PRE-CLINICAL RESEARCH ON THE APPLICATION OF CAR-T THERAPY IN THE TREATMENT OF ACUTE LYMPHOBLASTIC LEUKEMIA

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Abstract

Objectives: To create chimeric antigen receptor (CAR) T cells and evaluate the effect on CD19+ B cells in animal models of acute lymphoblastic leukemia. ***Methods:*** An experimental research (*In vitro* and *In vivo*) was conducted at the Department of Pathophysiology, Military Medical University, from June 2019 to 2022. ***Results:*** Successfully transformed and created CAR-T cells by nucleofection technology; the survival rate of cells after transformation was 48.9 - 60.7%. The expression of CAR molecules on the T cell surface after 21 and 28 days of culture were 66.23% and 97.34%, respectively. CAR-T cells could proliferate vigorously when co-cultured with cancer cells carrying CD19+, and the ability to lyse cancer cells carrying CD19+ after 6 and 24 hours (lysis efficiency was 24.8 - 71.8%, respectively); this ability increased with the proportion of CAR-T cells present in the medium and with the time of co-culture. CAR-T cell block restricted the proliferation of CD19+ cancer cells (through Luciferase activity), prolonged the survival time, and increased the survival rate of the murine model of acute lymphoblastic leukemia. ***Conclusion:*** CAR-T cells can grow when co-cultured with CD19 receptor-expressing cells and CD19+ cancer cell lysis. The excellent effect when using generated CAR-T cells to treat a mouse model of acute leukemia was assessed by Luciferase activity associated with CD19+ cancer cells, survival time, and mouse weight.

Keywords: CAR-T therapy; Acute lymphoblastic leukemia.

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INTRODUCTION

Artificial chimeric antigen receptor-expressing T (CAR-T) cells targeting CD19 are groundbreaking in treating lymphoblastic leukemia. The principle of CAR-T therapy in cancer treatment involves engineering T cells with artificial receptors capable of recognizing specific antigens on the surface of cancer cells, thereby activating the T cells' ability to eliminate cancer cells.

Compared to targeted biologic drugs such as monoclonal antibodies, CAR-T therapy possesses targeted therapeutic properties and exhibits superior effectiveness. In the case of acute lymphoblastic leukemia (ALL), CAR-T therapy has proven effective even in patients resistant to chemotherapy, radiation therapy, or metastasis. Moreover, CAR-T cells can proliferate and persist in the body for an extended period to eliminate recurrent cancer cells, which is a significant factor contributing to the failure of current cancer treatments.

Currently, there is no research conducted in Vietnam regarding the application of CAR-T therapy for treating lymphoblastic leukemia. Therefore, our project aims to "Investigate the application of CAR-T therapy in the pre-clinical treatment of lymphoblastic leukemia" with the following objectives: *To generate CAR-T cell mass and evaluate its effectiveness on CD19-positive B lymphoma cells and assess the efficacy of the CAR-T cell mass on an animal model inducing acute lymphoblastic leukemia.*

MATERIALS AND METHODS

1. Materials

- K562 cells expressing CD19, CD64, CD86, and CD137 markers designated as 1D2 are products of the research project "Application and Development of National-level Technology" with code KC 10.39/16-20.

- Daudi cells: Daudi Burkitt lymphoma cells (ATCC CCL-213) expressing CD19 (ATCC, USA).

- Peripheral blood was donated by healthy volunteers and collected in specialized tubes.

- NOD/scid mice: Immunodeficient mice lacking B lymphocytes, T lymphocytes, and natural killer (NK) cells, imported from Charles River Company (USA), n = 30.

- CAR-T CD19RCD137/pSB structure (CAR-T) is a product of the research project "Application and Development of National-level Technology" with code KC 10.39/16-20.

- CAR-T CD19RCD137/pMC structure (CAR-T) is a product of the research project "Application and Development

of National-level Technology" with code KC 10.39/16-20.

- SB mRNA (sleeping beauty transposase encoding mRNA) is transcribed in vitro at the Genetic Engineering Laboratory, Center for Research and Development in Biotechnology, Hanoi University of Science and Technology.

