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**RESEARCH ON THE SAFETY AND ANTI-LEUKEMIA
EFFECTS OF CAR-T CELLS COMBINED WITH ANTI-PD-1
ANTIBODIES *IN VITRO* AND *IN VIVO***

Major: Biomedical science

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SUMMARY OF THE DOCTORAL THESIS IN MEDICINE

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INTRODUCTION

Leukemia accounts for about 2.4% of all new cancer cases and 3.1% of cancer-related deaths in the world. Currently, chemotherapy is the most common treatment for leukemia. The treatment effect of this therapy is limited. After treatment, patients cannot completely recover from the disease, and the relapse rate is high. Recently, new treatment strategies such as targeted therapy, including CAR-T cell therapy, have been developed and have recorded positive results. PD-1 is a protein expressed on the surface of T cells. When PD-L1 binds to the PD-1 receptor, it causes T cell inactivation, inhibiting T cell immune activity. The presence of PD-1 on the surface of CAR-T cells is thought to contribute to the loss of function of CAR-T therapy. Therefore, blocking PD-1 on CAR-T cells with PD-1 inhibitors or antibodies is thought to enhance the tumor-killing effect of CAR-T cells. Anti-PD-1 antibody (PD-1 Ab) will activate CAR-T cells to help increase the effectiveness and function of CAR-T cells, thereby improving prognosis, reducing side effects, and increasing treatment effectiveness. PD-1 blockade is considered a promising new direction to help improve the function of CAR-T cells, bringing good treatment effects. However, in Vietnam, no studies have evaluated the effectiveness of combining CAR-T cells and anti-PD-1 antibodies on leukemia. Consequently, this study was undertaken with the following two objectives:

- 1. Evaluate the safety of CAR-T cells combined with anti-PD-1 antibodies in vitro and in vivo.*
- 2. Evaluate the anti-leukemia effects of CAR-T cells combined with anti-PD-1 antibodies in vitro.*

Significant contributions of the thesis:

This is the first study in Vietnam to evaluate the safety and anti-leukemia effects of CAR-T cells combined with anti-PD-1 antibodies in vitro.

Study results indicate that CAR-T cells and CAR-T combined with anti-PD-1 antibodies did not affect survival, body weight, hematopoietic function, liver function, kidney function, and liver and kidney histopathology of experimental mice after 4 weeks of monitoring.

CAR-T and CAR-T cells combined with anti-PD-1 antibodies had a higher lysing ability to CD19+ cancer cell lines (Daudi, Raji) than control PBMC cells. CAR-T combined with anti-PD-1 antibody increased 1.82 times the lysis effect on Raji cells by a Raji/CAR-T ratio of 1:10

Structure of the thesis:

The thesis comprises 133 pages, including the Introduction section, four chapters (Chapter 1: Literature review; Chapter 2: Subjects and Methods; Chapter 3: Results; Chapter 4: Discussions), Conclusions, and Recommendations. The thesis incorporates 18 tables, 35 figures, and 158 references.

CHAPTER 1: LITERATURE REVIEW

1.1. OVERVIEW OF LEUKEMIA

1.1.1. Epidemiology

1.1.1.1. Epidemiology of leukemia in the world

According to GLOBOCAN statistics for 2022, leukemia is the 13th most commonly diagnosed cancer and the 10th leading cause of cancer death worldwide, with 486,777 new cases and 305,033 deaths from leukemia.

1.1.1.2. Epidemiology of leukemia in Vietnam

According to GLOBOCAN, in 2022, Vietnam had 5,789 new cases, ranking 8th, and 4,330 deaths, ranking 6th due to leukemia.

1.1.2. Causes and pathogenesis of leukemia

1.1.2.1. Causes

Up to now, the causes of leukemia have not been identified. However, many risk factors increase the incidence of the disease, such as a history of exposure to ionizing radiation or chemicals, treatment with certain anti-cancer drugs, infection with certain viruses (HTLV-1 and HTLV-2, Epstein Barr), history of hematologic disorders, genetic conditions (Fanconi anemia, ataxic Bloom syndrome, Down syndrome, xeroderma factor, Li-Fraumeni syndrome).

1.1.2.2. Pathogenesis mechanism

Leukemia is a malignant blood disease that involves the overproduction of immature or abnormal white blood cells. The disease occurs when some blood cells have DNA mutations and certain abnormalities that cause the cells to grow and divide faster and continue living while normal cells die. Over time, these abnormal cells can crowd out normal blood cells in the bone marrow, leading to a decrease in normal white blood cells, red blood cells, and platelets, affecting the function of these organs and patient health.

1.2. CAR-T CELL THERAPY

1.2.1 Overview of CAR-T cell therapy

1.2.1.1. history of formation and development of CAR-T TB therapy

In 1989, Eshhar Z. and his colleagues created the chimeric T cell receptor gene (chimeric TCR gene). By 2017, the FDA authorized CAR-T cell therapy to treat ALL in adults and children. As of 2022, there are 377 clinical trials in the United States and 636 in China, while Europe has only 58 trials, still far behind these two leading countries. Clinical trials targeting CD19 account for more than 40% of CAR-T cell trials in the United States. The US Food and Drug Administration has licensed treatment for six CAR-T cell products, including four CAR-T CD19 products.

1.2.1.2. Generations of CAR-T cells

Five generations of CAR-T cells have been developed.

1.2.2. Toxicity of CAR-T cell therapy to the body

1.2.2.1. Cytokine release syndrome and neurotoxicity

The most common toxicity of CAR-T cell therapy is cytokine release syndrome (CRS), which is a systemic inflammatory response mediated by the overactivity of effector cells and large amounts of cytokines.

Immune Effector Cell-Associated Neurotoxicity Syndrome (ICANS) is the second most common acute toxicity observed with CAR-T cell therapy, with an incidence ranging from 2% to 64% for all levels of ICANS and 0-50% for severe ICANS.

