC and 1398 bp for the HC, adding 178 bp of the vector's sequences amplified by CMV primers).

The genes in the positive plasmids were confirmed by sequencing. The sequencing results revealed that plasmids pOp-CD3ε-LC, pOp-CD3ε- HC, pc- CD3ε- LC, and pc-CD3ε- HC harbored the genes encoding the HC and LC of mAb with the correct sequence and open reading frame (data not shown).

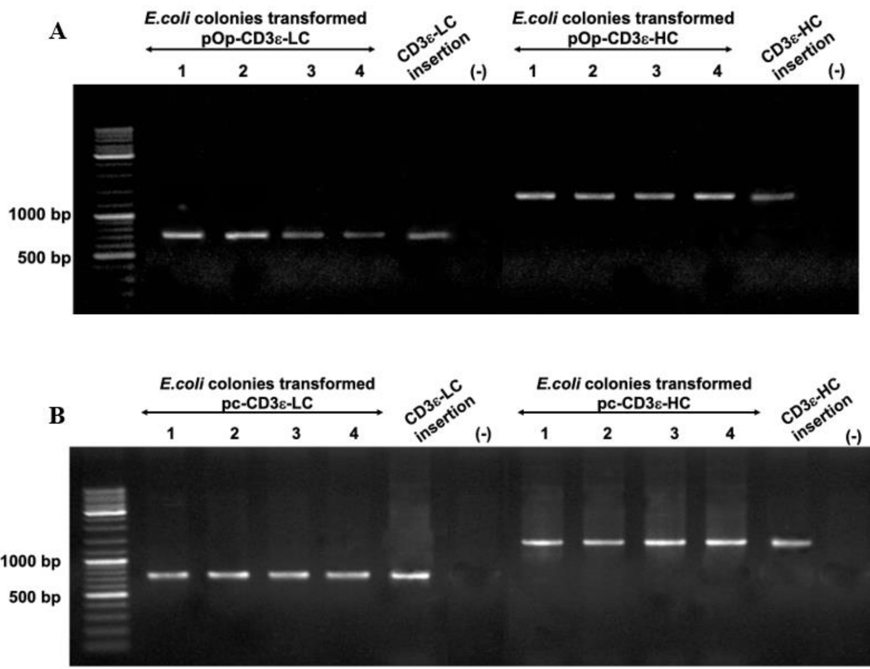

Figure 1. Analysis of amplicons amplified LC, HC genes from E. coli colonies transformed with recombinant vectors on agarose gels. (A) Colonies transformed with recombinant pOp-CD3ε- LC or pOp-CD3ε- HC. (B) Colonies transformed with recombinant pc-CD3ε- LC or pc-CD3ε- HC. Artificial gDNA encoding the heavy chain and lights chain of a monoclonal anti-CD3εmAb were used as a positive control for PCR colonies. H_2O was used as a negative control for PCR reactions.

Selection of the optimal ratio of heavy to LC genes for efficient recombinant mAb production by high protein concentrations and dimer structure

Four sets of constructs containing mAb HC and LC genes were tested to optimize the production of specific target antibodies. These sets included (1) pOp-CD3ε- HC + pc-CD3ε-LC, (2) pc-CD3ε- HC + pOp-CD3ε-LC, (3) pOp-CD3ε- HC + pOp-CD3ε-LC, and (4) pc-CD3ε- HC + pc-CD3ε-LC. Protein concentrations and dimer structure were analyzed across ratios of HC to LC genes encoded on separate plasmids, ranging from 12:1 to 1:12.

Results of ELISA analysis (Figure 2) recorded at a single wavelength of 450 nm showed that the mAb concentration obtained in the cell population transformed with different combinations of backbone vectors, pOp-CD3ε- HC + pc-CD3ε-LC or pc-CD3ε-HC + pOp-CD3ε-LC, was higher than those of other combinations, pOp-CD3ε- HC + pOp-CD3ε-LC and pc-CD3ε- HC + pc-CD3ε-LC. In the set of pOp-CD3ε- HC + pc-CD3ε-LC, the anti-CD3ε mAb yielded a maximum mAb titter at an HC gene ratio of 8:5 to 3:10. In the set ofpc-CD3ε- HC + pOp-CD3ε-LC, the appropriate ratio of heavy to LC genes was from 9:4 to 4:9. Meanwhile, mAb concentrations obtained from two populations of CHO-DG44 cells transformed with sets with the same backbone vector, pc-CD3ε- HC + pc-CD3ε- LC or pOp-CD3ε-HC + pOp-CD3ε- LC, showed no significant difference compared to the control CHO-DG44 cells carrying the empty vector.

