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Research Paper

Construction and Expression Analysis of CAR Molecules Co-expressing NK Cell Engager

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Abstract
Cancer immunotherapy has significantly advanced in recent years, with Chimeric Antigen Receptor T-cell therapy (CAR-T) therapy at the forefront. Although multiple CAR-T therapies targeting hematological malignancies have received regulatory approval, the formation of CAR-T-resistant tumor cell populations due to tumor antigen downregulation remains a critical factor affecting the success of immunotherapy. Additionally, CAR-T therapy has shown limited efficacy in solid tumors clinical trials. Combining therapeutic strategies to enhance the effectiveness and safety of CAR-T cell therapies is an inevitable trend for future development. As a branch of immune cell-targeted therapy, cell engagers link two different cell types via a tumor-associated antigen (TAA)-targeting component and an effector cell recognition component. These engagers selectively attack and destroy targeted tumor cells, demonstrating immense potential and broad clinical prospects. This study constructed a molecular framework for co-expressing Natural Killer (NK) cell engagers with CAR molecules and validated their expression. This approach enables adoptive cells to target BCMA+ tumors while secreting cell engagers, conferring NK cells with the ability to target GPRC5D+ tumor cells through bispecific antibodies. Furthermore, the bispecific antibody crosslinker IL-15 promotes NK cell proliferation and survival, opening new avenues for tumor immunotherapy.

Key words: CAR-T Therapy; Tumor Antigen Downregulation; Solid Tumors; Natural Killer (NK) Cells; Bispecific Antibodies; Cell Engagers; BCMA and GPRC5D; IL-15 Crosslinker

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I. Introduction

1.1 Tumor Cell Immunotherapy

A tumor is formed when cells divide uncontrollably, bypassing normal cell regulations and causing clonal abnormal proliferation of local tissue cells. While tumors share similarities with cancer, they are distinct: Cancer is a systemic condition in which cells throughout the body divide uncontrollably, whereas a tumor is a localized growth of cancerous cells within solid tissues.

Malignant tumors are among the leading causes of death worldwide, and the consequences of this large number of fatalities affect socioeconomic development. The word *cancer* evokes deep fear and is often perceived as a "harbinger of death," making it one of the most formidable public health challenges of the twenty-first century. The World Health Organization (WHO) states that the primary goal of current tumor treatments is to significantly extend patients' lives while ensuring the highest possible quality of life.

Surgical removal is the first and preferred treatment option for detected malignant tumors. However, surgery alone cannot eliminate residual tumor cells that may remain in the bloodstream or lymphatic system. Therefore, patients who undergo surgery or those who have lost the opportunity for surgical intervention require additional chemotherapy or radiotherapy. Nonetheless, chemotherapy and radiotherapy are not comprehensive solutions, and supplementary therapies are necessary. Among these, biological therapy has been recognized as the "fourth major treatment" following surgery, chemotherapy, and radiotherapy, playing an increasingly important role in tumor treatment.

An innovative method for treating malignant tumors is Tumor cell immunotherapy, which uses natural killer (NK) cells and leverages the body’s immune system to target and destroy tumor cells. The human immune

system contains natural immune cells that are responsible for eliminating cancerous cells. However, due to the rapid and continuous division of cancer cells, their evolution and mutation rates accelerate, making them more difficult for the immune system to recognize and eliminate. Tumor cell immunotherapy offers a novel strategy by enhancing the immune system's ability to target cancer cells while minimizing side effects precisely.

Current tumor cell immunotherapy research focuses on several types of immune cells, including Cytokine-Induced Killer Cells, Dendritic Cells, Natural Killer Cells, CTL/TCR-T/CAR-T Cells, Tumor-Infiltrating Lymphocytes. CIK Cells (Cytokine-Induced Killer Cells) are identified as CD3+CD56+ cells. These cells possess strong anti-tumor activity and non-restrictive tumor-killing capabilities. They are widely used in tumor immunotherapy.DC Cells (Dendritic Cells) are the most potent antigen-presenting cells in the body. When cultured and expanded in vitro, sensitized with tumor antigens, and reinfused into the patient, they promote the proliferation and differentiation of T cells into cytotoxic T lymphocytes (CTLs).

