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Overcoming the Challenge of Transduction of Human T-cells with Chimeric Antigen Receptor (CAR) Specific for ERBB2 Antigen

(Mengatasi Cabaran Transduksi Sel-T Manusia dengan Reseptor Kimera Antigen (CAR) Khusus kepada Antigen ERBB2)

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ABSTRACT

Breast cancer is one of the most common malignancies among woman. Decades of scientific study have linked the overexpression of ERBB2 antigen to aggressive tumors. To target aggressive breast cancer, chimeric antigen receptor (CAR) technology can be utilized. For this, human T-cells are transduced with a gene sequence encoding a CAR that is specific for tumor-associated antigens (TAAs). These genetically-engineered CAR transduced T-cells (CAR-T cells) are able to target the tumor antigen without the need for major histocompatibility complex (MHC) recognition, rendering it a potentially universal immunotherapeutic option. However, efficient transduction of therapeutic gene into human T-cells and further cell expansion are challenging. In this study, we reported a successful optimization of a transduction protocol using spinoculation on CD3+ T-cells with different concentrations of lentiviral plasmid encoding the CAR gene. CD3+T-cells were isolated from the peripheral blood mononuclear cells (PBMCs). The constructed CAR gene was inserted into a lentiviral plasmid containing the green fluorescent protein (GFP) tag and lentiviral particles were produced. These lentiviral particles were used to transduce activated T-cells by spinoculation. T-cells were activated using Dynabead-conjugated CD3/CD28 human T-cell activator and interleukin-2 (IL-2) before transduction. CD3+ T-cells were selected and GFP expression, which indicated transduction, was observed. Future studies will focus on in vitro *and* in vivo *models to determine the efficiency of CAR-T cells in specifically targeting ERBB2-expressing cells.*

Keywords: Breast cancer; CD3+ T-cells; chimeric antigen receptor (CAR); immunotherapy

ABSTRAK

Kanser payudara adalah salah satu kanser yang kerap melanda kaum wanita. Kajian saintifik telah mengaitkan lebihan ekspresi antigen ERBB2 pada barah kanser yang lebih agresif. Untuk menangani masalah ini, teknologi reseptor kimera antigen (CAR) boleh digunakan. Untuk ini, sel T manusia ditransduksi dengan urutan gen pengekodan CAR yang khusus untuk antigen berkaitan-barah (TAA). Sel T yang ditransduksi dengan CAR (CAR-T) secara genetik dapat mensasar kepada antigen kanser tanpa memerlukan pengenalan kompleks kehistoserasian utama (MHC), menjadikan ia pilihan terapi imun berpotensi umum. Walau bagaimanapun, transduksi gen terapeutik ke dalam sel T manusia dan pengembangan sel selanjutnya adalah mencabar. Dalam kajian ini, kami berjaya melaporkan pengoptimuman protokol transduksi menggunakan spinokulasi sel T CD3+ dengan kepekatan plasmid lentiviral pengekodan gen CAR yang berbeza. Sel T CD3+ telah diasingkan daripada sel-sel mononuklear darah periferi (PBMCs). Gen CAR yang dibina dimasukkan ke dalam plasmid lentiviral mengandungi protein pendarfluor hijau (GFP) dan zarah lentiviral penuh dihasilkan. Zarah lentiviral digunakan untuk transduksi spinokulasi T-sel yang telah diaktifkan. Sel T diaktifkan menggunakan CD3 berkonjugasi Dynabead/pengaktif manusia sel T CD28 dan interleukin-2 (IL-2) sebelum transduksi. Kejayaan transduksi dilaporkan apabila ekspresi GFP diperhatikan di dalam sel T CD3+. Kajian masa depan akan memberi tumpuan kepada model in vitro *dan* in vivo *untuk menentukan kecekapan sel CAR-T dalam mensasarkan dan membunuh sel kanser yang mempunyai ekpresi ERBB2 berlebihan.*

Kata kunci: Kanser payudara; reseptor kimera antigen (CAR); sel CD3+ T; terapi imuno

INTRODUCTION

T-cells of the immune system can eradicate disease at extreme specificity by identifying the antigen expressed by the antigen presenting cells (Maher 2012). T-cell receptors (TCRs) present on the T-cells engage antigens that are presented by the major histocompatibility complex (MHC) molecules (Maher 2012). In most inflammatory conditions, an adaptive immune response is triggered following the presentation of peptides (non-self) by MHC molecules to immune cells. However, during tumor progression, the

immune system often perceives tumor antigens as 'self' rather than foreign, hence becomes tolerant to tumors (Whiteside 2010).