- Consumables required for cell culture: Petri dishes, pipettes, and culture medium containers.

- Laboratory facilities for cell culture: Cleanroom, CO2 incubator, inverted microscope, centrifuge, 4°C refrigerator, -20°C, and -80°C freezers, liquid nitrogen storage tank, 4D Nucleofector core Unit (Lonza), cell viability analyzer (Countess II FL), and flow cytometer BD FACSLyric (BD Bioscience).

2. Methods

* *Research methods:* An experimental research was conducted in the laboratory of the Department of Pathophysiology, Vietnam Military Medical University, from August 2020 to May 2023.

* *Techniques used in the research:*

- Cell culture technique, observation of cells under a microscope.

- Electroporation technique for cell transfection: PBMCs after activation using Dynabeads™ Human T-Activator CD3/CD28 were optimized for transfection in terms of transfection system, cell quantity, and DNA amount per transfection reaction. This mixture, then, was transferred into a specialized cuvette (Lonza Biosciences) and electroporated using the EO-115 program of the Amaxa™ 4D-Nucleofector system.

- Peripheral blood mononuclear cell (PBMC) collection technique using density gradient centrifugation: Blood from voluntary human donors was anticoagulated, diluted with 1X PBS (pH 7.2), and then gently layered into a 50mL Falcon tube containing 15mL of Ficoll-Paque PREMIUM. Centrifuge and collect the layer of liquid located between the plasma and the Ficoll.

- Flow cytometry technique for cell analysis: The harvested transferred cells were stained with CD19 FITC-conjugated antibody (rCD19-FITC, abcam, #ab246020) following the manufacturer's instructions. After staining, the cells were washed with 1X PBS and resuspended in 1X PBS supplemented with 1% FBS before being analyzed using the BD FACSLyric™ Clinical System.

- Care and maintenance of experimental mice.

- Tumor cell implantation technique in mice: NOD scid mice were maintained in aseptic conditions. They were injected with Daudi cells into the

peritoneal cavity at a dose of 10 cells/1mL. Treatment was initiated after 3 days.

* *Statistical analysis:*

Data analysis was performed on SPSS 20.0 and FlowJo 10.4 software. The statistical difference is considerably significant ($p < 0.05$).

3. Ethics

This study was approved by the Ethical Committee of Military Hospital 103 (No.180/CNChT-HĐĐĐ) on 10th August 2021.

RESULTS AND DISCUSSION

We performed the transfection of the corresponding plasmid and minicircle structures to generate CAR-T cell mass. The results showed significant differences in transfection efficiency and cell viability post-transfection between plasmid + plasmid and mRNA + minicircle approaches. Specifically, the plasmid + plasmid structure exhibited a cell viability rate of 48.9% post-transfection.

On the other hand, the mRNA + minicircle structure showed a higher cell viability rate of 60.7% post-transfection. The improved transfection efficiency observed with minicircle structures compared to plasmids could be attributed to the size of the structures and the use of SB100X mRNA for faster transposase gene expression and reduced cytotoxicity, thereby enhancing transfection efficiency.

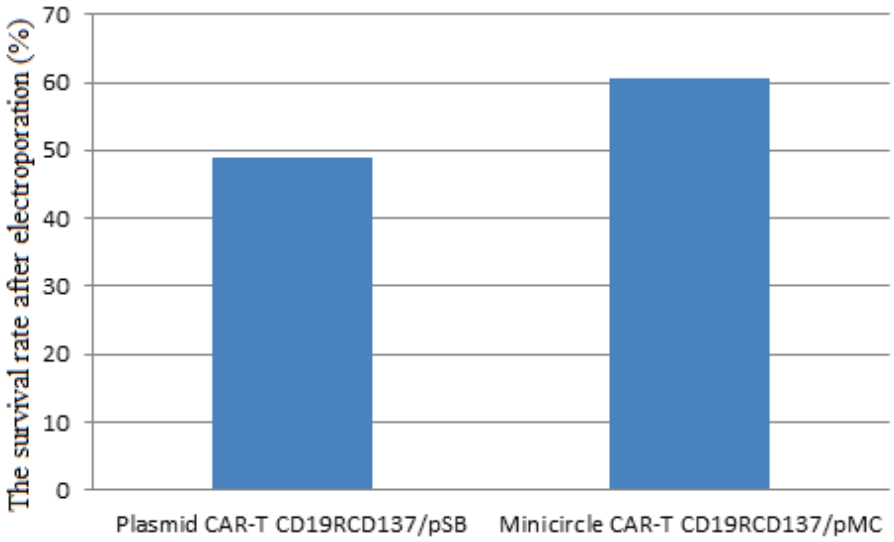

Figure 1. Cell viability rate after transfection with plasmid and minicircle structures for CAR-T cell generation.