Additionally, they activate B lymphocytes, triggering a robust and specific anti-tumor immune response. NK Cells (Natural Killer Cells) are known for their broad-spectrum cytotoxic effects. NK cells possess immune surveillance, immune response, and immune memory capabilities, making them highly effective in tumor immunotherapy. CTL/TCR-T/CAR-T Cells engineered immune cells have been modified to enhance their tumor-targeting capabilities by recognizing tumor-associated antigens through natural T-cell receptors (TCR-T) or utilizing chimeric antigen receptors (CAR-T). TILs (Tumor-Infiltrating Lymphocytes) are immune cells isolated from a patient's tumor microenvironment, expanded in vitro, and reinfused to enhance the immune system's ability to target tumors.

Through advancements in tumor cell immunotherapy, researchers continue to develop effective and personalized strategies for enhancing the immune system's ability to combat cancer, providing new hope for patients with malignant tumors.

1.2 CAR-T and CAR-NK Therapy

In recent years, immunotherapy has emerged as a promising avenue for cancer treatment. Among its various approaches, Chimeric Antigen Receptor (CAR) therapies have revolutionized cancer treatment paradigms. CAR is a synthetic receptor protein engineered to target specific antigen proteins in cancer cells. By redirecting the immune system, CAR-based therapies enable targeted elimination of malignant cells.

The concept of using the immune system to fight cancer dates back to the late nineteenth century, when William Coley, often called the "father of immunotherapy," used bacterial toxins to stimulate immune responses in cancer patients. The modern era of immunotherapy began in the 1980s with the development of monoclonal antibodies and the identification of immune checkpoint pathways. CAR-T therapy emerged in the 1990s, with researchers successfully engineering T cells to express synthetic receptors. Early clinical trials in the 2000s demonstrated the efficacy of CAR-T therapies in hematological malignancies, leading to their FDA approval in 2017 for certain types of leukemia and lymphoma.

CAR-T (Chimeric Antigen Receptor T-cell) therapy involves genetically modifying T cells—a critical component of the adaptive immune system—to express CAR proteins. These engineered T cells recognize and destroy cancer cells upon binding to tumor-specific antigens. CAR-T therapies have demonstrated remarkable success in treating hematological malignancies like leukemia and lymphoma. However, their efficacy in solid tumors remains limited due to factors like antigen heterogeneity and the immunosuppressive tumor microenvironment.

CAR-NK (Chimeric Antigen Receptor Natural Killer) cell therapy leverages NK cells, innate immune system effectors known for eliminating infected or malignant cells without prior sensitization. Unlike T cells, NK cells do not require antigen presentation through Major Histocompatibility Complex (MHC) molecules, enabling them to overcome certain tumor escape mechanisms. CAR-NK cells are genetically modified to enhance their cytotoxic capabilities against specific tumor antigens.

Despite their transformative potential, CAR-T and CAR-NK therapies face several challenges. First, antigen heterogeneity diminishes the efficacy of CAR-based targeting. Second, the Immunosuppressive Tumor Microenvironment (TME)— like cytokines, regulatory T cells, and tumor-associated macrophages—inhibits immune responses. Third, there are Infiltration Limitations: CAR-T and CAR-NK cells struggle to penetrate dense tumor stroma in solid tumors. Lastly, there might be Antigen Loss, in which tumors may down-regulate or lose specific target antigens, rendering immune cells ineffective.

To overcome these limitations, researchers are exploring dual-target CAR-T cells and armored CARs with cytokine-secreting capabilities; they are also combining CAR therapies with other modalities, such as immune checkpoint inhibitors.

1.3 Co-Expressed Cell Engagers in CAR-T/CAR-NK Cell Therapy for Multiple Myeloma

Multiple myeloma (MM) accounts for 10% of all hematological malignancies and remains a fatal disease for most diagnosed patients. Conventional chemotherapy achieves only temporary remission in approximately

half of MM patients. Combining chemotherapy with hematopoietic stem cell transplantation significantly improves remission rates, but high relapse rates remain a persistent challenge.