To overcome this challenge, researchers designed genetically-modified T-cells to specifically target the desired tumor-associated antigens (TAAs) (Jena et al. 2010). The T-cells were equipped with a Chimeric Antigen Receptor (CAR). A CAR links an antigen specific single-chain antibody fragment (scFv) to intracellular signalling domains, CD28 and CD3-ζ chain of the T-cell receptor (TCR) (Porter et al. 2011). The scFv segment of the CAR is able to directly target and bind to the TAA on the tumor cells, bypassing the MHC immune recognition (Maher 2012). Thus, genetically-modified T-cells to express CAR retain its immunogenic functions against tumor cells.

Human epidermal growth factor receptor 2 (ERBB2) is an antigen of interest for CAR technology because ERBB2 overexpression in breast cancer (occurring in 15-25% of cases) (Iqbal & Iqbal 2014) is associated with increased tumor aggression, resistance to chemotherapeutics and ultimately poor patient prognosis (Eroles et al. 2012). ERBB2 overexpression is also observed in endometrial cancer (12% of cases) (Elsahwi & Santin 2011), gastric and esophageal cancer (15-20% of cases) (Iqbal & Iqbal 2014), in addition too varian (Teplinsky & Muggia 2015), stomach (Iqbal & Iqbal 2014), bladder (Iqbal & Iqbal 2014) and lung carcinomas (Iqbal & Iqbal 2014) (Figure 1). ERBB2 positive breast cancer patients are treated with anti-ERBB2 therapy in addition to surgery, chemotherapy and radiation (Carey 2011). Treatment options for anti-ERBB2 therapies include the monoclonal antibodies trastuzumab and pertuzumab (Scott et al. 2012). Hormone therapies such as tamoxifen (Indah et al. 2015) and aromatase inhibitors (Dieci et al. 2016) are also available.

Current clinical trials trend is moving towards immunotherapeutic strategies such as checkpoint inhibitors, oncolytic viruses and anti-ERBB2 intracellular single-chain antibodies (Marchini et al. 2016). Novel avenues such as ERBB2-specific tyrosine kinase inhibitors (i.e. emodin) (Schroeder et al. 2014), ERBB2-targeting antisense oligonucleotides (Han et al. 2013) and rationally designed anti-ERBB2 peptide mimetics (Govindarajan et al. 2012) are also undergoing different stages of clinical trials (Figure 1). The limitations of chemotherapy and radiation include the fact that they not only affect cancerous cells, but also target all actively dividing healthy cells. This can lead to prominent side effects including loss of appetite, hair loss, nausea and vomiting, extreme fatigue, mouth soreness, bleeding and low immunity against infections (Carey 2011) (Figure 1). Thus, ERBB2 is a suitable TAA for use in CAR technology.

The genetic-modification of T-cells to express CAR involves the delivery of *CAR* gene via viral transduction into autologous human T-cells. A huge problem concerning T-cell manipulation involves the foremost step of isolating a pure population of T-cells from patient's blood for use in immunotherapy (Stemberger et al. 2014). Previous studies adopting the CAR method have largely manipulated the CD8+ T-cells sub-population within the CD3+ T-cells (Jensen et al. 2010; Zhong et al. 2009). However, achieving clinically significant numbers via *ex vivo* expansion of this modified population of CD8+ T-cells are difficult (Maher 2012). Furthermore, primary human T-cells are resistant to transduction by virus, as naïve T-cells are non-proliferating or quiescent cells *in vitro*. *CAR* gene integration into the