* *Analysis of CAR expression by real-time PCR:*

To confirm the proliferative capacity of CAR-T cells when co-cultured with CD19-positive cells, we co-cultured non-transfected PBMC (PBMC), CAR-T CD19RCD137 with aAPC 1D2 (K562 cells expressing CD19), and Daudi cells. After 3, 7, and 14 days, 1×10^6 viable cells were collected, mRNA was extracted, and real-time PCR was performed using specific primers for the CAR expression region (Wang et al., 2018).

The results showed an increased expression of CAR when co-culturing CAR-T cells with CD19+ cells. On day 3, after 35 cycles, the signal began to separate from the baseline. In contrast, on day 7, only after 15 cycles, and on day 14, even before 15 cycles, similar signals were observed as on day three after 35 cycles.

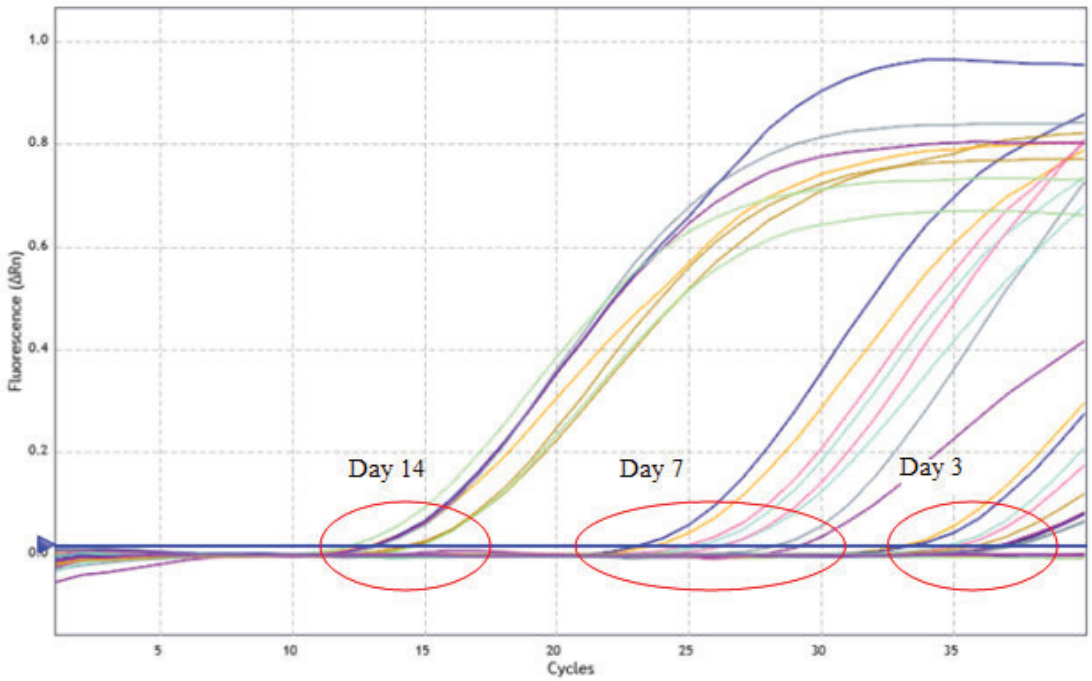

Figure 2. Analysis of CAR expression after 3, 7, and 14 days of co-culture between CAR-T cells and aAPC using real-time PCR.

* *Analysis of CAR surface expression by flow cytometry*:

After 21 and 28 days of proliferation, the expression of CAR on CAR-T CD19RCD137 cells was evaluated using a CD19-FITC binding assay and

analyzed by flow cytometry. The results showed CAR expression levels of approximately 66.23% and 97.34%, respectively.