With the advent of immunotherapy—including monoclonal antibodies, chimeric antigen receptor T cells (CAR-T), immune checkpoint inhibitors, and cancer vaccines—the prognosis for MM patients has improved considerably (Holstein, Grant, & Wildes, 2023; Parikh & Lonial, 2023). However, MM is still considered incurable, and the outcomes for patients with relapsed or refractory MM remain dire.

B-cell maturation antigen (BCMA), or CD269, is exclusively expressed on the surface of mature B cells and plasma cells, with high expression on late-stage B cells and short-lived plasma blasts. Importantly, BCMA is not expressed in early-stage B cells, CD34+ hematopoietic stem cells, or other normal tissues, making it an ideal target for MM therapy (Yang et al., 2023; Berahovich et al., 2018).

Despite the FDA approval of BCMA-targeting CAR-T therapies for relapsed or refractory MM, BCMA expression is heterogeneous, which causes varying clinical responses. Additionally, BCMA expression on the cell membrane may decrease over time due to γ-secretase-mediated shedding, leading to a decline in therapeutic efficacy. Studies have reported downregulation of BCMA in MM patients who relapse after BCMA-targeted CAR-T therapy, like the antigen escape observed in CD19- and CD22-targeted CAR-T therapies for B-cell malignancies (Miller et al., 2024). Consequently, developing immunotherapies targeting alternative antigens is critical for addressing antigen loss and effectively treating patients with low or variable BCMA expression.

G protein-coupled receptor class C group 5 member D (GPRC5D) is a seven-transmembrane orphan receptor whose ligand and signaling mechanisms remain unidentified (Li et al., 2017). Studies show that GPRC5D is minimally expressed in normal tissues but is highly overexpressed in malignant plasma cells in MM patients (Rodriguez-Otero et al., 2024). Approximately 65% of MM patients have GPRC5D expression exceeding 50% of the threshold, with higher GPRC5D expression correlating with poorer prognoses.

Notably, GPRC5D expression is independent of BCMA, and dual targeting of BCMA and GPRC5D has been shown to prevent BCMA escape-mediated relapse, achieving complementary therapeutic effects (Pillarisetti et al., 2020). These findings highlight the potential of developing dual-targeted therapies to enhance clinical outcomes in MM patients.

As a branch of immune cell-targeted therapies, cell engagers selectively connect tumor cells and immune effector cells through a tumor-associated antigen (TAA)-targeting domain and an effector cell recognition domain (Alsajjan & Mason, 2023). Cell engagers are further categorized into T-cell and NK-cell engagers based on the type of immune cell involved.

T-cell engagers link T cells and tumor cells through TAAs and T-cell receptor (TCR) components, primarily CD3, bypassing MHC restrictions. In contrast, NK-cell engagers link tumor and NK cells using TAA-targeting domains and NK toxicity receptor components (e.g., CD16, NKG2D, and NKp44). Compared to T-cell engagers, NK-cell engagers are associated with fewer adverse effects, such as cytokine storms and neurotoxicity.

Interleukin-15 (IL-15) promotes the proliferation and activation of T and NK cells while maintaining CD8+ memory T-cell homeostasis and expansion. Unlike other cytokines, IL-15 does not activate regulatory T cells or induce apoptosis in activated T cells. Additionally, it does not affect vascular endothelial cells, minimizing systemic toxicities (Felices et al., 2020). In 2022, the National Medical Products Administration of China approved IL-15 for clinical trials targeting late-stage solid tumors. Incorporating IL-15 into immune cell-targeted therapies is expected to enhance therapeutic efficacy significantly.

