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Original Article

Generation of CD19-Targeted Chimeric Antigen Receptor T Cells

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Abstract

Background: Current advancements in the field of chimeric antigen receptor (CAR) therapy, particularly U.S. FDA approval of Kymriah and Yescarta, heralds a new era of cancer treatment. This rapid progress in technology has urged more countries and institutions to keep pace with the fast-growing and developing technology of producing CAR T cell-based therapies in the race to develop new cancer-targeting drugs. Hence, for stepping in line with global advances and to pave the way for subsequent preclinical and clinical studies, we have established a development protocol for a cancer-targeting CAR T cell; we have chosen CD19 CAR T cell as a well-defined model to set-up T cell expansion, activation, and viral transduction as the prerequisites for diverse CAR T cell therapies.

Methods: T cells from peripheral blood mononuclear cells (PBMCs) were activated and expanded. CD19 CAR lentiviral particles were produced in the Lenti-X™ 293T Cell Line using PolyFect Transfection Reagent.

Results: Activation protocol resulted in (65 ± 4%; $P = 0.046$) increase in the rate of activated T cells 24 hours after the initiation of the procedure. The expansion methodology resulted in a high purity of the T cell population (96 ± 3%) in the pool of PBMCs within 14 days of the procedure. Finally, 35 ± 6% of T cells were transduced with CD19 lentivirus with MOI of 3.

Conclusion: Collectively, the results of this study prove that we have successfully overcome the first hurdle on the road to reach CAR T cell technology which is the prerequisite for developing preclinical and clinical phases of CAR therapy in settings with basic resources.

Keywords: B-cell malignancies, CAR T cell, CD19, Chimeric antigen receptor

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Introduction

In recent years, scientists have learned to direct the immune potency of T cells against desired antigens in the form of an engineered T cell named chimeric antigen receptor (CAR) T cell.[1] These new engineered T cells are capable of targeting non-immunogenic and even self-antigens that are expressed by normal tissues as well as cancer cells, a feature that broadens the range of target antigens that could be struck by an immune response.[2] This versatility in targeting originates from the modular design of CAR molecules comprised of a single chain fragment variable (scFv) recognizing the desired antigen and a multi-segmented signaling moiety that activates the T cell and promotes its function.[3] Moreover, unlike naive T cells, CAR T cells have the merit of not relying on MHC molecules to recognize their antigenic targets, enabling them to target intact antigenic molecules.[4] All these capabilities have boosted research on designing CAR T cells that strike different antigenic targets for a variety of malignancies. While few well-defined antigens are available for most malignancies, CD19 has been known for years as an appropriate tumor-associated antigen (TAA) for B cell malignancies and thus, it is an ideal target for immunotherapies.[5] This intriguing perception has resulted in the FDA approval of two CD19-specific CAR T cells for acute lymphoblastic leukemia (ALL) (Kymriah™)[6] and relapsed or refractory large B-cell lymphoma (Yescarta™).[7]

Regarding the rapid progression of CAR T cell-based therapies worldwide, it seems inevitable for healthcare systems, especially in developing countries, to adapt their research and development platforms for the advent of new technology. The first hurdle is to establish robust protocols for expansion and gene manipulation in immune cells. We have tried to set-up T cell expansion, activation, and viral transduction as the prerequisites for diverse CAR T cell therapies. As a model, we have chosen well-established CD19 CAR to pave the way for subsequent preclinical and clinical studies.

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Materials and Methods

Peripheral Blood Mononuclear Cell Isolation and T Cell Expansion

Peripheral blood mononuclear cells (PBMCs) were obtained by density-gradient centrifugation of a blood sample from a healthy donor over Ficoll-Paque-Plus (GE Healthcare, UK). Then, the resultant PBMC layer was further washed with PBS 2 times at 200 g for 10–15 minutes at 20°C to eliminate platelets. To expand T cells, in a 24-well plate, 1.6 × 10^6 PBMCs were plated at a 2:1 ratio with 0.8 × 10^6 anti-CD2/CD3/CD28-coated MACSiBead™ particles (Miltenyi Biotec, Germany) in 2 mL TexMACS medium (Miltenyi Biotec, Germany) containing 10% heat-inactivated FBS (Gibco, USA), 100 U/mL penicillin/streptomycin (Bioidea, Iran) and 20 IU/mL recombinant human IL-2 (Miltenyi Biotec, Germany). The cell culture medium was replaced on a three-day schedule for 28 days with the aforementioned medium formulation. Half of the propagating T cells were removed from each well whenever their cell density reached 3.2 × 10^6 cells to prevent overgrowth. Additionally, MACSiBead™ particle restimulation was done after every three rounds of medium exchange while keeping bead-to-cell ratio of 2:1.