1.2.3. Application of CAR-T TB therapy in the treatment of leukemia

1.2.3.1. Clinical CAR-T cell production process

- Collection of mononuclear cells from peripheral blood.
- T cell activation.
- Transforming the gene encoding CAR and increasing cell proliferation.
- Infuse CAR-T cells back into the patient.

1.3. ANTI-PD-1 ANTIBODIES

1.3.2. Anti-PD-1 antibodies

PD-L1 is a transmembrane protein present in the membrane of cancer cells, while the PD-1 receptor is present on the surface of immune cells such as T cells, B cells, NK cells, and macrophages. When PD-L1 binds to the PD-1 receptor, it causes T cell inactivation, inhibiting T cell immune activity. Therefore, antibodies that inhibit PD-1 or PD-L1 are used to inhibit the PD-1/PD-L1 axis, activating the T cell's immune system and attacking the cancer cells.

CHAPTER 2: STUDY SUBJECTS AND METHODS

2.1. RESEARCH TIME AND LOCATION

2.2. RESEARCH SUBJECTS AND MATERIALS

2.2.1. CAR-T cells

The 2nd generation CD19R/pSB CAR-T cell blocks were transformed and proliferated from Peripheral Blood Mononuclear Cells (PBMCs) of healthy, voluntary individuals. This is the product of the national-level technology application and development research project code KC10.39/16-20 at the Department of Pathophysiology - Vietnam Military Medical University. CAR-T cell blocks meet basic standards: expressing CD19 CAR structure, capable of lysing CD19+ cancer cells, and not infected with bacteria or fungi.

2.2.2. Anti-PD-1 antibodies

Anti-PD-1 antibody (ATC:L01XC18) concentration 25mg/mL.

2.2.3. Leukemia cell lines

Daudi cells (ATCC CCL-231) are a CD19+ Burkitt lymphoma cell line (ATCC, USA), Raji cells (ATCC CCL-86) are a CD19+ B lymphoblastic leukemia cell line (ATCC, USA), K562 cells (ATCC CCL-243) CD19- chronic myeloid leukemia cell line (ATCC, USA).

2.2.4. Animal

Sixty white mice, weighing $30\text{g} \pm 5\text{g}$, provided by the Experimental Animal Research Center, Vietnam Military Medical University, qualified for experimentation.

2.3. RESEARCH METHODS

2.3.1. Research design

Experimental research on cells and animals (white mice). Prospective intervention study with control group comparison.

2.3.2. Research content

2.3.2.1. Evaluating the safety of CAR-T cell block combined with anti-PD-1 antibody in experiments

- Evaluate the quality of CAR-T cells combined with anti-PD-1 antibodies: sterility (bacterial culture), CAR expression rate after proliferation culture, and Endotoxin content.

- Evaluate the safety of CAR-T cell block combined with anti-PD-1 antibodies on experimental animals: 60 white mice were divided into 4 groups, each group with 15 animals.

+ Group 1 (control): Inject 0.1 ml PBS/mouse via peritoneal route + Inject 0.1 ml PBS/mouse via tail vein.

+ Group 2 (CAR-T): Inject 10^6 CAR-T cells/0.1ml/mouse via peritoneal route + Inject 0.1 ml of PBS/mouse via tail vein.

+ Group 3 (PD-1 Ab): Inject 0.1 ml of PBS/mouse via peritoneal route + Inject PD-1 Ab (250 μ g)/0.1ml/mouse via tail vein.

+ Group 4 (CAR-T + PD-1 Ab): Inject 10^6 CAR-T cells/0.1ml/mouse via peritoneal route and Inject PD-1 Ab (250 μ g)/0.1ml/mouse via tail vein.

Monitor mice for 4 weeks to evaluate body condition, weight, and injection site in mice. At the end of 4 weeks, blood and liver and kidney tissues of mice were taken to evaluate:

+ Concentrations of IL-2, IL-6, and TNF- α were measured by ELISA test. (Mouse IL-2 ELISA kit MBS2701039, Mouse IL-6 ELISA kit MBS2508516, Mouse TNF- α ELISA kit MBS2500421 from MyBiosource).

+ Liver function (AST, ALT), kidney (Urea, Creatinine), liver and kidney histopathology, and hematopoietic organs (hematological index) were measured.

2.3.2.2. Evaluation of the anti-leukemia cell lines effect of CAR-T cell block combined with anti-PD-1 antibody

- Evaluate the effects of CAR-T cell block combined with PD-1 Ab on leukemia cell lines (Daudi, Raji, K562):

+ Designed research to evaluate lysis on 4 groups: PBMC, PD-1 Ab, CAR-T, and CAR-T combined with PD-1 Ab. With the ratio of Daudi/ Raji/ K562 (Target) cancer cells supplemented with PBMC and CAR-T (Effector) cells at the target: effector (T:E) ratio of 1:2, 1:5, 1:10. Anti-PD-1 antibody was added to the wells at a concentration of 20µg/mL.

+ After 6 hours of co-culture, evaluate the rate of lysed cells using the Flow Cytometry technique, using CFSE fluorescent cell dye to mark leukemia cells. The fluorescent nuclear dye 7-Amino Actinomycin D (7-AAD) was then used to examine the ratio of lysed cancer cells to the CFSE-positive leukemia cell population.

- Evaluation of cytokine secretion ability in vitro: cytokine concentration released into the cell culture medium when co-culturing cancer cells with 4 groups of PBMC, PD-1 Ab, CAR-T and CAR-T PD-1 Ab combination at two time points 24 hours and 48 hours (n = 3) (Human IL-2 Uncoated ELISA Kit 2237558-007, Human IL-6 Uncoated ELISA Kit 298722-008, Human TNF- α Uncoated ELISA Kit 279143-009 from Thermo company Fisher Scientific).

2.4. METHODS OF RESULTS ANALYSIS AND DATA PROCESSING

Research data were analyzed using IBM SPSS 22.0 and GraphPad Prism 8.0 software. Compare the median of 2 independent groups using the Mann-Whitney U test. Compare the means of more than 2 independent groups by one-way ANOVA test, with poshoc analysis using Turkey test.

ns: $p > 0.05$ is considered statistically significant.