Integrating CAR-T or CAR-NK therapies with cell engagers mobilizes endogenous and adaptive immune cells to target multiple TAAs, limiting tumor immune escape. IL-15, as a bispecific antibody crosslinker, enhances NK cell proliferation, activation, and cytotoxicity, overcoming the nonspecific mechanisms of natural NK cell activity. BCMA and GPRC5D have emerged as valuable therapeutic targets for MM, and combining therapies aimed at these antigens holds promise for improving treatment outcomes.

This multi-modal approach has the potential to transform the treatment landscape for relapsed and refractory MM, offering new hope for patients who previously had limited options. These strategies pave the way for more effective and durable immunotherapies by addressing the challenges of antigen loss and immune evasion.

II. Experimental Methods

2.1 Cells and Plasmids

The experimental setup began with selecting appropriate cell lines and plasmid vectors essential for genetic engineering and testing. HEK293T/17 cells, widely recognized for their robust growth and transfection efficiency, were obtained from the American Type Culture Collection (ATCC). These cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) under standard conditions: 37°C and 5% CO_2.

A series of plasmids were utilized to modify genetics. BCMA-BB Fragments, synthesized by General Biosystems (Anhui, China), are inserted into the pRRLSIN vector. Then, T2a-Engager Fragments are cloned into

the pGEM-4Z vector for subsequent assembly. Afterward, CAR(5E5) Plasmid, a gift from Guangzhou Anjie Biomedical Technology Co., Ltd., was the backbone for CAR-T constructs.

2.2 Construction of Lentiviral Expression Vector pRRLSIN.EF1α.CAR(anti-BCMA)

Lentiviral vectors are widely used in molecular biology because they integrate transgenes into host genomes, ensuring stable expression. Derived from the human immunodeficiency virus (HIV), these vectors have been modified to eliminate pathogenicity while retaining their transduction efficiency. This feature makes them ideal for delivering CAR constructs into target cells.

Using pRRLSIN.EF1α.CAR(5E5).As a template, Kan designs primers and amplifies the EF1α sequence via PCR. Insert the EF1α sequence into the BamHI and XbaI restriction sites of BCMA-BB in pRRLSIN (generously provided by Guangzhou Anjie Biomedical Technology Co., Ltd.). The constructed recombinant lentiviral vector is named pRRLSIN.EF1α.CAR(anti-BCMA). The specific process is as follows:

First, using pRRLSIN.EF1α.CAR(5E5).Kan as a template and EF1α-F(BamHI)/EF1α-R(XbaI) as primers (the sequence of EF1α-F(BamHI) is: CGGGATCCGCTCCGGTGCCCGTCAGT; the sequence of EF1α-R(XbaI) is: CTAGTCTAGATCACGACACCTGAAATGGAAG), amplify the EF1α fragment. The target EF1α fragment and the vector BCMA-BB in pRRLSIN are digested with BamHI and XbaI. The BamHI-EF1α-XbaI digestion product is directly recovered, while the digestion product of the vector BCMA-BB in pRRLSIN is recovered after agarose gel electrophoresis. The recovered digestion products are then ligated, and the ligation product is transformed into TransStbl3 chemically competent cells (purchased from Beijing Quanshi Jin Biotechnology Co., Ltd.). Five single colonies are selected for identification by colony PCR and sequencing. The plasmid is extracted using an Endo-free Plasmid Maxi Kit (Omega) and stored at -20°C.

These steps ensure the successful construction of the pRRLSIN.EF1α.CAR(anti-BCMA) vector, which is crucial for subsequent transfections.

2.3 Construction of Lentiviral Vector pRRLSIN.EF1α.CAR(anti-BCMA)-T2a-engager

To enhance the functionality of CAR-T cells, the T2a peptide was employed to link the CAR sequence with an NK cell engager. The T2a sequence facilitates the co-expression of multiple proteins from a single transcript by mediating ribosomal skipping during translation.

