

A Novel Dual CAR-T Cells Specifically Recognize MUC1 and HER2 on Human Pancreatic Cancer with Their Activation

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Abstract: Aim: The objective of this study was to develop a novel tandem chimeric antigen receptor T (CAR-T) cells targeting both Mucin1 (Muc1) and Human Epidermal Growth Factor Receptor 2 (HER2) on the surface of pancreatic tumor to enhance CAR-T cell specificity and efficacy against this challenging malignancy. Methods: To establish dual-CAR-Jurkat T cells, lentivirus carrying the designed CAR genes was used to infect Jurkat T cells and validated under an inverted fluorescence microscope. Then, in situ immuno-fluorescence staining was utilized to confirm recognition and binding of dual-CAR-Jurkat T cells to Capan-2 cells. Finally, activation of dual-CAR-Jurkat T cells was verified by ELISA after stimulation of Capan-2 cells. Results: The M1H2 CAR-Jurkat T cells targeting Muc1 and HER2 were validated through in situ immuno-fluorescence staining and ELISA. The results showed that compared with the control cells that lack the M1H2 CAR molecules, the many M1H2 CAR-Jurkat T cells adhered tightly to Capan-2 cells, suggesting that the M1H2 CAR-Jurkat cells specifically recognize pancreatic cancer cells. Additionally, the M1H2 CAR-Jurkat cells released higher levels of T cell-activating cytokine IL-2 and cytotoxic cytokine IFN- γ , indicating that the both may maintain the activation and function of the M1H2 CAR-Jurkat T cells. Conclusion: The genetically engineered and modified tandem M1H2 CAR-T cells are successfully established. They can specifically identify and effectively bind pancreatic cancer cells expressing Muc1 and HER2. This might enable men to overcome limitation of current therapeutic approaches and increase therapeutic effects on refractory pancreatic cancer.

Keywords: Dual CAR-T Cells; Pancreatic Cancer; Specifically Recognize; MUC1 and HER2.

1. Introduction

Pancreatic cancer is highly malignant and extremely aggressive with a poor prognosis, and often diagnosed at its advanced stage due to its subtle symptoms and deep location. Pancreatic ductal adenocarcinoma (PDAC)(1), the most common form, originates from ductal cells and is associated with high mortality. Genetic mutations, particularly in KRAS(2), TP53(3), CDKN2A(4), and SMAD4(5), play a significant role in its development, along with environmental factors like smoking, chronic pancreatitis, and diabetes. Symptoms such as abdominal pain, weight loss, and jaundice appear late, complicating early detection. Imaging techniques like CT, MRI, and EUS are used for diagnosis, but early detection remains challenging. Treatment varies by stage, with surgical resection for localized disease and chemotherapy for advanced cases. Newer approaches, including targeted therapies and immunotherapies, are under investigation to improve outcomes. Despite progress(6), pancreatic cancer's 5-year survival rate remains under 10%, highlighting the need for profound research and development of effective screening and treatment strategies.

Chimeric Antigen Receptor T (CAR-T) cell therapy represents a significant evolution in adoptive cell transfer therapy by addressing the limitations of T Cell Receptor Engineered T (TCR-T) cell therapy. Unlike TCR-T cells, which require Major Histocompatibility Complex (MHC) molecules to present tumor antigens for recognition, CAR-T cells are designed to recognize and bind directly to tumor antigens through the chimeric antigen receptor (CAR) without MHC involvement(7, 8). This direct recognition is

facilitated by CARs with an antigen recognition domain that specifically targets the tumor antigen, a hinge region that connects the antigen recognition domain to the transmembrane domain and helps form the immunological synapse, and a transmembrane domain that links the extracellular and intracellular parts of the CAR(9-11). Additionally, CARs include intracellular signaling domains that are crucial for T cell activation. These domains typically involve co-stimulatory molecules like CD28, 4-1BB, or ICOS, which provide necessary second signals for T cell activation and prevent anergy or apoptosis(12-14). Most CAR designs also incorporate the CD3 ζ chain for delivering the primary activation signal via immunoreceptor tyrosine-based activation motifs (ITAMs). This combination of components allows CAR-T cells to overcome the MHC restrictions and antigen processing issues faced by TCR-T cells, making them effective in targeting and eliminating cancer cells. CAR-T cells have displayed significant potential not only in treating various cancers but also in potentially addressing other diseases such as diabetes and autoimmune disorders, expanding their therapeutic applications and impact(15).