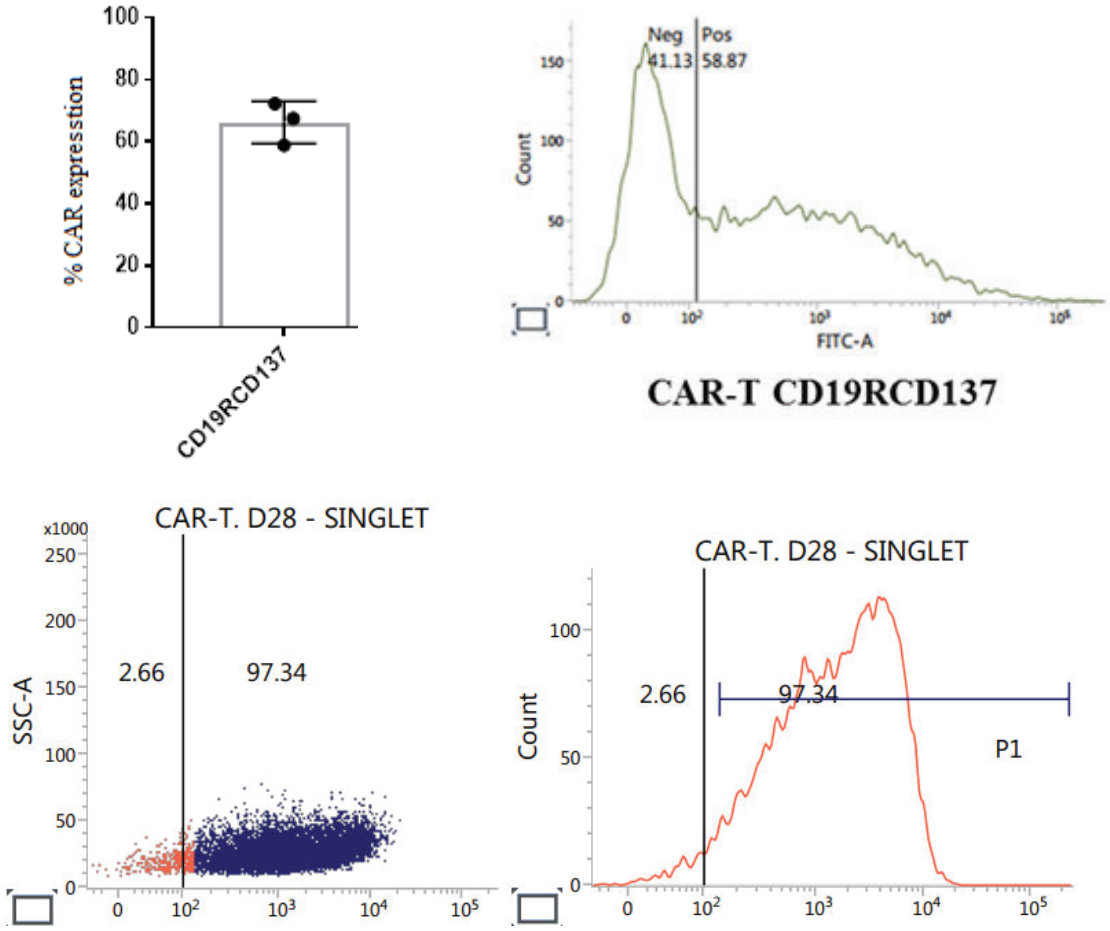

Figure 3. The proportion of cells expressing CAR receptor and analysis of expression by flow cytometry after 21 and 28 days of proliferation.

** Assessment of the proliferative capacity of CAR-T cells when co-cultured with CD19(+) cancer cell lines:*

CAR-T cells were co-cultured with the Daudi, 1D2 (CD19+), and K562 (CD19) cell lines, which were irradiated with X-ray radiation (100 Gy at a 1:1 ratio).