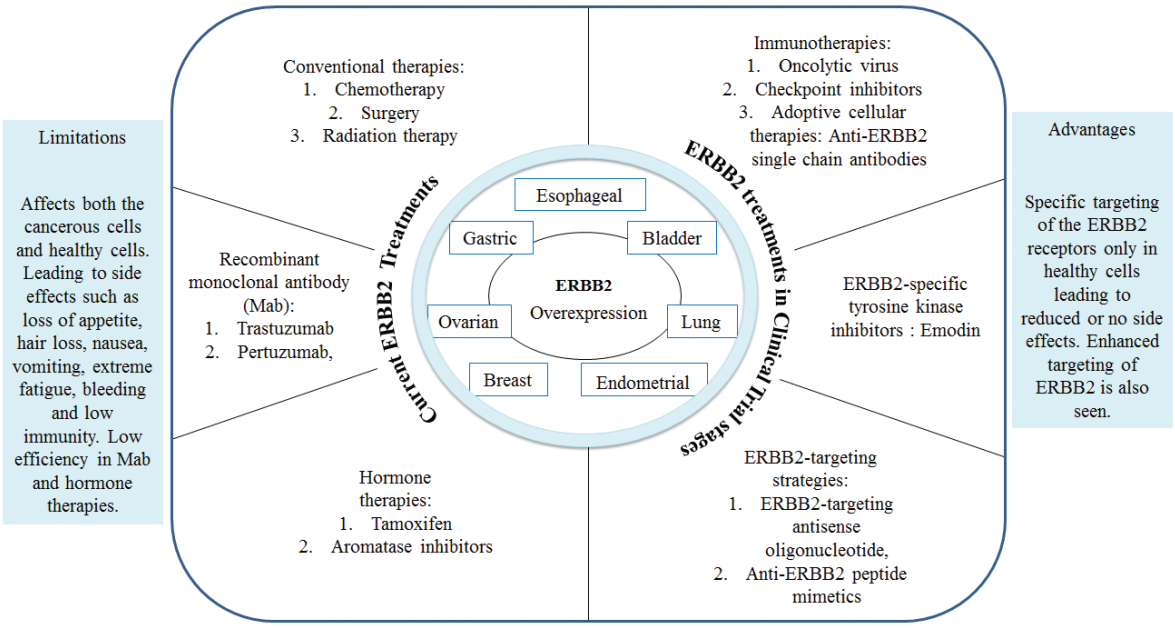

FIGURE 1. The schematic diagram portraying the trends of therapeutic options in ERBB2 overexpressing cancer: advantages and limitations. The diagram shows overexpression in multiple cancers, the current treatment options for ERBB2-positive cancers (breast cancer), and clinical trials for targeting ERBB2 with its limitations and advantages

genome of the transduced T-cells will also require prior activation through the TCR and cytokines (Zack et al. 2013). In order to overcome the multitude of challenges involved in the *CAR* gene delivery into T-cells, we optimized a robust protocol for the fast and efficient transduction of *CAR* into human T-cells.

In the current study, we aimed to optimize a transduction protocol to efficiently deliver *CAR* gene into human CD3+ T-cells. The *CAR* gene consists of an anti-ERBB2 scFv extracellular domain followed by the CD8 transmembrane domain and two co-stimulatory domains, namely, CD28 and CD3-ζ (ERBB2 scFv-CD8a-CD28-CD3-ζ). This would be able to specifically target ERBB2 antigens on tumor cells with increased efficiency and reduced side effects.

Material and Methods

HUMAN T-CELLS CULTURE AND EXPANSION

Ten milliliters of collected blood samples from healthy donors (National Medical Research Register, Malaysia; ethical approval number: NMRR-14-587-20201) were processed to isolate peripheral blood mononuclear cells (PBMCs) by density gradient separation using Ficoll-Paque PLUS (1.077 g/mL, Amersham Biosciences, Sweden). Isolated human PBMCs were cultured in advanced RPMI 1640 media (Gibco/Invitrogen, Massachusetts, USA), supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (Pen/Strep), and 0.1% gentamycin at 37°C and 5% CO_2 concentration. CD3+ cells were then isolated from PBMCs using an EasySep™ Human T Cell Enrichment Kit (Stem Cell Technologies,Vancouver, Canada) following manufacturer's protocol. The isolated CD3+ T-cells in complete culture media were mixed with Dynabead-conjugated CD3/CD28 human T-cell activator (1:10 ratio) (Gibco/Invitrogen, Massachusetts, USA) and 50 U/mL of interleukin-2 (IL-2) (PeproTech, Rocky Hill, USA) for activation.

FLOW CYTOMETRIC ANALYSIS

To detect surface antigens, aliquots of cultured T-cells were washed twice with phosphate-buffered saline (PBS) (Gibco/Invitrogen; Massachusetts, USA), pH 7.2 after centrifugation at 1000 × g for 5 min. The cells were incubated with fluorescein isothiocyanate (FITC), allophycocyanin (APC), phycoerythrin (PE) or phycoerythrin Cyanine7 (PE-Cy7) conjugated monoclonal antibodies on ice for 45 min. The antibodies used were CD3, CD4, CD8, CD25 and CD69, all purchased from Affymetrix eBioscience, Inc. (San Diego, USA). After incubation, the cells were washed twice by centrifugation at 300 × g for 5 min and subjected to flow cytometric analysis. In parallel, unstained and fluorochrome matched, non-specific isotype labeled cells were used as controls. The stained samples were assessed using BD FACS Canto II (Becton Dickinson (BD) BioSciences; New Jersey, USA). Gating at FACS acquisition was applied to exclude any dead cells and cell debris. Ten thousand events were acquired and the data from stained cells were acquired using FACSDiva 6.1.3 software (Becton Dickinson (BD) BioSciences; New Jersey, USA).