Currently, CAR-T cell therapies are predominantly effective for treating blood tumors, but have shown limited success in combating solid tumors, particularly pancreatic cancer, which is known for its high malignancy and resistance to treatment. A major challenge in applying CAR-T therapy to solid tumors is the suppression of CAR-T cells by immune checkpoint molecules like PD-1 within the tumor microenvironment (TME)(16). To address this issue and enhance the therapeutic potential of CAR-T cells, we have developed a novel tandem CAR-T approach. This method

involves targeting two tumor-associated antigens (TSA) simultaneously to improve the efficacy and persistence of CAR-T cells against tumors that may evade detection by a single antigen-targeted approach(17).

In our study, we utilized a human pancreatic cancer cell line Capan-2 as a tumor model. We engineered tandem CAR-T cells to simultaneously target Mucin1 (Muc1) and Human Epidermal Growth Factor Receptor 2 (HER2). Mucin1 is a well-characterized cancer-associated glycoprotein overexpressed in many tumors, including pancreatic cancer, while HER2 is another critical antigen involved in various cancers(18, 19). By incorporating both Mucin1 and HER2, our tandem CAR-T cells were designed to enhance their ability to recognize and eliminate Capan-2 cells, overcoming potential antigen escape and ameliorating curative effects. This dual-target approach represents a promising strategy to increase the effectiveness of CAR-T therapy in treating difficult-to-cure solid tumors like pancreatic cancer.

2. Materials and Methods

2.1. Materials and reagents

Human IL-2 and Human IFN- γ ELISA kits were purchased from Jiangsu Boshin Biotechnology Co., Ltd. M1H2 CAR-T plasmid was acquired from Wuhan Jinkairui Bioengineering Co., Ltd. Lentiviral packaging vector psPAX2, envelope vector pMD2G, and overexpression plasmid pCDH were offered by Thermo Scientific, USA. Puromycin was procured from Beijing Solarbio Science & Technology Co., Ltd. MUC1 and HER2 antibodies were sourced from Huahan Bio Co., Ltd. Alexa Fluor 647-conjugated anti-rabbit secondary antibody was purchased from SAB, USA.

2.2. Cell culture

Capan-2 cells and HEK-293T cells were maintained in our laboratory under specific culture conditions. Capan-2 cells were cultured in McCoy's 5A medium with 10% fetal bovine serum (FBS). HEK-293T cells were grown in DMEM medium with 10% FBS. Jurkat T cells were cultured in RPMI-1640 medium supplemented with 10% FBS. For CAR-Jurkat T cells, culture medium is further supplemented with 1 μ g/mL Puromycin to create a selective medium. All cells were incubated at 37°C with 5% CO₂.

2.3. Lentivirus Packaging

HEK-293T cells were seeded in a 10 cm dish at 1×10^7 cells the day before transfection to achieve 70 - 90% confluence. Lollowing day, medium was replaced, and in two sterile 2 mL EP tubes, 750 μ L of serum-free DMEM was mixed with 15 μ g of plasmid DNA in a tube and then with 30 μ L of Lipo6000 transfection reagent in another. After a 5-minute incubation at room temperature, DNA solution was combined with Lipo6000 reagent, mixed gently, and cultured for 20 minutes. This mixture was added to a 10-cm dish, and after 6 hours it was replaced by fresh complete medium. After 24 - 48 hours, supernatant with viral particles was collected and concentrated using PEG. For titer determination, HEK-293T cells were seeded in a 96-well plate, and virus was subjected to 10-fold serial dilution. The diluted virus was added to the wells, incubated overnight, and then replaced with fresh medium. Fluorescence expression was observed, and the titer was calculated based on the number of wells showing positive fluorescence.

2.4. Lentiviral Infection of Jurkat T Cells

On the first day, 1×10^6 Jurkat T cells were seeded into each well of a new six-well plate and cultured in a 37°C incubator with 5% CO₂ overnight. The next day, concentrated lentivirus particles were gently added to center of each well, avoiding direct contact with T cell clusters. Microplates were gently swirled to ensure thorough penetration of the lentivirus particles and incubated under the same condition overnight. On the third day, the infection procedure was repeated as described.