*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$

2.5. ETHICS OF ANIMAL RESEARCH

Animals are raised in conditions that ensure adequate temperature, light, food, and water. Experimental procedures on animals are carried out in accordance with the standards and regulations of the Experimental Animal Research Center - Vietnam Military Medical University.

CHAPTER 3: RESULTS

3.1. QUALITY OF CAR-T CELL BLOCKS AND SAFETY OF CAR-T CELLS COMBINED WITH ANTI-PD-1 ANTIBODIES IN VITRO

3.1.1. Quality of CAR-T cell mass

- Sterility: Testing for culturing and identifying aerobic and anaerobic bacteria on the automated system of both control, PBMC, CAR-T, PD-1, and CAR-T + PD-1 Ab groups all had negative results.

- The expression rate of CAR on the surface of T cells analyzed by flow cytometry was 26.13% of T cells expressed CAR on their surface.

- CAR-T cells expressed the CAR gene when assessed using a real-time PCR technique.

- Endotoxin content of CAR-T cell and CAR-T combined PD-1 Ab media samples were < 0.1 EU/ml within the allowable limit.

3.1.2. Safety of CAR-T cell block and CAR-T combined with anti-PD-1 antibody in experiments

3.1.2.1. Whole body condition of mice during the experiment

- General condition: during the 4-week monitoring period, the mice ate, drank, and moved normally. They were agile, had smooth fur, clear eyes, dry anus, and no loose stools. The survival rate of mice in 4 groups was 100% until the last day of the experiment.

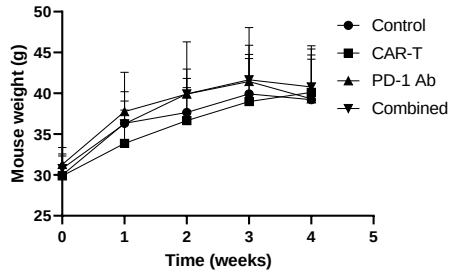


Figure 3. 3. Effect of CAR-T cells combined with PD-1 Ab on mouse weight

At day 0, day 7, day 14, day 21, and day 28, the weight of mice in the CAR-T, PD-1 Ab, and CAR-T combined PD-1 Ab groups had no significant difference compared to the control group ($p > 0.05$).

3.1.2.2. Affects the hematopoietic function of mice

The indexes of red blood cells, white blood cells, and platelets in all 3 groups of CAR-T, PD-1 Ab, and CAR-T combined with PD-1 Ab did not have statistically significant differences compared to the control group ($p > 0.05$).

3.1.2.3. Affects AST, ALT, and liver histopathology indices

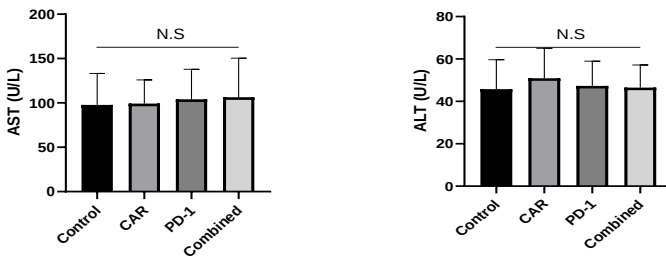


Figure 3. 6. Characteristics of AST and ALT indexes between groups

The AST and ALT indexes in all 3 groups of CAR-T, PD-1 Ab, and CAR-T combined with PD-1 had no significant difference compared to the control group ($p > 0.05$).

* Histopathology: liver microscopic morphology had no difference in all groups, and liver parenchyma was normal.

3.1.2.4. Effects on creatinine index, urea, and kidney histopathology

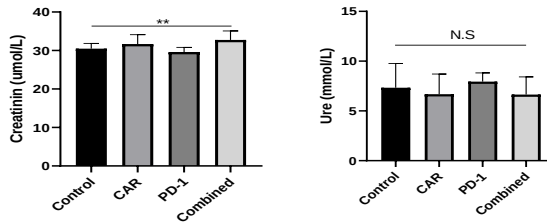


Figure 3. 8. Urea and Creatinine index results between groups

$(p(1)(4) < 0.05; p(3)(4) < 0.001)$

Urea concentrations in all 3 groups of CAR-T, PD-1 Ab, and CAR-T combined with PD-1 Ab had no significant difference compared to the control group ($p > 0.05$). There was a difference in creatinine concentration of the CAR-T combined PD-1 Ab group compared to the control and PD-1 Ab groups ($p < 0.05$). However, the creatinine index of the groups was still within normal limits.

- Histopathology: kidney microscopic morphology had no difference in all groups, and kidney parenchyma was normal.

3.1.2.5. Effects on cytokine concentrations

Table 3. 7. IL-6 cytokine concentrations of the study groups

Index Group	n	IL-6 (pg/mL)	p #
		Median (IQR)	
Group 1 (Control)	15	129.01 (93.52 - 158.32)	$p(1)(2) > 0.05$ $p(1)(3) < 0.001$ $p(1)(4) < 0.005$ $p(2)(4) > 0.05$ $p(3)(4) > 0.05$
Group 2 (CAR-T)	15	97.32 (86.03 - 141.41)	
Group 3 (PD-1 Ab)	15	86.03 (82.34 - 93.52)	
Group 4 (CAR-T+PD-1 Ab)	15	89.75 (86.03 - 97.32)	

Mann-Whitney U test

IL-6 concentration in the CAR-T group did not differ from the control group. IL-6 concentrations of the PD-1 Ab and CAR-T combined with PD-1 Ab groups were statistically significantly lower than the control group ($p < 0.05$).