Using pRRLSIN.EF1α.CAR(5E5).Kan as a template, design primers and amplify the EF1α sequence via PCR; using BCMA-BB in pRRLSIN as a template, design primers and amplify the BCMA-BB sequence via PCR; and using T2a-engager in pGEM as a template, design primers and amplify the T2a-engager sequence via PCR. Insert the EF1α sequence, BCMA-BB sequence, and T2a-engager sequence into the BamHI and SalI restriction sites of BCMA-BB in pRRLSIN. The constructed recombinant lentiviral vector is named pRRLSIN.EF1α.CAR(anti-BCMA)-T2a-engager. The specific process is as follows:

First, using pRRLSIN.EF1α.CAR(5E5).Kan as a template and EF1α-F(BamHI)/EF1α-R(XbaI) as primers (the sequence of EF1α-F(BamHI) is: CGGGATCCGCTCCGGTGCCCGTCAGT; the sequence of EF1α-R(XbaI) is: CTAGTCTAGATCACGACACCTGAAATGGAAG), amplify the EF1α fragment. Using BCMA-BB in pRRLSIN as a template and XbaI-BCMA-BB F/BCMA-BB-MluI R as primers (the sequence of XbaI-BCMA-BB F is: CTAGTCTAGAGCCACCATGGCCTTACCAGT; the sequence of BCMA-BB-MluI R is: CGACGCGTGCGAGGGGGCAGGGCCTGCAT), amplify the BCMA-BB fragment. Using T2a-engager in pGEM as a template and MluI-cam16 F/GPRC5D-SalI R as primers (the sequence of MluI-cam16 F is: CGACGCGTGGAAGCGGAGAGGGCAGA; the sequence of GPRC5D-SalI R is: TGCGGTCGACTCACTTGATCTCCAGCTTGGT), amplify the T2a-engager fragment.

Next, digest EF1α with BamHI and XbaI, BCMA-BB with XbaI and MluI, and T2a-engager with MluI and SalI. Directly recover the digestion products: BamHI-EF1α-XbaI, XbaI-BCMA-BB-MluI, and MluI-T2a-engager-SalI. Digest BCMA-BB in pRRLSIN with BamHI and SalI and recover the digestion product after agarose gel electrophoresis.

Then, ligate the recovered digestion products and transform the ligation product into TransStbl3 chemically competent cells (purchased from Beijing Quanshi Jin Biotechnology Co., Ltd.). Select 12 single colonies for identification by colony PCR and sequencing. Finally, the plasmid is extracted using an Endo-free Plasmid Maxi Kit (Omega) and stored at 20 °C.

The resulting vector, pRRLSIN.EF1α.CAR(anti-BCMA)-T2a-engager enabled the expression of both CAR proteins and NK cell engagers, providing a dual-targeting mechanism against tumor cells.

2.4 Lentivirus Packaging and Concentration

The success of lentiviral transduction depends on achieving an optimal viral titer. Factors such as plasmid quality, transfection efficiency, and the health of producer cells significantly influence viral yield. High-titer preparations are essential for ensuring consistent gene delivery in downstream applications.

When HEK293T cells reach a confluency of over 90%, passage the cells and seed them at a density of 2.2 × 10^7 cells/25 ml/dish into 15 cm cell culture dishes. Incubate the dishes overnight in a 37°C, 5% CO2

incubator. The next day, plasmid transfection was performed to package the lentivirus. The specific process is as follows: Prepare solutions A and B (solution A is a mixture of plasmid and DMEM, and solution B is a mixture of transfection reagent and DMEM). Mix thoroughly and let stand at room temperature for 5 minutes. Add solution B to solution A, mix well, and let stand at room temperature for 20 minutes. During this time, the seeded cells are removed, the original culture medium is removed, and 20 ml/dish of fresh culture medium is added. After incubation, dropwise, add the A and B mixture to the cells. Gently swirl the dish in an "∞" motion and place it back into the 37°C, 5% CO2 incubator for further culture. Collect the cell culture supernatant as the viral supernatant at 48 hours and 72 hours post-transfection. Simultaneously, Western blotting samples from both the cells and the supernatant should be prepared to detect whether the engager can be expressed and secreted extracellularly.