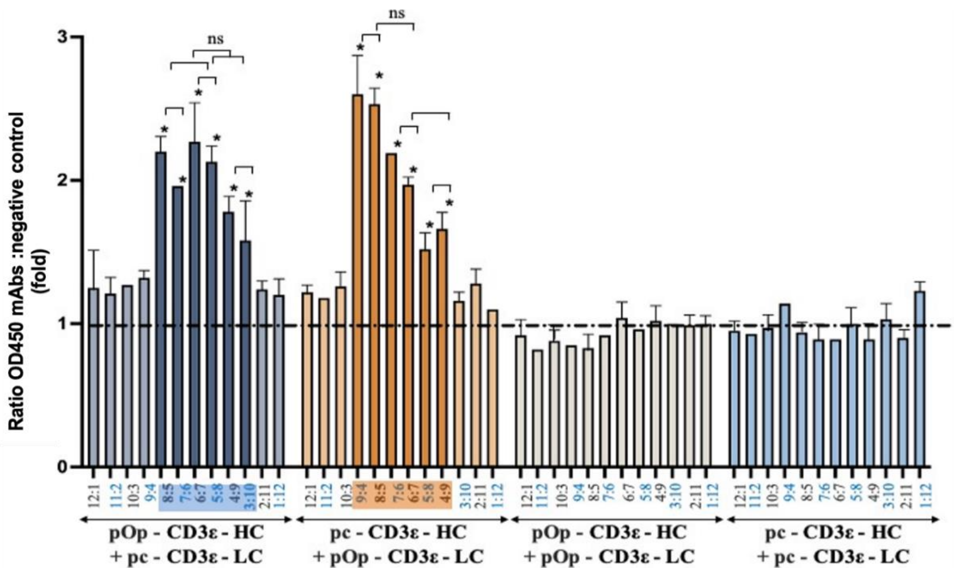

Figure 2. Comparison of the recombinant anti-Cd3 mAbs levels between CHO-DG44 populations transformed with combination sets of four constructs containing mAb heavy and LC genes by ELISA. Averages and SE bars are shown (n = 3). ns: non-significant, *p < 0.5.

The dimeric structure of anti-CD3ε antibodies is composed of two identical heavy and two identical LCs assembled via sulfide bonds of two adjacent Fc domains. The dimer structure of the recombinant anti-CD3ε mAb is crucial for the molecule's function. The purified mAb from culture supernatants at the optimal HC: LC ratios expressing the highest concentration were examined by SDS- PAGE Western blotting with anti-Fc IgG1 Ab (Invitrogen) under reducing (with β-mercaptoethanol) and non-reducing (without β-mercaptoethanol) conditions. The combination groups of 6 pOp-CD3ε-HC: 7 pc-CD3ε- LC and 7 pc-CD3ε- HC: 6 pOp-CD3ε- LC exhibited a high content of dimers and monomers, corresponding to 210 kDa for dimeric form, 55 kDa (HC) and 25 kDa (LC) for monomer, compared with other transformation rates (Figure 3).

Many studies have demonstrated that HC: LC ratios can play profound roles influencing on expression, aggregation, glycosylation, and conformation stability of proteins (Lee *et al.*, 2009; Schlatter *et al.*, 2005; Gonzalez *et al.*, 2002; Li *et al.*, 2007; O'Callaghan *et al.*, 2010). Excessive expression of HCs in CHO-DG44 cell lines stimulated aggregation, leading to proteasome overload and cellular stress. Additionally, the dimeric structure of the anti-CD3εmAb, composed of two HCs and two LCs linked via disulfide bonds, plays a crucial role in the biological activity of antibodies. By selecting the optimal HC: LC ratio in both mAb expression and structure, a ratio of 6:7 for pOp-HC: pc-LC and 7:6 for pc-HC: pOp-LC was chosen to generate mAb-producing cell lines.