After four days, the CAR-T cell count was performed, and the results demonstrated the proliferative capacity of CAR-T cells when co-cultured with CD19(+) cells. Conversely, no proliferative capacity was observed, and there was a tendency for a decrease in cell count when co-culturing with K562 (CD19-) cells.

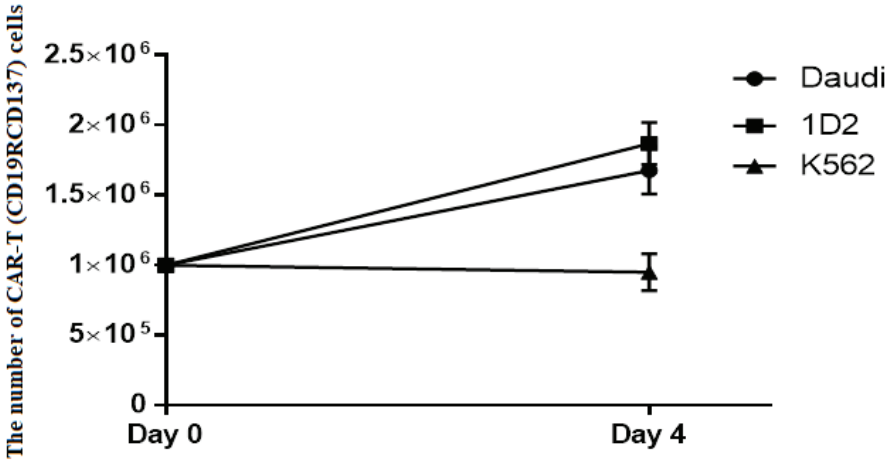

Figure 4. Proliferative capacity of CAR-T cells when co-cultured with CD19(+) cells.

** Assessing the ability of CAR-T cells to eliminate CD19+ cancer cell lines:*

The three cell lines used for evaluating the lytic ability of CAR-T cells are Daudi (CD19+), 1D2 (CD19+), and K562 (CD19-).

The results indicate that CAR-T cells do not exhibit significant lytic capability against the original K562 cell line, which does not express CD19.

For CD19+ cells, CAR-T cells expressing CD19RCD137 demonstrate good lytic capability. The percentage of lytic cells increases with the ratio of co-cultured CAR-T cells to CD19+ cells. Specifically, the lytic rates achieved for Daudi cells range from 24.8% - 71.8% with CD19RCD137 CAR-T cells. In contrast, activated PBMCs exhibit minimal lytic activity (<11%). Similar results were observed when co-culturing 1D2 cells with CAR-T cells. While the lytic efficiency of activated PBMCs remained below 11%, CD19RCD137 CAR-T cells achieved lytic rates of 45.43% - 76.68% for 1D2 cells.

These findings demonstrated that the CAR-T cells generated in this study can lyse cancer cells, mainly exhibiting selectivity towards CD19+ cancer cells. These results aligned with the experimental design and were consistent with other studies (such as Bruddo and Sommermeyer) in which CAR-T cells were engineered to target CD19+ cancer cells.

** The results of evaluating luciferase activity in the mouse blood across different groups:*

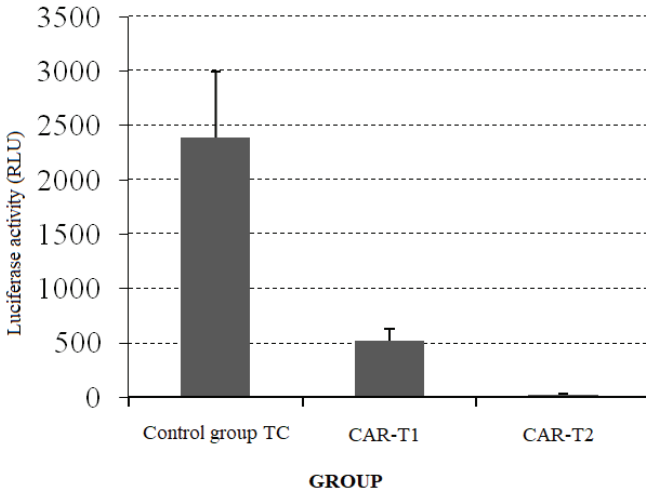

Figure 5. Depicts the results of luciferase activity assessment in mice across different groups.