PLASMID CONSTRUCTION

pReceiver-Lv 183 lentiviral expression plasmid (GeneCopoeia Rockville, USA) containing a green fluorescent protein (GFP) selection marker located downstream of the inserted *CAR* target gene was used. The *CAR* gene was synthesized according to the WO2012031744 (NCBI accession number: JB399738) patent number and consisted of 1515 bp.

LENTIVIRAL PARTICLE PRODUCTION

The Lenti-Pac™ HIV Expression Packaging Kit from GeneCopoeia (Rockville, MD, USA) was used in combination with the lentiviral expression plasmid (pReceiver-Lv 183) containing the *CAR* gene to produce complete viral particles. The lentiviral vectors were produced using the packaging cell line, 293FT. A total of 2.5 µg, 4.0 µg or 5.5 µg of lentiviral expression plasmid and 5.0 µL (0.5 µg/µL) of Lenti-Pac HIV mix were diluted in a sterile tube containing 200 µL of Opti-MEM® I (Gibco/Invitrogen; Massachusetts, USA). In a separate tube, 15 µL of EndoFectin Lenti reagent was diluted into 200 µL of Opti-MEM I. The diluted EndoFectin Lenti reagent was then mixed with the DNA solution before adding it to the 293FT cells. Cells were then incubated in a CO_2 incubator at 37°C. After 12 h, the overnight culture medium was replaced with fresh DMEM with 5% FBS and 1% Pen/Strep. The pseudovirus-containing culture medium was collected at 48 h post-transfection. The supernatant was then concentrated using amicon viral concentration ultrafiltration 100 kDa tubes (Merck Milipore; Darmstadt, Germany) with centrifugation at 1200 × g for 15 min at 4°C. The freshly collected concentrated virus supernatant was used for transduction of T-cells.

TRANSDUCTION OF *CAR* GENE INTO T-CELLS VIA LENTIVIRUS EXPRESSION SYSTEM

A total of 2×10^5 T-cells were plated on a 24-well plate 24 h before viral infection. Viral media were prepared with 0.5 mL of virus suspension diluted in complete medium with polybrene (8 µg/mL). The target CD3+ T-cells were infected by adding the viral media. For the control well, 0.5 mL of complete medium with polybrene only was added. The cells were spinoculated at 800 × g for 1.5 h at 32°C. The plates were incubated overnight in a CO_2 incubator. The culture media were replaced with fresh complete media (without polybrene) 12 h after transduction. The infected target T-cells were analyzed for transgene expression using the internal GFP control via fluorescence microscopy.

Results

Efficient isolation of CD3+ T-cells from human PBMCs using magnetic selection

Fresh blood was collected and the PBMC layer was isolated by density gradient centrifugation. To further enhance the lymphocyte concentration for downstream experimental work, the PBMC layer was subjected to magnetic separation of CD3+ T-cells using the EasySep™ Human T Cell Enrichment Kit. Our results showed an increase in CD3+ T-cells after magnetic selection (Figure 2). The selection process was extremely efficient in enriching for a homogenous T-cells population by depleting all other PBMCs and as a result, the T-cells population was increased from 78.2% to 99.4%. The isolated CD3+ cells were activated for transduction and expansion.

Successful activation of isolated CD3+ T-cells

Morphological observation of CD3+ T-cells showed that non-activated T-cells appeared as single spherical suspension in culture. Meanwhile, activated T-cells supplemented with Dynabead-conjugated CD3/CD28 human T-cell activator and IL-2 appeared as clumped cells (Figure 3(A)). Figure 3(B) shows the flow cytometric analysis of T-cell activation. Non-activated CD3+ T-cells showed presence of 62.1% of CD4 and 32.6% of CD8+ cells. The number of cells expressing CD25 and CD69 activation markers were 0.1% and 0.2%, respectively. Activation of CD3+ did not affect changes in the population of CD4 (65.1%) and CD8 cells (34.4%). However, the percentage of cells expressing of CD25 and CD69 activation markers have increased to 15.5% and 14.7% (Figure 3(B)). This indicates that the activation protocol employed was successful in inducing activation of T-cell, priming them for efficient transduction. This result was consistent between variations in variables including the donor and passage of the cells (data not shown). All flow cytometry dot plots were compared to specific isotype controls.