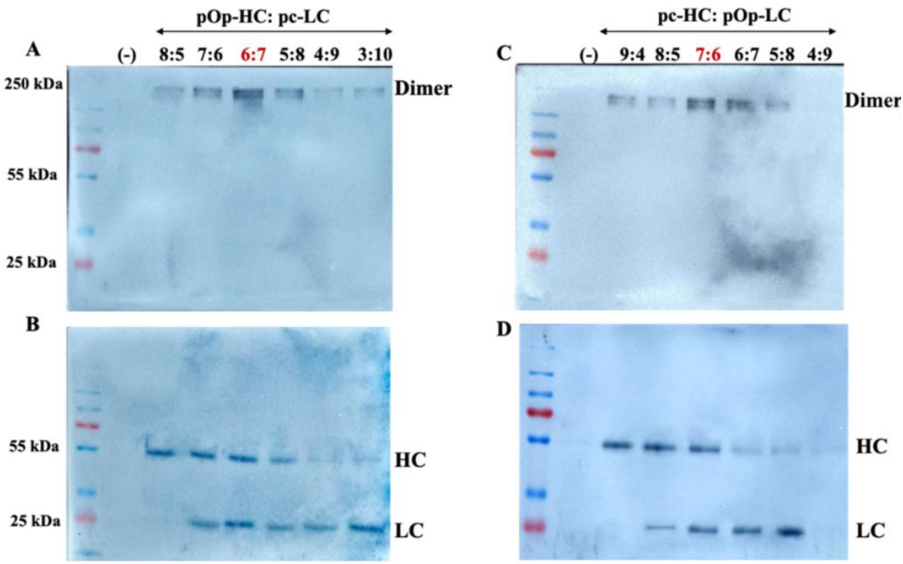

Figure 3. Investigation of recombinant anti-CD3εmAb structures by Western blot. A, C: Proteins were electrophoresed under non-reducing condition (without β-mercaptoethanol); B, D: Proteins were electrophoresed under reducing condition (with β-mercaptoethanol).

Selection of stable expressed CHO-DG44 cell lines with activity of anti-CD3εmAb quantification of T cell subsets profile in peripheral blood by using flow cytometry

We next evaluated the activity of the recombinant anti-CD3ε antibodies collected from two stable cell lines transfected with a 6:7 ratio of pOp-HC: pc-LC and 7: 6 of pc-HC: pOp-LC. The effect of the anti-CD3ε antibodies was analyzed in the quantification of human peripheral blood mononuclear cells (PBMCs) isolated from the blood of healthy donors. The combination of the cellular components measured by side scatter and CD45-positive cells was used for gating the T lymphocyte subpopulation. The efficiency of CD3ε antigen recognition by the recombinant anti-CD3εmAb produced in this study was compared with commercial anti-CD3ε antibodies (BD, US) and results of $CD3^{-}$ $CD4^{-}$ CD8 count diagnostic test conducted at the hospital on the same blood sample.

The results (Figure 4) indicated that the anti-CD3ε mAb obtained from the cells transfected with the pOp-HC + pc-LC plasmids quantified 81% of $CD45^{+}/CD3^{+}$ double-positive T cells, which is not significantly different with the result obtained using the commercial anti-CD3 reagent (83%). Meanwhile, only 46% of $CD45^{+}/CD3^{+}$ peripheral blood lymphocytes were counted by antibodies collected from the set of pc-HC + pOp-LC plasmids. $CD3^{+}$ T cells were not observed in negative controls, including the unstained PBMCs, PBMCs stained with secondary mAb, and PBMCs stained by mouse isotype IgG (Thermo, US).

The numbers of CD3 T cells quantified by the recombinant anti-CD3 mAb in the project were further compared to the results of the $CD3^{-}$ $CD4^{-}$ CD8 count diagnostic test from Cho Ray Hospital (data not shown). There was no significant difference between the number of $CD3^{+}$ T cells between the

hospital's results and quantification results of the anti-CD3ε expressed from a set of pOp-HC + pc-LC plasmid, which were 78.69% and 81%, respectively.

Functional differences in the quantification of CD3 antigen on peripheral blood T cells of the recombinant anti-CD3εmAb collected from two CHO-DG44 populations transfected set of pOp-HC + pc-LC or pc-HC + pOp-LC plasmids may be related to mAb purification. Our preliminary analysis showed that the purity of the anti-CD3 antibodies would be different depending on the set of co-transformed plasmids. The purity level of the anti-CD3εmAb from a set of pOp-HC + pc-LC plasmids was higher than that of a set of pOp-HC + pc-LC plasmids (data not shown).

These data suggested that the recombinant anti-CD3εmAb from stably expressed CHO-DG44 cell lines transfected with the set of 6 pOp-HC: 7 pc-LC plasmids has biological activity equivalent to commercial reagents when using flow cytometry. These antibodies were selected for further analysis.