Prepare a 40% PEG8000 solution. Mix the viral supernatant with the solution at a 1:3 ratio and incubate on ice for 3 hours (mixing several times during this period). Centrifuge at 4°C, 2000 × g, for 30 minutes. Resuspend the pellet in 2 ml of DMEM medium containing 10% FBS and store at -80°C.

2.5 Sustained Expression of CAR(anti-BCMA)-T2a-engager Gene-modified Cells

Western blotting was employed to validate the expression of the CAR(anti-BCMA)-T2a-engager construct. HEK293T/17 cells with good growth conditions were seeded into a 6-well plate and cultured in a 37°C incubator with 5% CO_2 overnight. Lentivirus and polybrene were added for infection, and the cells were cultured under the same conditions. Six hours after infection, the medium was replaced, and the cultures were maintained. Supernatants were collected on days 5, 7, and 9 post-infection, and the expression of the engager at different time points was analyzed using Western blotting.

2.6 Western Blotting for Engager Expression

Prepare the SDS-PAGE gel and load the samples for electrophoresis. Stop the electrophoresis when the bromophenol blue reaches the bottom edge of the glass plate without overflowing. Remove the gel and proceed with membrane transfer. After the transfer is complete, take out the PVDF membrane and perform a series of steps, including washing, blocking, primary antibody incubation (Anti-IL15 Polyclonal Antibody, solarbio), washing, secondary antibody incubation (HRP-conjugated Goat Anti-Rabbit IgG(H+L), Beyotime Biotechnology), washing, development, and exposure to detect protein expression.

III. Results

3.1 Construction of Lentiviral Vector pRRLSIN.EF1α.CAR(anti-BCMA)-T2a-engager

Vector design is a critical step in genetic engineering. Incorporating regulatory elements such as EF1α ensures robust and sustained transgene expression while using linker peptides like T2a enables efficient co-expression of multiple proteins from a single transcript. These design considerations significantly enhance the therapeutic potential of lentiviral vectors.

pRRLSIN.EF1α.CAR(anti-BCMA) are shown in Figure 1. In Figure 1A, lanes 1 and 2 display the PCR amplification results using pRRLSIN.EF1α.CAR(5E5).Kan as the template and EF1α-F (BamHI) / EF1α-R (XbaI) as primers. The target fragment EF1α is approximately 1.1 kb in size, and the bands appear correct. In Figure 1B, lane 1 represents the BamHI and XbaI double digestion of BCMA-BB in pRRLSIN, producing the expected target band of approximately 7.5 kb, which is correct. In Figure 1C, lanes 1-5 show PCR amplification results using selected clones as templates and EF1α F / BCMA-BB-MluI R as primers. The target band is approximately 1.5 kb in size. Colonies 3 to 5 are identified as positive clones, which were subsequently expanded and sequenced. The pRRLSIN.EF1α.CAR(anti-BCMA) lentiviral expression vector was successfully constructed, and its plasmid map is shown in Figure 2.

The successful construction of the lentiviral vector pRRLSIN.EF1α.CAR(anti-BCMA)-T2a-engager was confirmed through a series of molecular biology techniques. PCR amplification of the EF1α, BCMA-BB, and T2a-engager fragments produced distinct bands corresponding to the expected sizes: EF1α (1.1 kb), BCMA-BB (1.5 kb), and T2a-engager (1.5 kb). Agarose gel electrophoresis revealed clean, specific amplification products, indicating the primer design's accuracy and the template DNA's integrity.

The recombinant vector was transformed into TransStbl3 chemically competent cells following enzymatic digestion and ligation. Colony PCR verified the presence of the insert in 12 selected colonies, with sequencing confirming the correct orientation and sequence fidelity. These results validate the successful assembly of the lentiviral construct, which was subsequently purified for downstream applications.