Production of CAR lentiviral particles and human T-cell transduction

We transfected 293FT packaging cells using three different concentrations of the *CAR* transfer plasmid (2.5, 4.0 and 5.5 µg) to produce lentivirus particles (Figure 4). From Figure 4, we observed that GFP expression using 5.5 µg of the *CAR* transfer plasmid was visibly higher than that of 2.5 µg or 4.0 µg of the *CAR* transfer plasmid in the 293FT cells 48 h post-transfection. Following that, the supernatants were

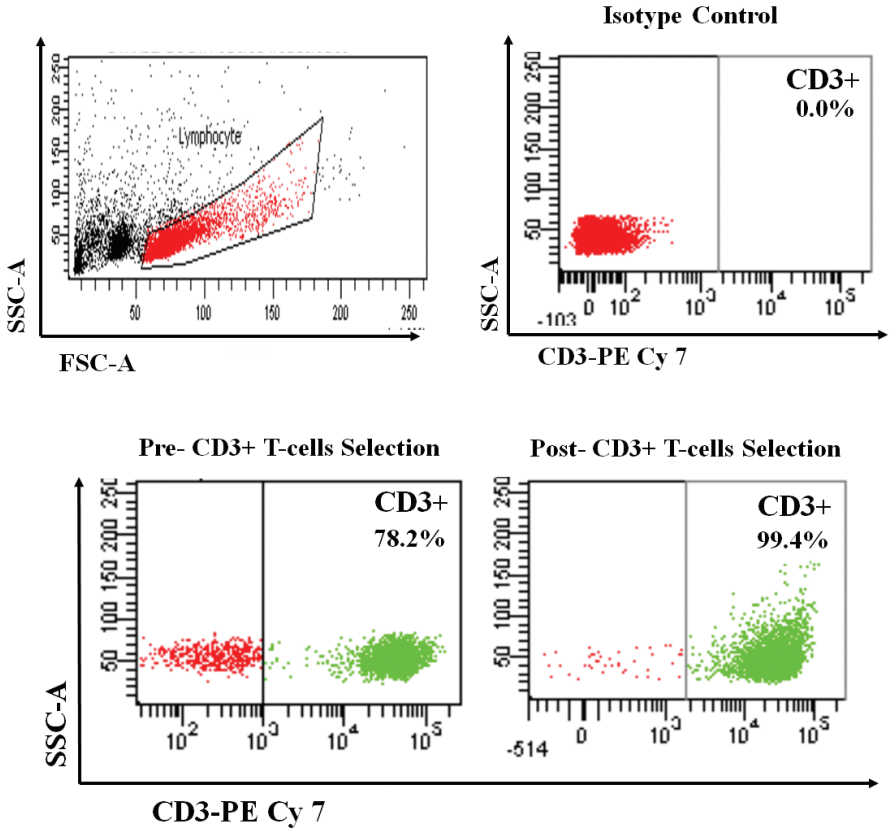

FIGURE 2. Immunophenotyping of peripheral blood mononuclear cells (PBMCs) pre- and post-CD3+ magnetic selection. Fresh PBMCs were subjected to CD3+ magnetic selection and immediately characterized by flow cytometry. An increase in CD3+ T-cells from 78.2% pre-magnetic selection to 99.4% post-magnetic selection was seen. CD3+ isotype-matched antibody was used as a negative control. All dot plots shown were gated from the lymphocyte population of the FSC-A *vs*. SSC-A plot