CD19 CAR Design

The designed CD19 CAR has a modular structure consisting of CD8A leader sequence, CD19-scFv, CD8A hinge and transmembrane regions, and cytoplasmic domains of 4–1 BB and CD3ζ in 5' to 3' order. All protein segments had a human origin with the exception of anti-CD19 scFv for which a mouse monoclonal antibody (FMC63) was engineered. All designed structure was synthesized and cloned in a lentiviral-based vector with a GFP tag.

Production of Lentiviral Vectors

Lentiviral vectors were produced using PolyFect Transfection Reagent (Qiagen, Iran) was used according to the manufacturer's protocol. In brief, pMD2.G (3.45 µg), psPAX2 (6.91 µg) and CD19 CAR vectors (6.91 µg) were used to produce the recombinant lentiviruses in the Lenti-X™ 293T Cell Line (Cat NO. 632180, Clone Tech) in 10 cm plates. The supernatant media were collected at 56 and 80 hours post-transfection. Then, all virus-containing media were pooled, filtered through a 0.45 µM polyethersulfone filter, and concentrated on sucrose gradient media. For this aim, 2 mL of 20% sucrose solution was carefully laid on the bottom of a pre-sterilized ultracentrifuge tube and then overlaid by 20 mL of pooled media so that a clear layer of sucrose cushion would appear. Then, the tubes were centrifuged for 3 hours, 4°C, 50 000 ×g. The resultant pellet was diluted in 100 µL TexMACS medium (Miltenyi Biotec, Germany). Finally, the titer of the concentrated lentivirus was determined by transducing HEK 293T cells and reporting the percent of GFP positive HEK 293T cells by flow cytometry. All steps were similarly performed for a mock control virus lacking the CD19 CAR sequence in vector construction.

Immunophenotyping

The phenotype of expanded T cells was analyzed on the day of isolation (day 0) and 1, 14, 28 day (s) after the initial expansion. For each time point, 2 × 10^5 cells were re-suspended in 100 µL PBS containing 3% bovine serum albumin (Sigma, USA) and incubated with 5 µL of anti-human CD3- PerCP (Miltenyi Biotec, Germany), anti-human CD4-FITC/CD8-PE (BD Biosciences, USA) and anti-human CD69-PE (eBioscience, USA), mouse IgG2a Isotype PerCP (Miltenyi Biotec, Germany), mouse IgG1 isotype-FITC (BD Biosciences, USA), and mouse IgG1 isotype-PE (BD Biosciences, USA) at 4°C in the dark. Cell surface markers were analyzed with BD FACSCalibur™ flow cytometer and then processed with the FlowJo software.

T Cell Transduction and CD19 CAR Expression Verification

Twenty-four hours post-activation with MACSiBead™ particles, T cells were transduced by either CD19 CAR or mock virus at multiplicity of infection (MOI) of 3. At day 5 post-transduction, the amount of CAR-positive cells was measured by F(ab')2 fragment specific antibody (Jacksonimmuno, UK) in the GFP positive cell population.

Data Presentation

All data were presented as mean ± standard deviation. *P* values were reported up to three decimals.

Results

T Cell Expansion and Immunophenotyping

To reach sufficient numbers of T cells, we expanded and subsequently inspected the immunophenotype of expanding T cells within a 28-day time period for characterization. Our results showed that a portion of T cell population (CD3 positive cells) had sharply increased in the bulk of mononuclear cells over the time of expansion, resulting in a pure population of T cells even on day 14 (96 ± 3% T cell purity) (Figure 1A). The expression of CD69 (an immediate marker of T cell activation)[8] indicated that 65 ± 4% (*P* = 0.046) of the T cells were activated at Day 1 (Figure 1B). While $CD4^+$ T cells were dominant compared with $CD8^+$ T cells on the day of PBMC isolation (day 0) and day 1, the proportion of CD8+ T cells increased over time, resulting in approximately 3- and 18-fold increase in number of $CD8^+$ to $CD4^+$ T cells on days 14, and 28 post-activation, respectively (Figure 1C). The number of seeded PBMCs and their ratio with MACSi bead particles resulted in a monolayer of cells with limited numbers of T cell-MACSiBead aggregates on day 0 of T cell expansion. Notably, after 24 hours, the number of T cell-MACSiBead aggregates was considerably increased. Over time, the quality of T cells was impaired with a noticeable decrease in the rate of response to bead reactivation and the formation of cytoplasmic granulation (Figure 1D).