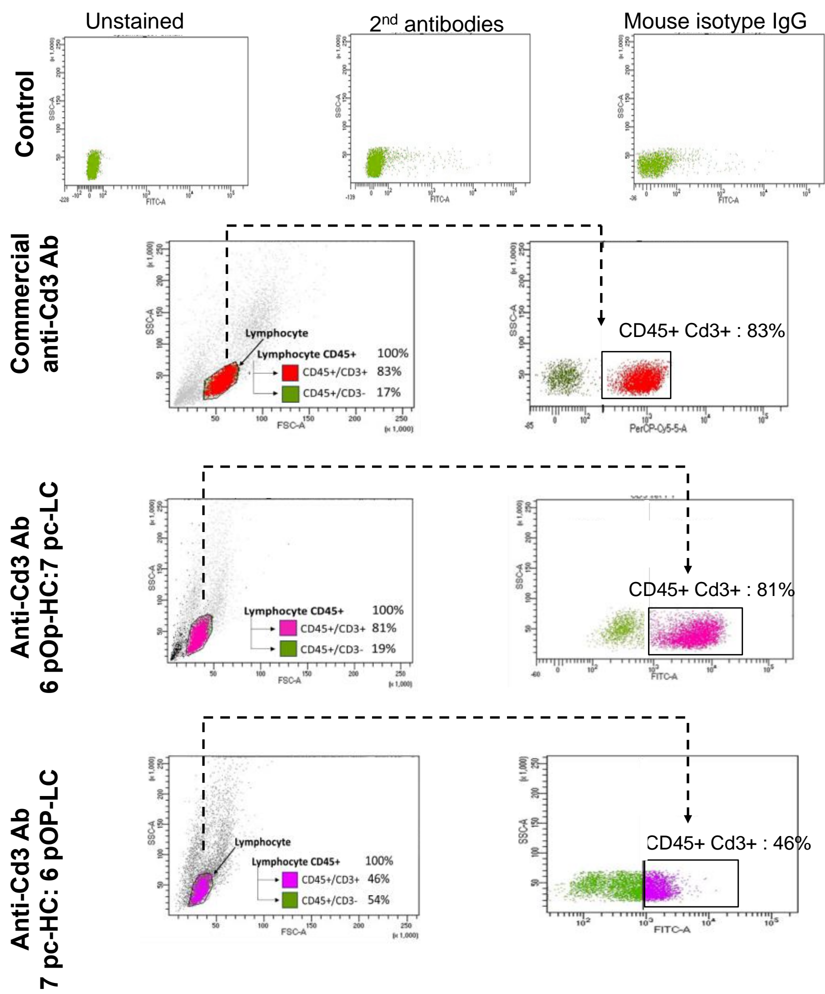

Figure 4. Selection of CHO-DG44 cell lines expressed the recombinant anti-CD3 antibodies with activity quantification of T cell subsets profile in peripheral blood by using flow cytometry.

Activity specifically to target antigen binding of the recombinant anti-CD3ε antibodies

Human peripheral blood comprises numerous cell types, including lymphocytes (T, B, and natural killer (NK) cells), monocytes, and dendritic cells. We investigated the efficiency of the anti-CD3εmAb on the recognition of antigen-specific T cells derived from PBMCs. The SUP -T1 cell line (ATCC, US) and Raji B cells (ATCC, US) were used as positive and negative controls. SUP-T1, human T cells lymphoblastic cells, expressed surfaceT cell-specific antigens such as Leu-6 $(CD1a)^+$ markers; Leu-4 $(CD3)^+$; Leu-3 $(CD4)^+$; Leu-1 $(CD5)^+$; Leu-9 $(CD7)^+$; Leu-2a $(CD8)^+$; OKT-10 (CD38) (Product information: ATCC, CRL-1942). In contrast, Raji B, human B lymphoblastoid cells, express antigens specific to B lymphocyte populations such as CD19, CD20, etc. (Bae *et al*., 2005; Product information -ATCC, CCL-86).

In this experiment, PBMCs were stained with anti-CD3ε antibodies obtained from CHO-DG44 transfected with a set of pOp-HC: pc-LC plasmids and/or commercial antibodies against common biomarkers to identify lymphocytes (anti-CD20 mAb (BD, US) for the B-cells, anti-CD56 mAb (BD, US) for NK cells), and monocyte populations (anti-CD14 mAb (BD, US)).