Table 3. 8. IL-2 cytokine concentrations of the study groups

Group \ Index	n	IL-2 (pg/mL)	p #
		Median (IQR)	
Group 1 (Control)	15	39.84 (35.70 - 52.96)	$p(1)(2) > 0.05$ $p(1)(3) > 0.05$ $p(1)(4) < 0.01$ $p(2)(4) < 0.01$ $p(3)(4) < 0.01$
Group 2 (CAR-T)	15	38.79 (35.70 - 43.02)	
Group 3 (PD-1 Ab)	15	44.10 (35.70 - 52.39)	
Group 4 (CAR-T+PD-1 Ab)	15	33.67 (31.68 - 38.27)	

Mann-Whitney U test

The IL-2 concentration of the CAR-T and PD-1 Ab groups did not have a statistically significant difference compared to the control group ($p > 0.05$). The CAR-T and PD-1 Ab combination group had statistically significantly lower IL-2 concentrations than the control, CAR-T, and PD-1 Ab groups ($p < 0.01$).

Table 3. 9. TNF- α cytokine concentrations of the study groups

Group \ Index	n	TNF-α (pg/mL)	p
		Median (IQR)	
Group 1 (Control)	15	127.29 (110.26 - 185.68)	$p(1)(2) > 0.05$ $p(1)(3) > 0.05$ $p(1)(4) < 0.001$ $p(2)(4) < 0.001$ $p(3)(4) < 0.001$
Group 2 (CAR-T)	15	183.58 (117.76 - 255.48)	
Group 3 (PD-1 Ab)	15	83.22 (74.62 - 226.50)	
Group 4 (CAR-T+PD-1 Ab)	15	383.76 (278.30-418.89)	

* Mann-Whitney U test

The TNF- α concentration of the CAR-T and PD-1 Ab groups did not have a statistically significant difference compared to the control group ($p > 0.05$). However, the combination group of CAR-T cells and PD-1 Ab had statistically significantly higher TNF- α concentrations than the control group, CAR-T group, and PD-1 Ab group ($p < 0.001$).

3.2. ANTI-LEUKEMIA EFFECTS OF CAR-T CELL BLOCK COMBINED WITH PD-1 Ab

3.2.1. Results of evaluating in vitro cell lysis effects

3.2.1.1. Lysis effect of Daudi cells (CD19+)

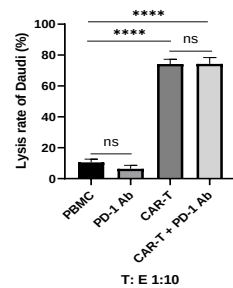
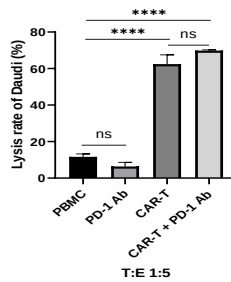
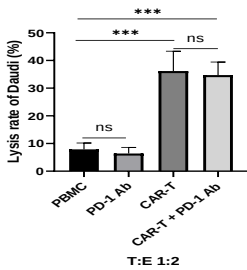


Figure 3. 10. Lysis of Daudi cells in 4 groups with T:E = 1:2

Figure 3. 11. Lysis of Daudi cells in 4 groups with T:E = 1:5

Figure 3. 12. Lysis of Daudi cells in 4 groups with T:E = 1:10

The group of CAR-T cells and CAR-T combined with PD-1 Ab showed good lytic ability against Daudi cells (CD19+). The cell lysis rate increased as the CAR-T/Daudi co-culture ratio increased, reaching 36.19% - 74.21%. Combining CAR-T cells with PD-1 Ab achieved a lysis rate of 34.73 - 74.28%. There was no statistically significant difference in the lysis rate between the CAR-T and CAR-T combined with the PD-1 Ab group.

3.2.1.2. Raji cell lysis effect (CD19+)

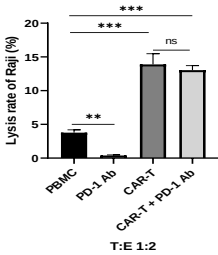


Figure 3. 13. Lysis of Raji cells in 4 groups with T:E = 1:2

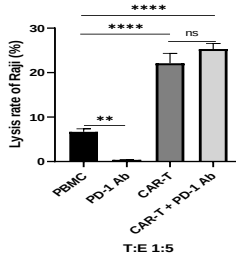


Figure 3. 14. Lysis of Raji cells in 4 groups with T:E = 1:5

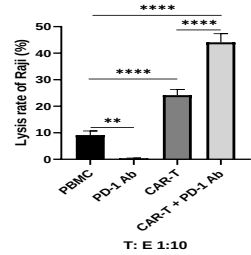


Figure 3. 15. Lysis of Raji cells in 4 groups with T:E = 1:10

CAR-T cells also lysed Raji cells (CD19+) better than PBMCs.

The rate of Raji cell lysis increased as the CAR-T/Raji co-culture ratio increased, reaching 13.89% - 24.2%. The lytic effect of CAR-T on Raji is much lower than that on Daudi (36.19% - 74.21%). However, in the CAR-T+PD-1 Ab combination group, there was a tendency to increase lysis efficiency when increasing the CAR-T/Raji ratio. In particular, with the ratio Raji/CAR-T = 1:10, the lysis efficiency increased from 24.2% to 44.11%, equivalent to an increase of 1.82 times.

3.2.1.3. Lysis effect on K562 (CD19-) cells

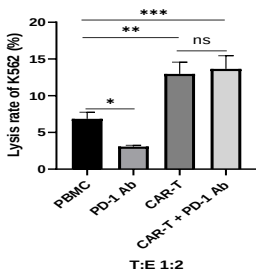


Figure 3. 16. Lysis of K562 cells in 4 groups with T:E = 1:2

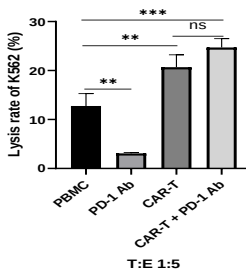


Figure 3. 17. Lysis of K562 cells in 4 groups with T:E = 1:5

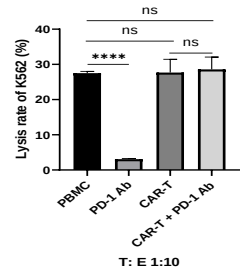


Figure 3. 18. Lysis of K562 cells in 4 groups with T:E = 1:10

For K562/CAR-T ratios 1:2 and 1:5, CAR-T and CAR-T + PD-1 had better cell lysis effects than PBMC, but both were at low levels. With the ratio K562/CAR-T = 1:10, the results showed that the rate of K562 cells lysed when co-cultured with PBMC was 27.52%, CAR-T was 27.67% and CAR-T combined with PD-1 Ab 28.59% were similar. The difference was not statistically significant ($p > 0.05$).