2.5. In Situ Immunofluorescence Staining

A new 24-well plate was prepared with 2×10^4 Capan-2 cells seeded into each well and cultured in a 37°C incubator with 5% CO₂ for 24 hours. On the following day, 2×10^5 effector cells were added to each well at 37°C for an additional 24-hour culture. Cells were then gently washed three times with PBS at 37°C, each wash lasting 5 minutes. Next, 300 μ L of 4% paraformaldehyde solution was added to each well for fixation at room temperature for 30 minutes. The wells were washed three times with PBS, with each wash lasting 5 minutes. Blocking was performed with 1% BSA at room temperature for 1 hour, followed by three washes with PBS, each lasting 5 minutes. Primary antibody diluted in PBST was then put and gently shaken in the rocking bed at room temperature for 1 hour. After three washes with PBS, secondary antibody diluted in PBST was added and shaken under the same condition for 1 hour, and then washed three times with PBS, each for 5 minutes, before observation and imaging under a fluorescence microscope.

2.6. ELISA

To perform ELISA, 1×10^5 Capan-2 cells were seeded into a new 96-well plate and cultured in a 37°C incubator with 5% CO₂ overnight. Experimental and control Capan-2 cells in the plate were mixed at different effector-to-target ratios using 200 μ L of serum-free RPMI-1640 medium, and incubated in a 37°C incubator with 5% CO₂ for 8 hours. The plate was centrifuged at 3000 rpm for 10 minutes to remove debris. 50 μ L of gradient concentrations of standard solution IL-2 or IFN- γ was added to standard wells, and 10 μ L of sample and 40 μ L of sample diluent were put into sample wells. Then, 100 μ L of HRP-conjugated antibody was added to each well, and kept at 37°C for 60 minutes. Each well was washed with buffer five times. 50 μ L of substrate A and 50 μ L of substrate B were added to each well and kept at 37°C in the dark for 15 minutes. Then, 50 μ L of stop solution was added at the end and OD values were determined at 450 nm within 15 minutes.

3. Statistical Analysis

Single factor analysis of variance (GraphPad Prism 6) was performed by Tukey's post-test. Data represented at least 3 independent replicates, and appeared as mean $\pm$ standard deviation ($\bar{x} \pm SD$). p-values less than 0.05 were considered to be statistically significant.

4. Results

4.1. Preparation of M1H2 CAR-T Cells

To generate M1H2 CAR-Jurkat cells, Jurkat cells were seeded in 6-well plates at a density of 10^6 cells per well. Concentrated viral solutions adjusted according to the

determined viral titer were added to the respective wells. After 72 hours of incubation, the proportion of cells expressing green fluorescent protein (GFP) was assessed. As shown in Figure 1, no GFP expression was detected in the untransfected control wells, while the wells with transfected cells exhibited an increasing number of GFP-expressing cells over time. Subsequently, the cells were subjected to

puromycin selection at a concentration of 5 $\mu\text{g/mL}$. Most cells in the non-transfected wells died, whereas the surviving cells all expressed GFP (Fig 1). These results confirm that the transfected cells successfully express both GFP and the puromycin resistance gene, validating the effectiveness of the lentiviral infection of the cells.

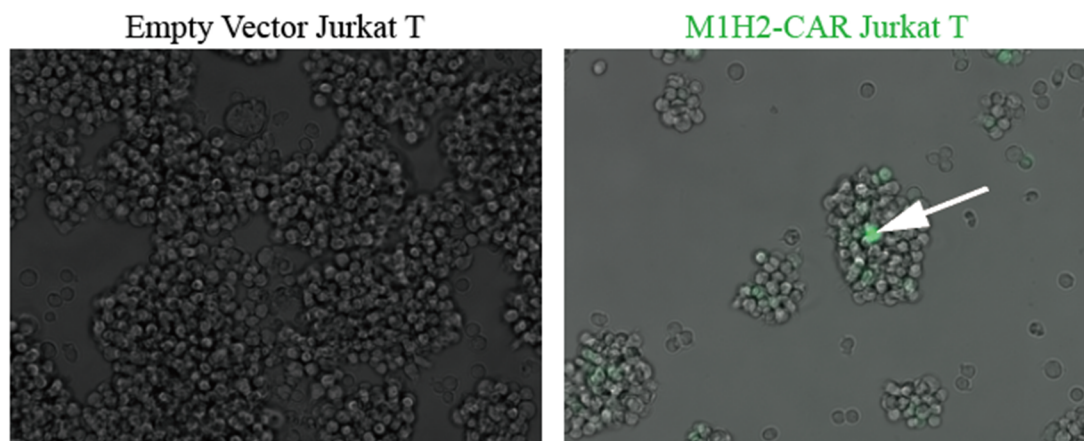


Fig 1. Preparation of M1H2 CAR-Jurkat T Cells

Jurkat T cells were transfected by lentivirus for 72 hours and observed under an inverted fluorescent microscope for the expression of green fluorescent protein on the genetically engineered and modified Jurkat T cells. Only the transfected cells emitted green fluorescence and survived the puromycin screening.