The FACS results (Figure 5) indicated that single-stained PBMCs identified 83% of $CD3^+$ T cells, 12.6% of $CD20^+$ B cells, 13.8% of $Cd56^+$NK cells, and 16.4% of CD14+monocytes. As expected, the combination of the recombination anti-CD3εmAb and commercial anti-CD20 Ab could differentiate T cells and B cells with 80.7% $CD3^+$ T cells and 10.1% $CD20^+$ B cells, equivalent to the percentage of these populations stained by only one mAb. Similar results were observed in evaluating the ability to differentiate $CD3^+$ T cells and $CD56^+$ NK cells, 80.7% for T cells and 14.9% for NK cells. Simultaneous staining of anti-CD3ε and anti-CD14 antibodies revealed two cell populations, corresponding to 79.3% $CD3^+$ T cells, and 16.7% $CD14^+$ monocyte. These percentages of cell subtypes match those of normal human peripheral blood from the Vietnam Ministry of Health (Vietnam Ministry of Health, 2014)

The FACS results (Figure 6) showed that 93% of the T-cell line SUP-T1, a positive control, was recognized by the recombinant anti-CD3ε mAb collected from stable CHO-DG44, equivalent to the commercial anti-CD3ε Ab. In contrast, the mAb did not identify $CD19^+$ B-cell line Raji B, a negative control (92.4% of Raji B cells were positive to the commercial anti-CD19 Ab).

Taken together, the recombinant anti-CD3ε mAb specifically recognizes T cells from other major human blood cell types, such as B cells, NK cells, and monocytes. These data collectively suggested that anti-CD3εmAb expressed by CHO- DG44 cells has the potential for further development towards the application of $CD3^+$ T cell quantification in disease diagnosis by using flow cytometry.