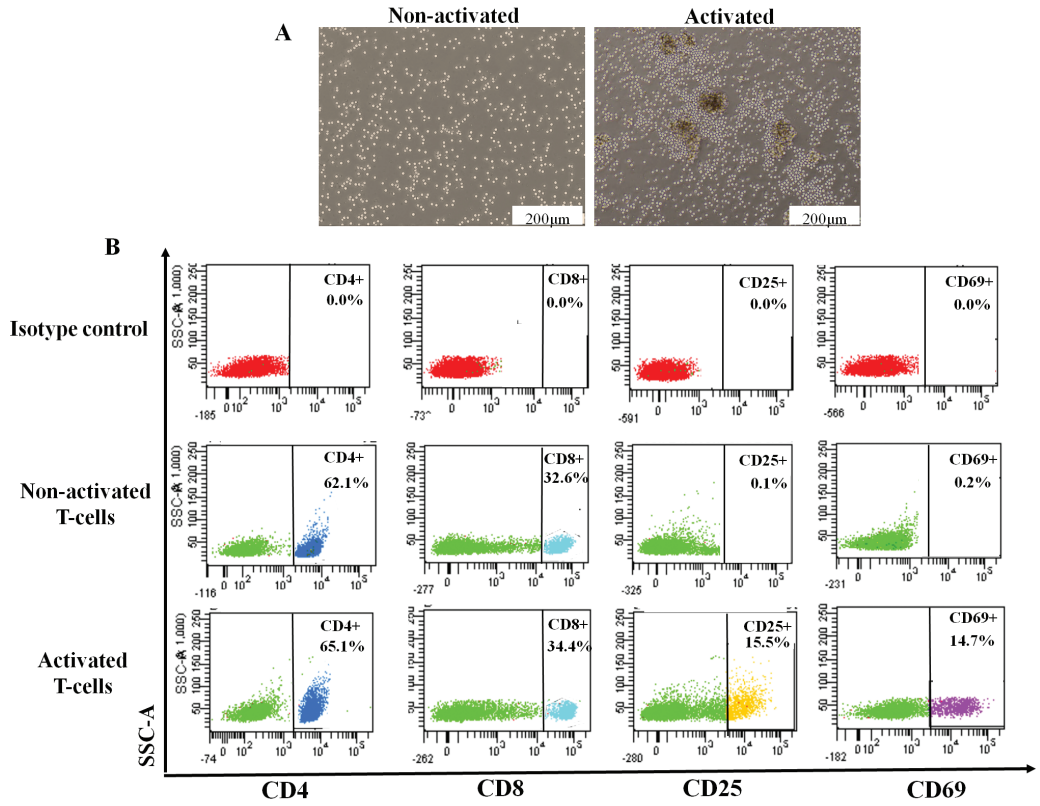

FIGURE 3. Activation in T-cells by Dynabead-conjugated CD3/CD28 human T-cell activator. (A) Morphological changes upon activation, (B) Flow cytometric analysis of T-cell activation surface markers. (A) Morphological observation of CD3+ T-cells: non-activated T-cells in culture appear as spherical shaped suspension cells while activated T-cells supplemented with Dynabead-conjugated CD3/CD28 human T-cell activator and IL-2 appear as darker clumped cells that formed suspension spheres, Figure 3(B) flow cytometric analysis of T-cell activation cell surface markers. Isotype control of flow cytometry results indicated for surface markers CD4, CD8, CD25 and CD69, showing 0.0%. Non-activated T-cells showed T-cell markers such as CD4 (62.1%) and CD8 (32.6%) while activation markers CD25 (0.1%) and CD69 (0.2%) are negative. Activated T-cells showed T-cell markers such as CD4 (65.1%), CD8 (34.4%) and activation markers CD25 (15.5%) and CD69 (14.7%) are shown to be significantly increased compared to the non-activated T-cells. All flow cytometry dot plots were compared to specific isotype controls. All microscopic images shown are at a magnification of 100× with scale bar = 200 µm

harvested from the 293FT cells culture and pooled for transduction into activated CD3+ T-cells via spinoculation. A relatively higher number of T-cells expressing green fluorescence were observed when transduced with supernatants collected from 5.5 µg compared to 2.5 µg or 4.0 µg of the *CAR* transfer plasmid.

DISCUSSION

The CD3 surface marker is expressed on all T-cells and is associated with TCR (Ahmadi et al. 2011). An approximate of 70-80% of human peripheral blood lymphocytes are composed of CD3+ T-cells whereas only 30% of the CD3+ T-cells is comprised of CD8+ T-cells (Rivkina et al. 2015; Sinon et al. 2013). The pre-selection of CD3+ T-cells to ensure a homogeneous T-cell population is vital, as the homogenous equilibrium of cells in patient's blood can be interrupted by malignant proliferative changes generated by cancer (Rivkina et al. 2015). Deriving blood from cancer patients for an autologous immunotherapy without extensive pre-selection will affect the efficiency and might result in unwanted complications during *in vivo* clinical applications. Thus, for this purpose, we have proposed that selecting T-cells based on CD3+ T-cell marker is not only sufficient but superior to selecting T-cells at the sub-population of CD8+. Selecting patient sample at CD3+ T-cells would overcome the challenge of limited cell availability and proliferative capability conferred by CD8+ T-cells. Furthermore, cancer immunotherapy is dependent on the ability of CD3+ T-cells to home and eradicate tumor cells *in vivo* (Bollard et al. 2012).

Transduction of primary human T-cells poses a problem as T-cells are non-dividing quiescent cells *in vitro* (Zack et al. 2013). Multiple strategies can be adopted to increase the viral titer and transduction efficiency, including optimization of the transfer plasmid and viral particle concentrations and modifications to the culture conditions such as spinoculation (Cribbs et al. 2013). Lentiviral vectors are favored for advantageous traits such as the ability to transfer genes into both dividing and non-dividing T-cells; in contrast, retroviral methods typically can only be used to transfer genes into dividing cells (Cribbs et al. 2013). As non-activated T-cells are primarily non-dividing cells, lentivirus allows for more stable and long-term transduction