4.2. Dual CAR Molecules Enhance Binding of M1H2 CAR-Jurkat T Cells to Target Cells

To visually assess ability of designed CAR molecule whether to facilitate binding between M1H2 CAR-Jurkat T

Cells and pancreatic cancer cell Capan-2, M1H2 CAR-Jurkat cells, Jurkat T cells, and Capan-2 cells were co-cultured at a ratio of 10:1. After 24 hours, the cells were examined using an inverted fluorescence microscope. In the control group without the CAR molecules, Jurkat T cells formed clusters around Capan-2 cells, but did not exhibit binding. In contrast, the CAR-expressing Jurkat T cells showed clear adherence to the surface of Capan-2 cells (Fig. 2). These results demonstrate that the designed CAR molecules effectively recognize the target tumor antigen and enable the M1H2 CAR-Jurkat cells to bind to the pancreatic cancer cells.

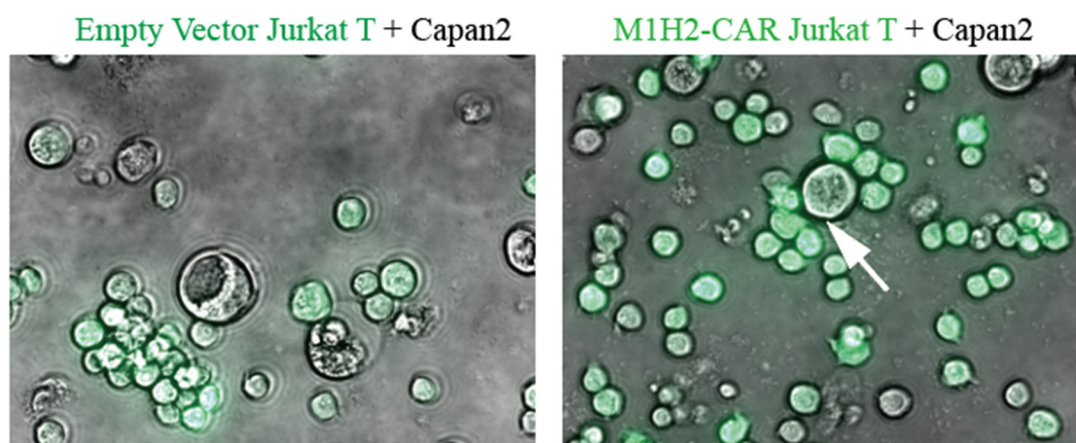


Fig 2. Bi-specific CAR molecules endow Jurkat T cells the ability to bind to Capan-2 cells

M1H2 CAR-Jurkat T cells were co-cultured with Capan-2 cells at a ratio of 10:1 for 24 hours. Then, they were examined using an inverted fluorescence microscope. The arrow shows that the cells with green fluorescence are M1H2-CAR Jurkat T cells that are binding Capan-2 cells.

4.3. Binding of CARs to Tumor Antigens on the target Cell Membrane

To confirm whether the designed CAR molecules can specifically identify MUC1 and HER2 antigens on Capan-2 cell membrane, we labeled co-cultured cells using anti-MUC1

and anti-HER2 antibodies, and then they were stained with Alexa Fluor 647-conjugated secondary antibodies to distinguish Capan-2 cells. In the control group, only Capan-2 cells showed strong red fluorescence and Jurkat T cells had no green fluorescence. In contrast, in the experimental group where Jurkat T cells were infected with a lentivirus containing the M1H2 CAR genes, we could simultaneously observe both red fluorescence from Capan-2 cells and green fluorescence from the CAR-expressing Jurkat T cells. The red fluorescence of Capan-2 cells was visible around their surface, while the M1H2 CAR-Jurkat T cells with the green fluorescent were

tightly bound to the surface of Capan-2 cells and not easily washed away (Fig. 3). These findings further demonstrate that MUC1 and HER2 antigens indeed present on the surface of

Capan-2 cells, and the M1H2 CAR molecules enable Jurkat T cells to specifically recognize and attach to tumor cells expressing the corresponding tumor antigens to function.