3.2.1.4. Comparison of the ability to lyse three leukemia cell lines of CAR-T cells and CAR-T cells combined with PD-1 Ab

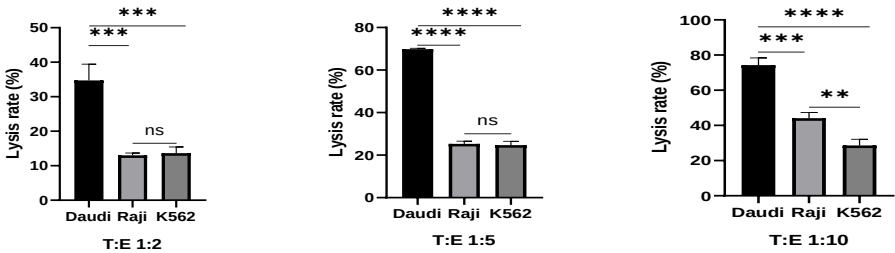


Figure 3.20. Comparison of the lysis rate of 3 leukemia cell lines of CAR-T cell block combined with anti-PD-1 antibody

Figure 3.20 shows that the Daudi cell lysis rate is statistically significantly higher than the Raji and K562 cell lysis rates in all three ratios. No statistically significant difference was seen between Raji and K562 cell lysis rates at T:E ratio 1:2 and 1:5 ($p > 0.05$). At a T:E ratio of 1:10, the Raji cell lysis rate of the combination therapy was statistically significantly higher than the K562 cell lysis rate ($p < 0.01$).

3.2.2. Changes in cytokine concentrations released in cell culture media

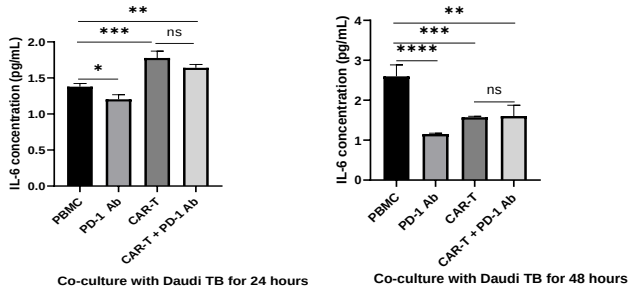


Figure 3. 21. Comparison of IL-6 cytokine secretion when co-cultured with Daudi cells in 4 groups after 24 and 48 hours

The results showed that at 24 hours, the IL-6 concentration in the culture fluid of Daudi cells co-cultured with CAR-T cells and CAR-T cells combined with PD-1 Ab were statistically significantly higher ($p < 0.01$) compared to the PBMC control group. By 48 hours, IL-6 concentrations in these two groups were statistically significantly lower than in the PBMC control group ($p < 0.001$).

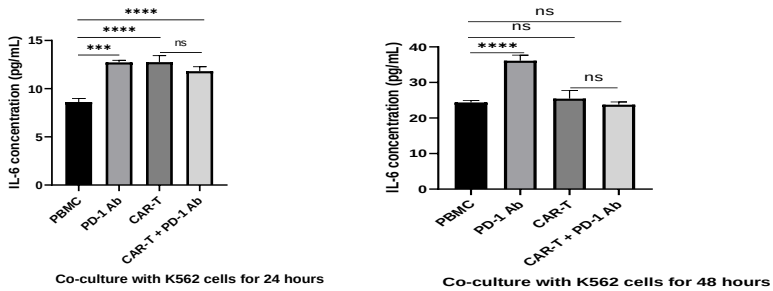


Figure 3.23. Comparison of IL-6 cytokine secretion when co-cultured with K562 cells in 4 groups after 24 and 48 hours

Figure 3.23 shows that at 24 hours, the concentrations of IL-6 in the culture fluid of CAR-T cells, PD-1 Ab, and CAR-T combined with PD-1 Ab when co-cultured with K562 cells were all significantly higher compared to the PBMC group ($p < 0.001$).

Moreover, at 48 hours, the IL-6 concentration of these two groups did not have a statistically significant difference compared to the PBMC cell control ($p > 0.05$).

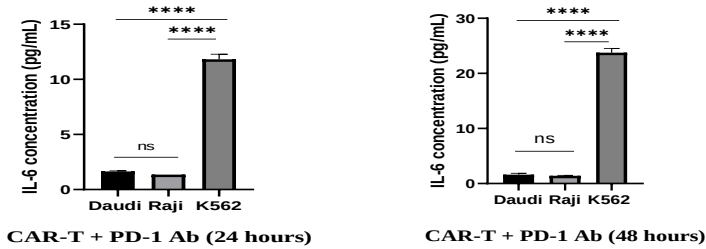


Figure 3.25. Comparison of IL-6 secretion ability of CAR-T cells combined with PD-1 Ab co-cultured with leukemia cells after 24 and 48 hours

Results in Figure 3.25 show that at 24 and 48 hours, the concentration of IL-6 in the culture fluid of CAR-T cells combined with PD-1 Ab, when co-cultured with Raji and Daudi cells, was significantly lower compared to when co-cultured with K562 cells ($p < 0.001$).