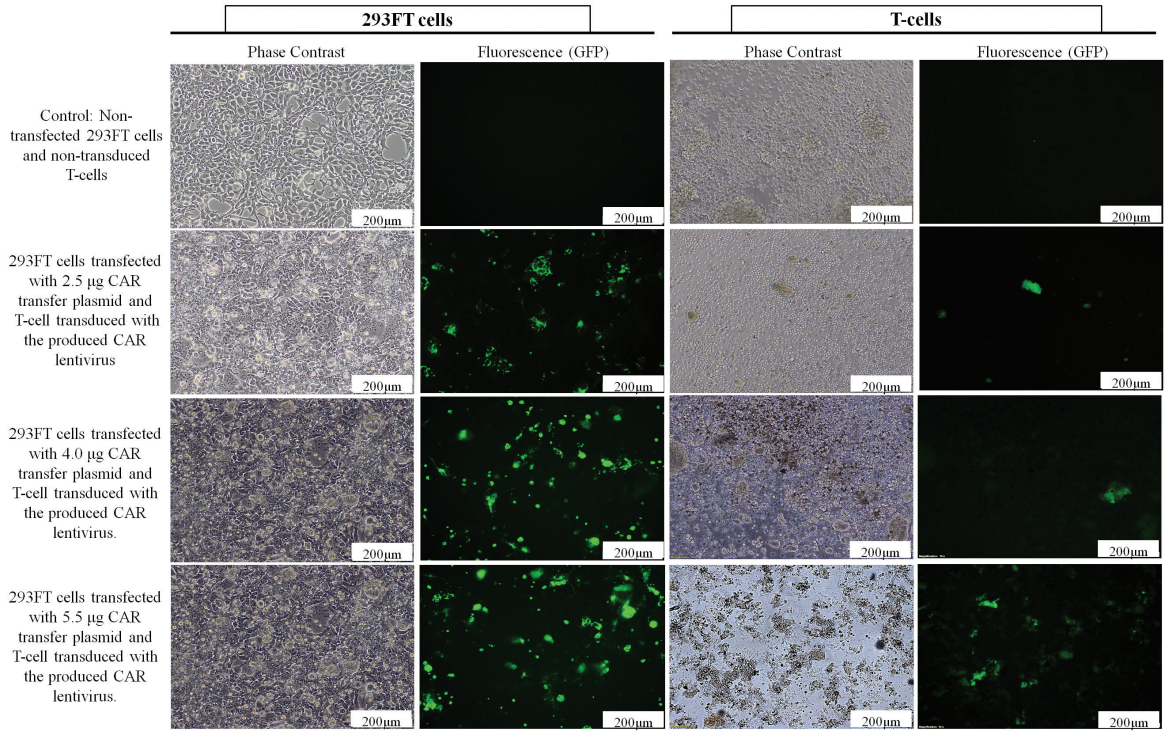

FIGURE 4. Phase contrast and fluorescence (GFP) imaging of 293FT cells and T-cells. 293FT cells were transfected with either control (non-transfection) or 2.5 µg, 4.0 µg and 5.5 µg of *CAR* transfer plasmid. The corresponding transduction of control (non-transduced), 2.5 µg, 4.0 µg and 5.5 µg of *CAR* lentivirus particles into T-cells is also shown. Increased GFP expression was seen when higher concentrations of *CAR* transfer plasmid were added (5.5 µg). The increase in T-cells expressing GFP indicated a higher rate of transduction into T-cells and the successful expression of GFP. All microscopic images shown are at a magnification of 100× with scale bar = 200 µm

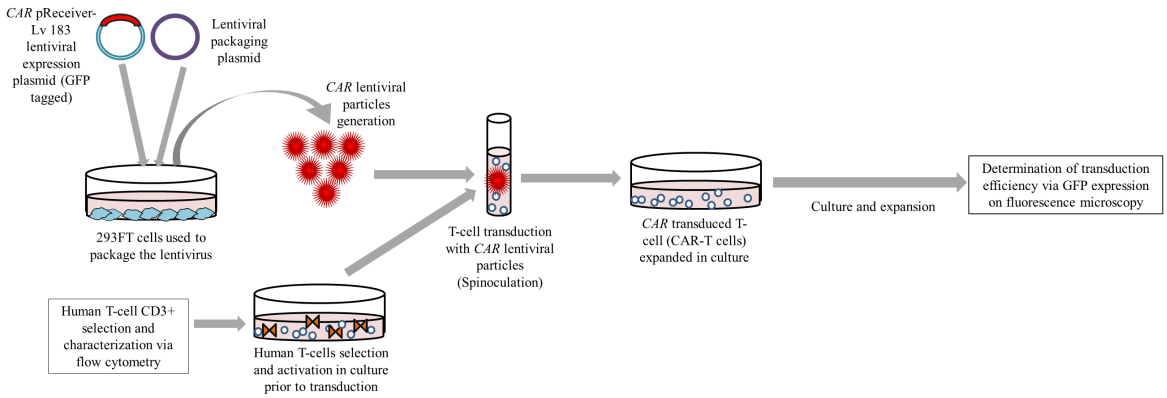

FIGURE 5. Schematic representation of the complete experimental protocols employed in this study. The protocols are schematically shown, including human T-cells isolation, activation, culture, transduction and ultimately determination of transduction efficiency via fluorescence microscopy. The *CAR* lentiviral particles generation is also portrayed

of cells with a lower tendency towards gene silencing, when compared to retroviruses (Shaw & Cornetta 2014).