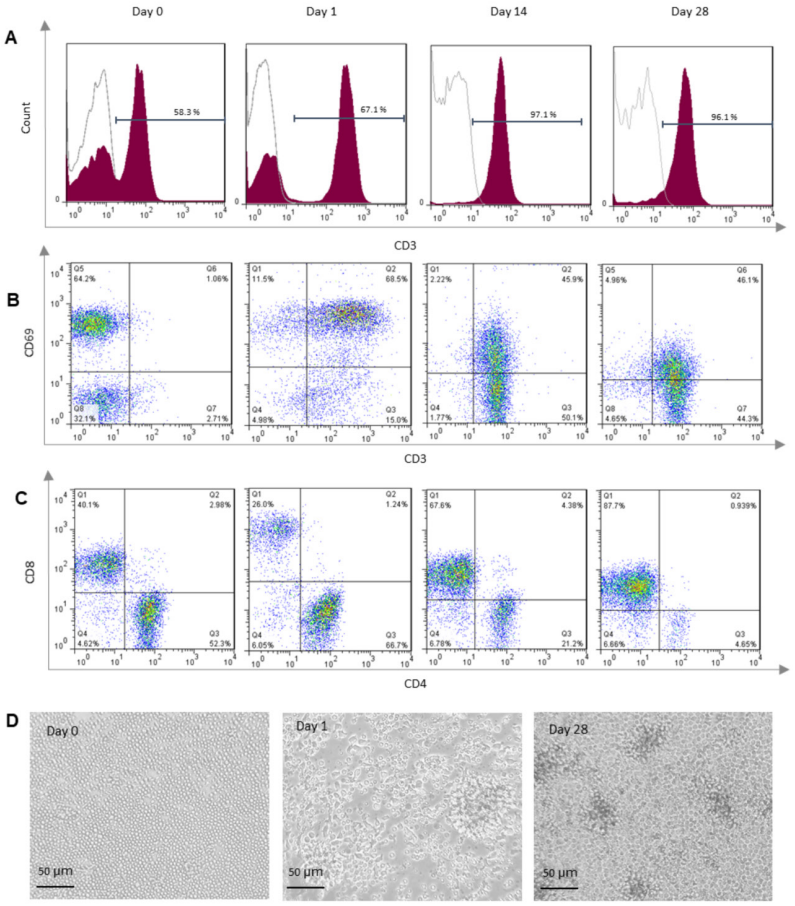

Figure 1. T Cell Expansion and Characterization. T cells were expanded from freshly isolated PBMCs under IL-2 and MACSi Bead stimulation. Cultures were monitored for 28 days both phenotypically and morphologically. The phenotype of expanded T cells analyzed by flow cytometry on the day of isolation (day 0) and 1, 14 and 28 days after stimulation. Representative flow cytometry plots are shown for each set of experiments. **A)** Percentage of CD3 T cells increased over time yielding a pure population of T cells. **B)** The expression of CD69 as an immediate marker of T cell activation began straight after stimulation and persisted till the end of culture although at lower levels. **C)** CD4 and CD8 phenotype of T cells were analyzed on $CD3^{+}$-gated cells. CD8 T cells were enriched and dominated the cultures over time. **D)** Microscopic observation of T cells on days 0, 1, 28 are shown.

Lentiviral Production

We used PolyFect Transfection Reagent (Qiagen) to produce the required lentiviruses. Moreover, to achieve a high titer lentivirus, we concentrated the supernatants containing viral particles with 20% sucrose. We used TexMACS medium for the final elution of concentrated viruses to prevent interference of other medium formulations on T cell activation and expansion. Our approach resulted in batches of virus containing 2.4 to 9 × 10^3 particles/µL. Aliquots of each batch were prepared and kept at – 80°C so that the content of each vial was allowed to thaw once.

CD19 CAR Design and T Cell Transduction

CD19 CAR structure has a modular design in nature, comprising compartments derived from several proteins. This type of design grants the CAR molecule different functions ranging from antigen recognition to signal transduction for subsequent T cell functions. The details of CAR design in this article are provided in Figure 2A. Light and fluorescent microscopy of expanded T cells five days after transduction showed GFP positive cells aggregated to each other through MACSiBead™ particles (Figure 2B, 2C). Flow cytometric analysis on these cells five days after cell transduction was indicative of 35 ± 6% CAR expression from which 30 ± 5% were double positive GFP and CD19 CAR molecule, and 7 ± 4% were merely positive for CD19 CAR (Figure 2D).