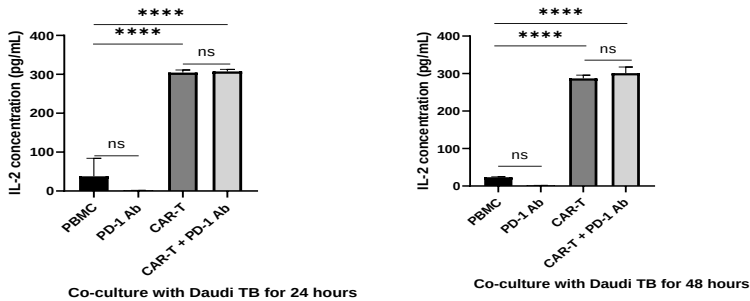


Figure 3.26. Comparison of IL-2 cytokine secretion when co-cultured with Daudi cells in 4 groups after 24 and 48 hours

Figure 3.26 shows that at both time points, the concentration of IL-2 in the culture fluid of CAR-T cells and CAR-T cells combined with PD-1 Ab when co-cultured with Daudi cells was statistically significantly higher compared to the PBMC cell group ($p < 0.001$).

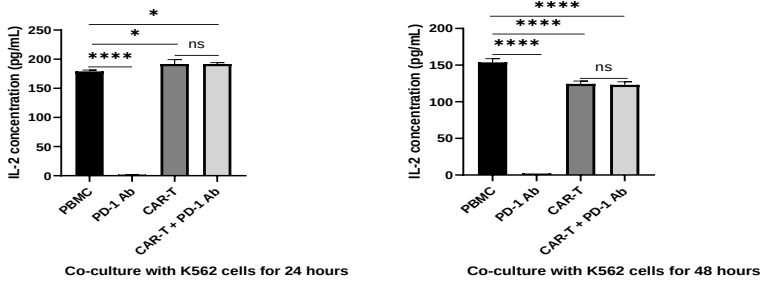


Figure 3.28. Comparison of IL-2 cytokine secretion when co-cultured with K562 cells in 4 groups after 24 and 48 hours

At 24 hours, the IL-2 concentration in the culture fluid of CAR-T cells and CAR-T combined with PD-1 Ab when co-cultured with K562 cells was statistically significantly higher than that of the PBMC group ($p < 0.05$). At 48 hours, the IL-2 concentration of these two groups decreased significantly compared to the IL-2 concentration in the co-culture fluid with PBMC cells ($p < 0.0001$).

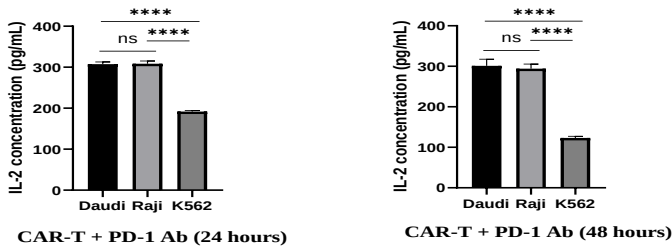


Figure 3.30. Comparison of IL-2 secretion ability of CAR-T cells combined with PD-1 Ab co-cultured with leukemia cells after 24 and 48 hours

Figure 3.30 shows that the concentration of IL-2 in the culture fluid of CAR-T cells combined with PD-1 Ab when co-cultured with Raji and Daudi cells at both 24 and 48 hours was significantly higher compared to co-culture with CD19- (K562) cells ($p < 0.0001$).

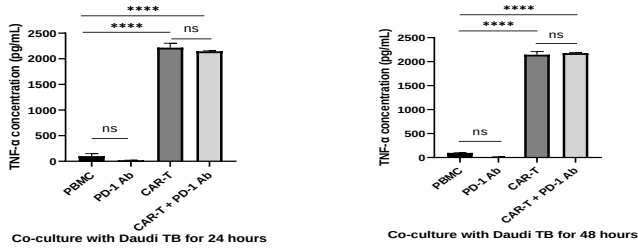


Figure 3.31. Comparison of the ability to secrete the cytokine TNF- α when co-cultured with Daudi cells in 4 groups after 24 and 48 hours

After 24 and 48 hours, the results showed that the concentration of TNF- α in the culture fluid of CAR-T cells and CAR-T combined with PD-1 Ab when co-cultured with Daudi cells was significantly higher than in the culture fluid of the control group of PBMC cells ($p < 0.05$).

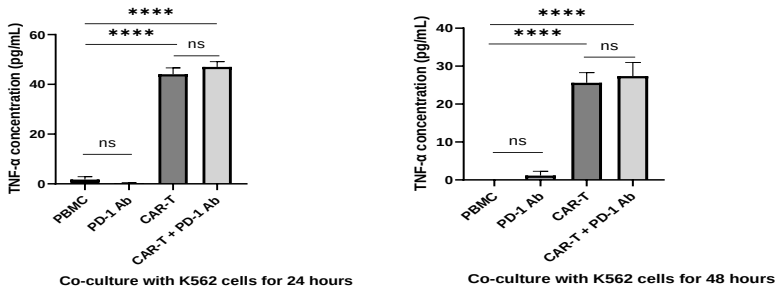


Figure 3.33. Comparison of the ability to secrete the cytokine TNF- α when co-cultured with K562 cells in 4 groups after 24 and 48 hours

TNF- α concentrations in the culture fluid of CAR-T cells and CAR-T combined with PD-1 Ab when co-cultured with K562 cells at both time points were statistically significantly higher ($p < 0.05$) compared to the TNF- α concentration in the culture fluid of the control group PBMC.

* At 24 and 48-hour, concentrations of cytokines IL-2, IL-6, and TNF- α of the CAR-T and CAR-T combined PD-1 Ab groups when co-cultured with all three cancer cell lines leukemia had no statistically significant difference ($p > 0.05$).