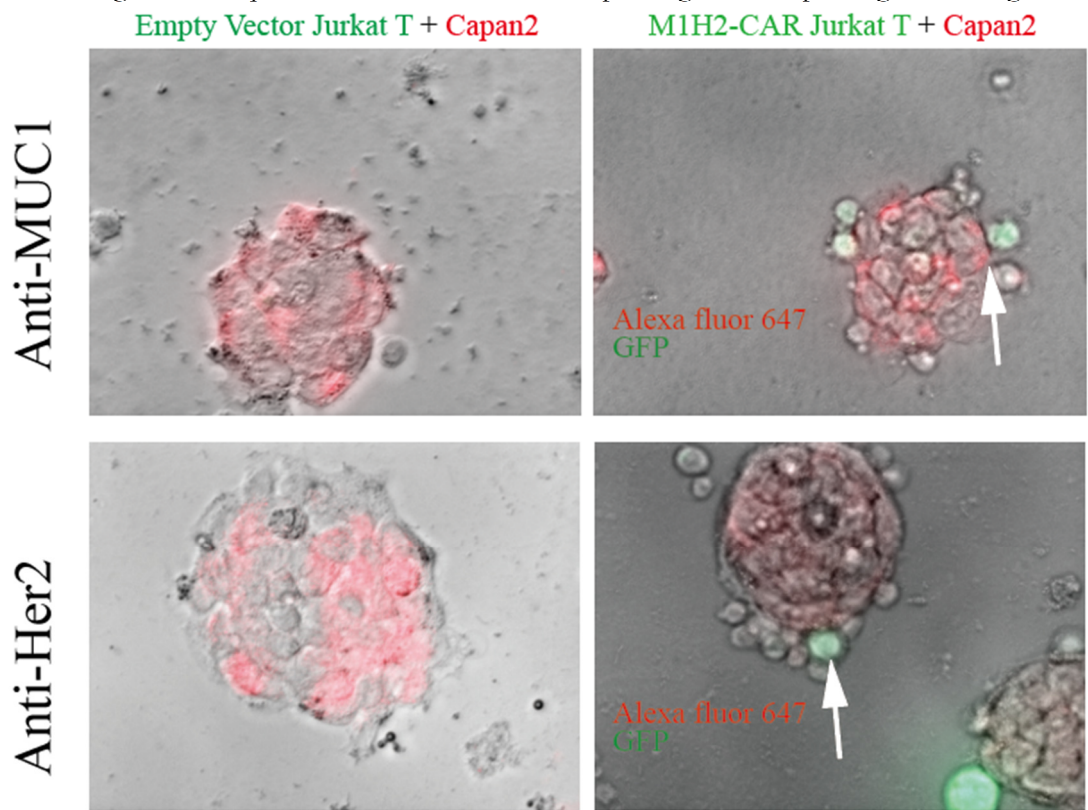


Fig 3. M1H2 CAR-Jurkat T cells specifically recognize Capan-2 cells highly expressing MUC1 and HER2

M1H2 CAR-Jurkat T cells could be recognized by their expressing EGFP. MUC1 and HER2 antigens on the surface of Capan-2 cells were identified by anti-MUC1 or anti-HER2 with the second antibody conjugated by Alexa fluor 647 to show Capan2 cells. Compared with the control group, M1H2

CAR-Jurkat T cells can specifically recognize and bind Capan-2 cells.