In the current study, isolated PBMCs were subjected to magnetic separation to generate a highly homogenous CD3+ T-cells population (Figure 2). The isolated CD3+ T-cells were activated and transduced with lentiviral particles encoding the *CAR* gene. Figure 5 shows a schematic representation of the complete experimental protocols employed in this study, including human T-cells isolation, activation, culture, transduction and ultimately determination of transduction efficiency via fluorescence microscopy.

Here, we demonstrated that activation of the isolated CD3+ T-cells with Dynabead-conjugated CD3/CD28 human T-cell activator and interleukin-2 (IL-2) before transduction and optimization of the *CAR* transfer plasmid at 5.5 µg of concentration in a fast and efficient method via spinoculation could result in increased transduction efficiency. Higher transduction efficiency is vital for downstream applications to evaluate CAR-T cell targeting

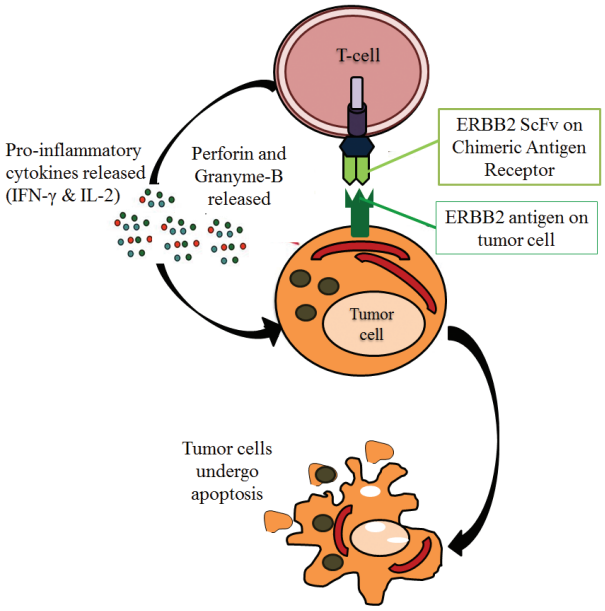

FIGURE 6. Schematic representation of the CAR-T cells killing mechanism of action. The apoptosis-inducing mechanism of CAR-T cells is triggered upon binding to the ERBB2 antigen on tumor cells, resulting in the release of perforin and granzyme B in addition to an increase in pro-inflammatory cytokine secretion, including interferon-gamma (IFN-γ) and interleukin-2 (IL-2). Copyright 2013. Modified with permission from Landes Biosciences & Creative Commons Attribution License (Oberoi & Wels 2013)

of ERBB2-overexpressing cancer cell lines. This, in turn, is crucial for the downstream application of evaluating the apoptosis-inducing function of CAR-T-cells on ERBB2-overexpressing cancer cell line.

CAR-T cells could mediate cytolysis predominantly by the action of granzyme-B and perforin (Chmielewski et al. 2013). Upon activation by a target cell via the binding of the anti-ERBB2 scFv segment on the CAR-T cells to the ERBB2 antigen on the tumor cells, the CAR-T cells trigger immune activity. CAR-T cells execute the exocytosis of secretory granules containing perforin and the pro-apoptotic serine protease, granzyme B (De Saint Basile et al. 2010), which induces apoptosis by cleaving critical substrates in tumor cells (Chmielewski et al. 2013). Figure 6 is a schematic representation of the apoptotic mechanism exerted by CAR-T-cells upon binding to ERBB2 antigens on a tumor cell. Following ligation, CAR-T cells release perforin and granzyme B to increase the secretion of pro-inflammatory cytokines such as interferon-gamma (IFN-γ) and interleukin-2 (IL-2).

CONCLUSION

The current study was able to optimize an efficient method for the transduction of the *CAR* gene specific for ERBB2 antigen into human CD3+ T-cells via spinoculation. We overcame the challenges of transducing primary T-cells by selecting at CD3+ T-cell marker, activating the T-cells prior to transduction and increasing the *CAR* lentiviral transfer plasmid concentration. Future studies should determine the efficiency of specific targeting and killing mediated by the transduced CAR-T-cells upon co-culture with the ERBB2-overexpressing cancer cell line.

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