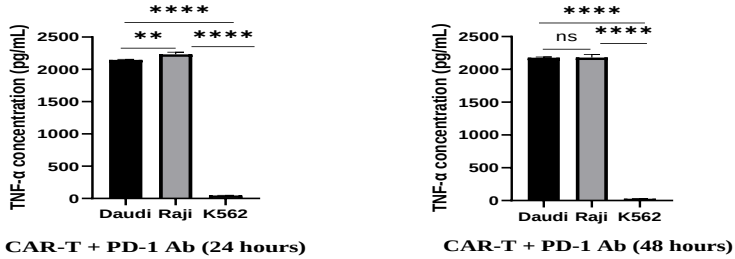


Figure 3.35. Comparison of TNF- α secretion ability of CAR-T cells combined with PD-1 Ab co-cultured with leukemia cells after 24 and 48 hours

TNF- α concentrations in CAR-T and CAR-T cells combined with PD-1 Ab when co-cultured with CD19+ cancer cells (Raji and Daudi) at 24 and 48 hours were significantly higher. Statistically significant ($p < 0.0001$) compared to when co-cultured with CD19- (K562) cells.

CHAPTER 4: DISCUSSION

4.1. QUALITY OF CAR-T CELL BLOCK AND SAFETY OF CAR-T CELL BLOCK COMBINED WITH PD-1 AB

4.1.1. Quality of CAR-T cells and CAR-T combined with PD-1 Ab

Our research results on CAR-T cell environment and CAR-T combined with PD-1 Ab all have endotoxin concentration < 0.1 EU/ml and no anaerobic or aerobic bacteria contamination. This shows that our laboratory's culture and testing process ensures standards and correct techniques.

Evaluating the expression rate of CAR-T cells using the Flow cytometry technique found that about 26.13% of T cells expressed CAR on their surface, and the expression of primer pairs in the design of plasmids transformed into T cells using the real-time PCR technique showed that CAR-T cell samples expressed CAR gene in all primer pairs.

4.1.2. Safety of CAR-T cell block and CAR-T combined with anti-PD-1 antibody in experiments

After injection, monitoring the mice daily for 4 weeks showed that 100% of mice in all groups were eating, drinking, and moving normally. They were agile, had smooth fur, clear eyes, dry anus, and no loose stools. The survival rate of mice in 4 groups was 100% until the last day of the experiment. Mouse weights at days 0, 7, 14, 21, and 28 in the CAR-T, PD-1 Ab, and CAR-T combined PD-1 Ab treatment groups were similar to the control group.

Our research results, when conducting tests to evaluate hematological indicators, liver and kidney function, liver and kidney histopathology, and IL-2 and IL-6 cytokine concentrations, all showed that groups using CAR -T, PD-1 Ab, and CAR-T combined with PD-1 Ab all had no statistically significant differences compared to the control group. Combination use of CAR-T and PD-1 Ab caused increased TNF- α levels compared to control groups and CAR-T cell, PD-1 Ab monotherapy. The results suggest a risk of excessive cytokine release syndrome when applying CAR-T and PD-1 Ab combination therapy. However, after monitoring the mice for 4 weeks, all mice were still eating, drinking, acting normally, and healthy until the end of the experiment.

Therefore, our study shows that CAR-T cells with a concentration of 10^6 cells/mouse and CAR-T cells combined with anti-PD-1 antibody at a dose of 250 μ g/mouse did not cause side effects in animals after 4 weeks of follow-up.

4.2. ANTI-LEUKEMIA EFFECTS OF CAR-T CELLS IN COMBINATION WITH PD-1 AB

4.2.1. Evaluation of *in vitro* cell lysis effect

The results showed that CAR-T cells and CAR-T cells combined with PD-1 Ab showed good lysis ability against Daudi

CD19⁺ cells. The lysis rate of these two groups was higher than that of the PBMC cell group. CAR-T cells in our study can target the CD19 antigen, bind to cells through this receptor, and cause apparent lysis of cancer cells that express CD19⁺, namely Daudi cells. The lytic ability of CAR-T cells and CAR-T combined with PD-1 Ab also increased with the increase of CAR-T ratio when co-cultured with cancer cells. This shows that the CAR-T cells created in this study have the effect of lysing cancer cells and are especially selective towards CD19⁺ cancer cells, as its mechanism of action suggests. This result is consistent with the design of the experiment and is similar to other studies when creating CAR-T cells to kill CD19⁺ cancer cells.

Similar to Raji CD19⁺ cells, the results also showed that CAR-T and CAR-T cells combined with PD-1 Ab showed relatively good lysis ability with Raji cells. These two groups' lysis rates were significantly higher than the PBMC cell group. The lytic ability of CAR-T cells and CAR-T combined with PD-1 Ab also increased with the increase in CAR-T ratio when co-cultured with cancer cells. Although the lysis rate was lower than that of Daudi cells, the group combining CAR-T with PD-1 Ab tended to increase lysis efficiency when increasing the CAR-T/Raji ratio. With Raji/CAR-T ratio = 1:10, the lysis efficiency increased from 24.2% to 44.11%, equivalent to a 1.82-fold increase in efficiency. Thus, combining PD-1 Ab with CAR-T increased the efficiency of Raji cell lysis.

To demonstrate the effectiveness of CD19 CAR-T cells according to the selective mechanism against CD19⁺ cancer cells, we evaluated the lysis effect of K562 CD19⁻ cells. For the ratio K562/CAR-T 1:2 and 1:5, CAR-T and CAR-T combined with PD-1 Ab had better cell lysis effects than PBMC but were both at low levels. The K562/CAR-T = 1:10 ratio showed that the percentage of

K562 CD19- cells lysed when co-cultured with PBMC was 27.52%, CAR-T was 27.67%, and CAR-T combined. PD-1 Ab combination 28.59% is similar. Thus, it can be seen that the K562 CD19 cells - CD19 CAR-T cells in our study had not clearly shown the ability to lyse. The lysing capacity was equivalent to that of PBMC cells at a ratio of 1:10. For the ratio K562/CAR-T = 1:2 and 1:5, CAR-T and CAR-T combined with PD-1 Ab had better cell lysis effects than PBMC, but both are at low levels.

Our above results are consistent with the mechanism of action of CD19 CAR-T cells. Initially, we found that CAR-T therapy combined with PD-1 Ab increased the lytic effect on Raji cells by 1.82 times at the ratio T:E = 1:10.