Discussion

The efficient expansion and transduction of T cells is the cornerstone of CAR T cell therapy in both preclinical and clinical settings. Here, we describe the generation of the CD19-specific CAR T cell for preclinical applications. Our observation showed that after 24 hours from the initial activation, 65 ± 4% (P = 0.046) of the cells present immediate activation phenotype based on CD69 expression while the initial PBMCs on day 0 had been in resting status as they presented approximately 1% CD69 expression. In other words, the activation protocol efficiently activated T cells by the time the cells were to be transduced. Moreover, the T cell population gained more purity during the expansion process, reaching 96 ± 3% purity 14 days after the initial expansion. Based on obtained data, 35 ± 6% of the T cell population were transduced by the CD19 CAR

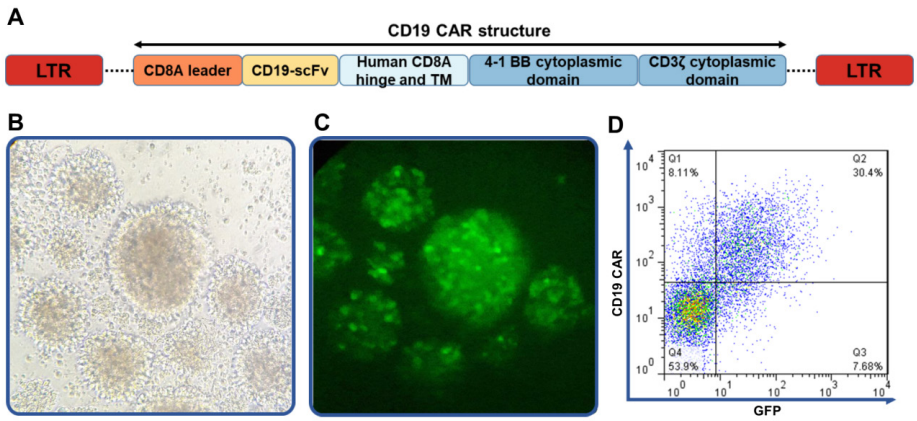

Figure 2. Construction and Expression of CD19-Specific CAR. **A)** Schematic diagram of the construction of the CD19-specific CAR composed of the CD8A signaling domain, anti-CD19 FMC63-derived scFv, human CD8A hinge and transmembrane domains, and the cytoplasmic domains of 4-1 BB and CD3ζ in 5′ to 3′ order. B) Light Microscopy of activated T cells transduced with CD19 CAR lentivirus **C)** Expression of the transduced construct assessed by fluorescence microscopy of GFP. **D)** Surface expression of anti-CD19 CAR assessed by flow cytometry using F(ab')2 fragment specific antibody.

lentiviral vector as judged by flowcytometry.

In Iran, the research on adoptive cell therapy (ACT) as a general term of immune cell therapy is quite late-arriving.[9] Despite 256 clinical trials and two FDA approvals for CAR T cell therapy[2] as a subcategory of ACT, Iranian scientists have had a very limited share of both publications and trials in this field. The few publications in the field are confined to experiments on the Jurkat cell line, hampering efficient translation of their findings to clinical demands. This lack of advancement stems from insufficient prioritization of immunotherapy in decision making and granting bodies, and subsequently inflating the scarcity of required infrastructure for CAR T cell research such as animal facilities, GMP virus production sites, and advanced imaging technologies.

Collectively, this research described the steps that we took to start CD19 CAR T cell therapy in Iran while focusing on important factors for its clinical translation such as T cell activation, expansion, and transduction. Hence, this research would be a foundation for CAR T cell therapy of diverse antigenic targets ranging from hematopoietic targets such as CD19 to targets with applicability for solid tumors.

Authors' Contribution

Study conception and design were performed by JK, HRA, and NA. Data acquisition was done by MN, MTR, AR, and EJ. MTR and EJ were also involved in analysis and interpretation of data. Manuscript was drafted by MN, MTR, and NA.

Conflict of Interest Disclosures

The authors declare that they have no conflict of interest.

Ethical Statement

This study was approved by the Research Review Board of Tehran University of Medical Sciences (Ethic No. IR.TUMS.DDRI.REC.1395.1).

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