4.4. Activation of M1H2 CAR-Jurkat T Cells Binding to Tumor Cells

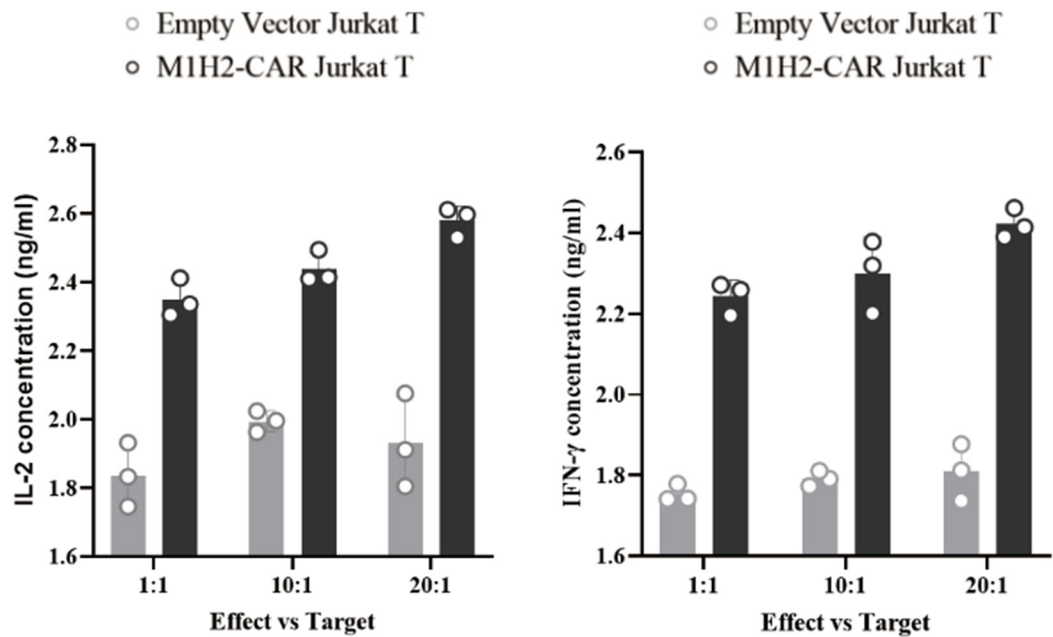


Fig 4. M1H2 CAR-Jurkat T cells show higher IL-2 and IFN-γ release levels

IL-2 and IFN-γ are key indicators of T cell activation, with IL-2 promoting T cell proliferation and IFN-γ serving as an inflammatory and cytotoxic cytokine. To further validate

activation of the constructed M1H2 CAR-Jurkat T cells, we measured release of their activation-related cytokines using ELISA after co-culturing effector cells with target cells. The

M1H2 CAR-Jurkat T cells were tested at three different effector-to-target (E: T) ratios 1:1, 10:1, and 20:1 for 24 hours, respectively. Compared to the empty lentivirus vector control group, the M1H2 CAR-Jurkat T cells exhibited higher levels of IL-2 and IFN- γ release (Fig. 4). These results indicate that M1H2 the M1H2 CAR-Jurkat T cells are activated upon binding to tumor cells, facilitating their proliferation and cytotoxic function.

M1H2 CAR-Jurkat T cells were co-cultured with Capan-2 cells at three different effector-to-target (E: T) ratios 1:1, 10:1, and 20:1 for 24 hours. IL-2 and IFN- γ were tested by ELISA. p -value < 0.0001 determined by two-way ANOVA test. $n = 3$.

5. Discussion

The use of dual CAR-T cells targeting MUC1 and HER2 represents a promising approach for treating pancreatic cancer, a notoriously aggressive and treatment-resistant malignant tumor. Both MUC1 and HER2 are naturally rich-expressed in human pancreatic cancer with MUC1 being a mucin that contributes to tumor progression and immune evasion and HER2 that is also implicated in some pancreatic tumors although more commonly associated with breast cancer. Therefore, we utilized this biological feature of pancreatic cancer to reconstitute T cells by a genetically engineering approach so as to strengthen their ability to recognize and bind pancreatic tumor cells for potentially overcoming their immunological escape. No report has been involved in this design strategy specific to pancreatic tumor until now. The current results demonstrate that the recognition and binding of the genetically engineered and modified T cells (named M1H2 CAR-Jurkat T cells) to the pancreatic tumor cells are largely elevated, and they are palpably in an activated state.

However, the dual-target approach presents a challenge in terms of design and safety. Genetically engineered CAR-T cells express the receptors specific to tumor-associated antigens both MUC1 and HER2, adding preparation complexity in the development process although this potentially improves therapeutic outcomes. Moreover, there are potential risks, including off-tumor toxicity due to possible expressions of these antigens in some normal tissues and immune-related cytokine release syndrome and neurotoxicity. To mitigate these risks, real time monitoring and advanced safety measures, such as suicide genes or inducible safety switches, will be essential.

Additionally, the immunosuppressive microenvironment in pancreatic tumor tissue further complicates CAR-T cell therapy, also directly impacting its treatment outcome. TGF- β , IL-10 and regulatory T cells in the tumor microenvironment can inhibit CAR-T cell activity, and the dense stroma in pancreatic tumor tissue may impede CAR-T cell infiltration. Strategies to enhance CAR-T cell resilience and penetration into tumor tissue are necessary(20). Clinical trials and preclinical studies are crucial to evaluate the safety, efficacy, and practical application of dual-targeting CAR-T cells, and will ultimately determine their feasibility as a viable treatment option for pancreatic cancer.

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