4.2.2. Changes in cytokine concentrations released in cell culture media

The concentrations of IL-6 in co-cultures of PBMCs with CD19+ leukemia cells after 24 and 48 hours were similar. In contrast, IL-6 concentrations were significantly higher in co-cultures of PBMCs and CD19-positive K562 cells. This suggests a higher IL-6 secretion in CD19- K562 cells than in CD19+ Raji and Daudi cells. Our results are consistent with CD19-positive K562 cells in that co-culture supplemented with PD-1 Ab increased IL-6 levels. In contrast, IL-6 levels were reduced in CD19+ cells (Daudi and Raji). This new finding suggests an unreported association between CD19 expression and the interaction of IL-6 and PD-1 signaling.

The results of this study indicated higher IL-2 concentrations when co-cultured PBMCs with K562 and Raji cells, compared to Daudi cells. Besides, in CD19+ cells Daudi and Raji, when co-cultured with CAR-T and CAR-T combined with PD-1 Ab, IL-2 concentration increased compared to the PBMC group at both 24 and

48 hours. In contrast, in CD19- K562 cells, the difference was insignificant between the CAR-T and CAR-T combined PD-1 Ab groups compared to the PBMC control group. Thus, IL-2 concentration increases when there is interaction between CAR-T carrying CD19 receptor and CD19 antigen on the surface of target leukemia cells (Daudi and Raji). The possible reason for the unknown change in IL-2 levels in K562 cells is the lack of target interaction of the chimeric receptor and the target antigen in K562 cells. When co-cultured with CAR-T cells and CAR-T cells conjugated with PD-1 Ab, CD19+ cells had statistically significantly higher IL-2 concentrations than CD19- cells. Our study results show that IL-2 concentrations increased significantly when co-cultured CD19+ leukemia cells with CAR-T CD19, suspected to be due to a specific interaction between the CD19 receptor on CAR-T and CD19 antigen on target cells. This effect was not significantly expressed in CD19- cells.

TNF- α concentrations in the culture fluid of CAR-T cells and CAR-T cells combined with PD-1 Ab when co-cultured with CD19+ Daudi and Raji leukemia cells were statistically significantly higher compared with PBMC cells and co-cultured with K562 CD19- cells. This result is consistent with previous reports on increased TNF- α levels after CAR-T cell therapy and the mechanism of action of CD19 CAR-T cell therapy, specifically on CD19+ cells. This result suggests that combined treatment on CD19+ cells has a stimulating interaction that increases the ability of CAR-T cells to secrete TNF- α .

CONCLUSIONS

1. Safety of CAR-T cell block combined with anti-PD-1 antibody in experiments

* Quality of CAR-T cells:

- CAR-T cells and CAR-T cells combined with PD-1 Ab ensured standards of endotoxin concentration and no infection.

- The CAR expression rate on T cells' surface analyzed by flow cytometry was 26.13%. The real-time PCR technique showed that CAR was expressed on transformed T cells.

* Safety of CAR-T cells combined with anti-PD-1 antibodies in experimental animals

CAR-T cells and CAR-T combined with PD-1 Ab did not affect survival, body weight, hematopoietic function, liver function, kidney function, and liver and kidney histopathology of experimental animals. Test after 4 weeks of follow-up.

2. Anti-leukemia effect of CAR-T cells combined with anti-PD-1 antibody in experiments

- CAR-T and CAR-T cells combined with PD-1 Ab had a higher ability to lyse CD19⁺ cancer cell lines (Daudi, Raji) than control group PBMC cells ($p < 0.05$).

- CAR-T therapy combined with anti-PD-1 antibody increased the lytic effect on Raji cells by 1.82 times at the ratio T:E = 1:10 ($p < 0.001$).

- There was no difference in the lysis rate of Daudi CD19⁺ cancer cells when co-cultured with CAR-T cells and combining CAR-T with PD-1 Ab ($p > 0.05$).

- The concentrations of cytokines IL-2, TNF- α in the culture fluid of CAR-T cells and CAR-T combined with PD-1 Ab when co-cultured with CD19⁺ cancer cells were higher than when co-cultured with CD19⁻ cells. There was no difference in IL-6, IL-2, and TNF- α concentrations in the group co-cultured with CAR-T cells and combining CAR-T with PD-1 Ab with $p > 0.05$.

LIST OF SCIENTIFIC PUBLICATIONS DERIVED FROM THE RESULTS OF THE THESIS

1. **Nguyen Hien Hanh**, Bui Khac Cuong, Nguyen Thi Mai Ly, Pham Chi, Nham Thi Phuong Linh, Ngo Thu Hang, Ho Viet Hoanh, Ta Viet Hung, Przemyslaw Bozko, Nguyen Linh Toan, Can Van Mao (2023). The safety of CAR-T cells and PD-1 antibody combination on an experimental model. *Biochemical and Biophysical Research Communications*, 649: 25-31.
2. **Nguyen Thi Hien Hanh**, Can Van Mao, Bui Khac Cuong (2023). Characteristics of some white blood cell indexes and IL-6 levels after treatment with CAR-T cell therapy combined with a monoclonal antibody that inhibits PD-1 experimentally. *Vietnam Medical Journal*, 530(1): 111-115.
3. **Nguyen Thi Hien Hanh**, Can Van Mao, Ngo Thu Hang, Dang Thuy Linh, Bui Khac Cuong (2024). Evaluation of the ability to lyse CD19(+) cancer cell lines of combination therapy of CAR-T cells with a monoclonal antibody that inhibits PD-1 in experiments. *Vietnam Medical Journal*, 535(1B): 265-269.
4. **Nguyen Thi Hien Hanh**, Can Van Mao, Bui Khac Cuong (2024). Survey of TNF- α levels and correlation with white blood cell indices in mice treated with a combination of PD-1 inhibition and CAR-T cells. *Journal of Medical Research*, 175(2): 164-171.