The results showed that mice in the control group, injected with Daudi cells but not treated, exhibited significantly high luciferase activity (2392.84 ± 600.27). In contrast, mice in the groups that were initially induced with the disease and subsequently treated with CAR-T cells at a dose of 1x106 TC or 5x106 TC demonstrated markedly lower luciferase activity compared to the control group. These findings indicate that Daudi cells, when introduced into the peritoneal cavity of the mice, developed and migrated into the bloodstream, resulting in mice carrying disseminated CD19+ cancer cell masses. The mice in the disease-induced groups, subsequently treated with CAR-T cells, exhibited significantly reduced luciferase activity compared to the control group. In summary, the passage demonstrates that the mice treated with CAR-T cells showed a notable decrease in luciferase activity compared to the control group, suggesting effective targeting and elimination of the CD19+ cancer cells by the CAR-T cells.

** The results of assessing the overall well-being of mice are as follows:*

- The overall condition of the mice before and during treatment:

Before treatment: After injecting Daudi cells at a dose of 1x106 cells/mouse into the peritoneal cavity of the mice, the mice maintained normal eating and drinking habits,

exhibited regular activity, had shiny fur, clear eyes, dry anus, and no signs of diarrhea. The survival rate of the mice reached 100%.

After treatment: During the treatment period, the control group of mice showed symptoms of fur loss, reduced appetite, and decreased mobility compared to the other groups. These results indicate that CAR-T cells did not induce systemic toxicity in the nude mice with leukemia. Throughout the study, the mice in all groups received identical experimental conditions and showed no other pathological signs leading to mortality.

- The results of survival time, survival rate, and cumulative survival rate of nude mice after treatment with CAR-T cells are as follows:

Table 1. Average survival time of the treatment groups of mice

The group	Number of Mice	Average Survival Time	Number of Surviving Mice	Survival Rate (%)	p
T-C (1)	10	23.90 ± 8.05	4	40	$p_{1-2} = 0.006$ $p_{1-3} = 0.001$
CAR-T1 (2)	10	28.90 ± 2.85	8	80	
CAR-T2 (3)	10	29.80 ± 0.63	9	90	

At all-time points during the study, the number and proportion of deaths in the control group were higher than those in the four CAR-T treatment groups. The difference was statistically significant from day 21 onwards, with $p < 0.01$. At the end of the experiment (on day 30 after treatment), the survival rate in the control group was 4/10 (40%), in the CAR-T1 group was 8/10 (80%), and in the CAR-T2 group was 9/10 (90%). Thus, the proportion of mouse deaths and the number of days survived depended on the development, progression, and consequences of leukemia and the outcome of the CAR-T treatment.

- Results of immune cell activation in peripheral blood and liver of mice:

The total white blood cell count and monocyte proportion tended to increase, while the neutrophil proportion tended to decrease in peripheral blood compared to the control group.

The total white blood cell count in the liver of mice treated with CAR-T tended to increase. The proportion of

neutrophilic multinucleated cells in the liver increased in the CAR-T1 treatment group and remained unchanged in the CAR-T2 treatment group. The proportions of lymphocytes and monocytes in mice treated with CAR-T tended to decrease. In contrast, the balance of eosinophilic multinucleated cells in the liver of mice treated with CAR-T tended to increase. These indices were compared to the control group.

CONCLUSION

CAR-T cell mass was successfully transfected using nucleofection technology. Optimal CAR-T cell proliferation was achieved after 21 days of culture with three additional aAPC supplements (on days 1, 7, and 14), with 66.23% expressing CAR molecules on the surface.

CAR-T cells demonstrated robust proliferation when co-cultured with CD19(+) cancer cells.

CAR-T cells could eliminate CD19(+) cancer cells after 6 and 24 hours. This ability increased with the proportion of CAR-T cells in the culture environment and the duration of co-culture.

Treatment with CAR-T cells limited the proliferation of CD19(+) cancer cells (Daudi luc+) (Luciferase activity in the CAR-T treatment groups was significantly lower than in the mouse leukemia model group).

CAR-T cells extended the survival time and increased the survival rate of mice with leukemia.

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