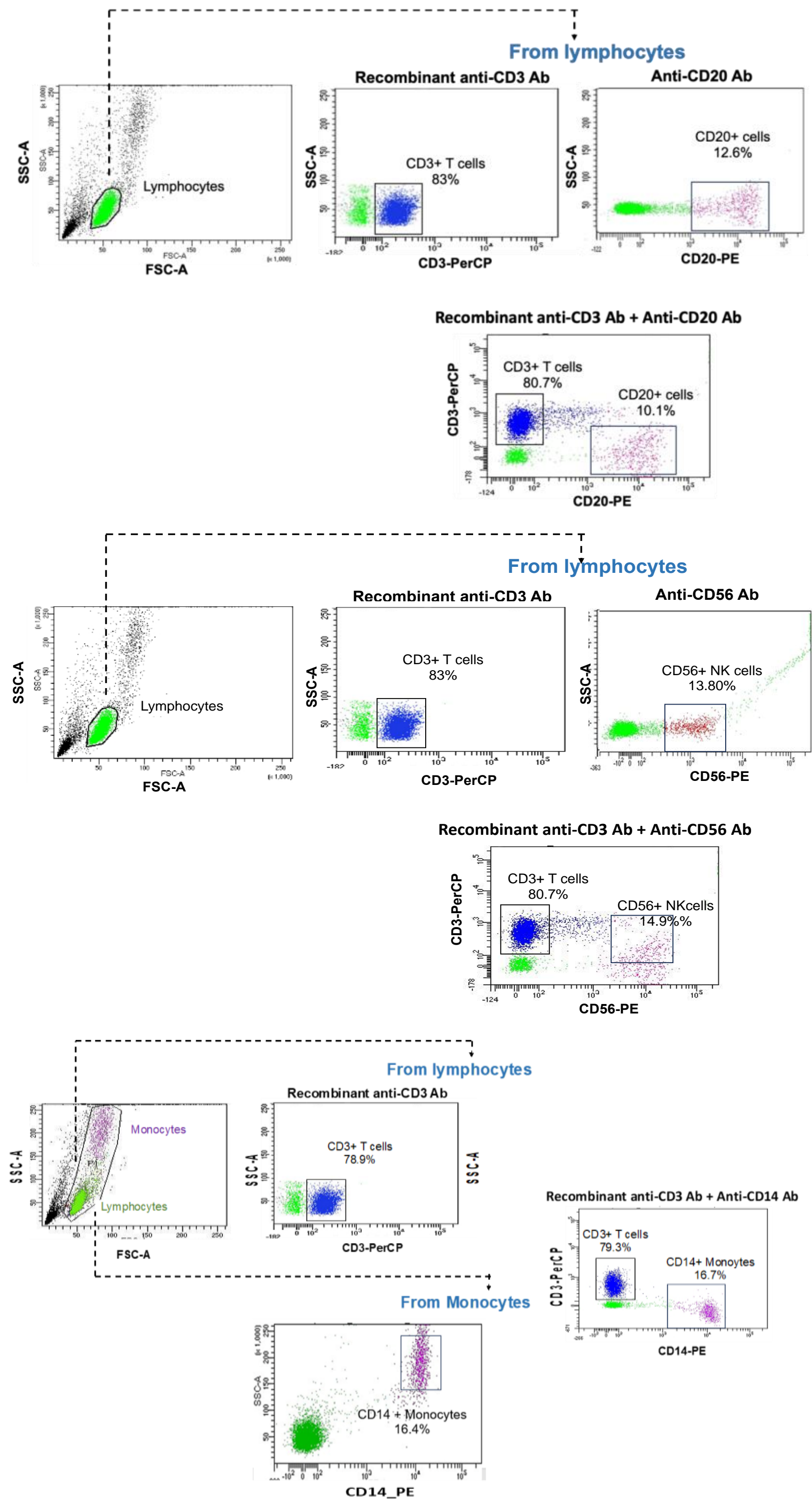

Figure 5. Activity specifically binding to T cells isolated from the normal human peripheral blood of the recombinant anti-CD3ε antibodies. PBMCs were stained by single or a combination of recombinant anti-CD3 mAbs and commercial antibodies against CD20, CD56, or CD14.

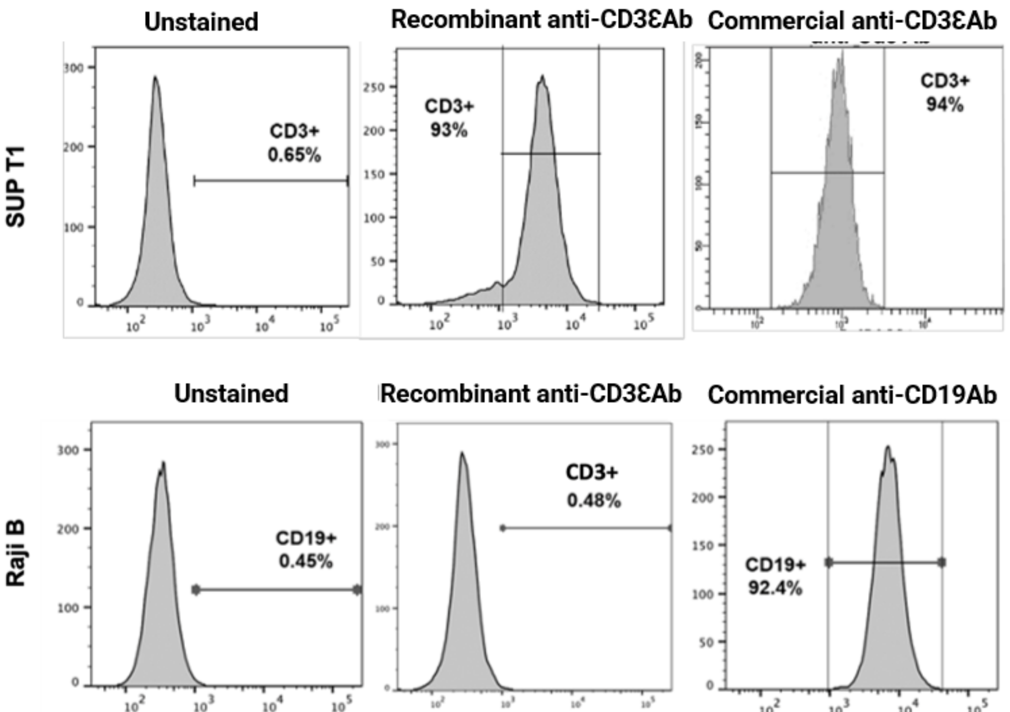

Figure 6. Activity specifically binding to the *cell-surface antigen* in vitro of the recombinant anti-CD3 antibodies. SUP-T1 and Raji B were stained by recombinant anti-CD3ε mAbs and commercial antibodies against CD3ε, and CD19.

ACKNOWLEGEMENT

The study received support from the Department of Science and Technology, Ho Chi Minh City, Vietnam (1046/QĐ-SKHCN signed Dec/29/2021). However, the funding bodies were not involved in the design of the study, collection, analysis, and interpretation of data, or in writing the manuscript.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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