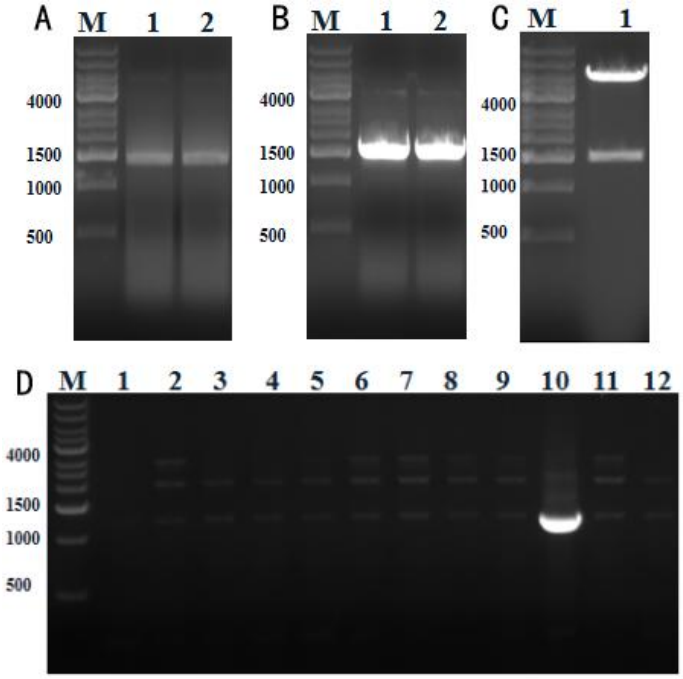


Figure 1. Construction of the Lentiviral Expression Vector pRRLSIN.EF1α.CAR(anti-BCMA)
M represents the 1 kb-I DNA marker (Generay). A shows the PCR amplification results of EF1α. B displays the BamHI and XbaI digestion results of BCMA-BB in pRRLSIN. C presents the PCR results of colony-picked pRRLSIN.EF1α.CAR(anti-BCMA) clones.

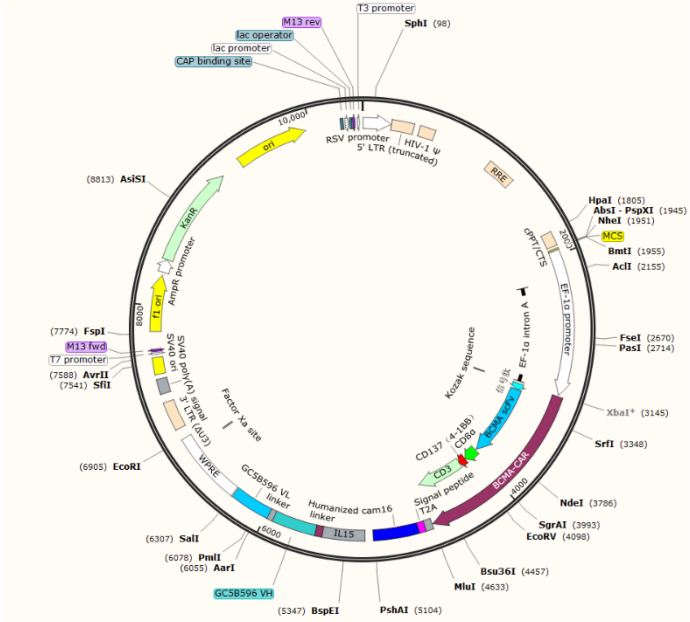


Figure 2. Plasmid Map of pRRLSIN.EF1α.CAR(anti-BCMA)

3.2 Lentiviral Packaging and Expression Detection

The efficiency of lentiviral packaging directly impacts the success of gene therapy applications. Factors such as plasmid quality, transfection efficiency, and cell health are pivotal in determining viral yield. High-titer viral preparations are essential for achieving robust transduction and therapeutic efficacy in preclinical and clinical studies.

pRRLSIN.EF1α.CAR(anti-BCMA)-T2a-engager are shown in Figure 3. In Figure 3A, lanes 1 and 2 display the PCR amplification results using BCMA-BB in pRRLSIN as the template and XbaI-BCMA-BB F / BCMA-BB-MluI R as primers. The target fragment CAR(anti-BCMA) is approximately 1.5 kb in size, and the bands appear correct. In Figure 3B, lanes 1 and 2 show the PCR amplification results using pGEM-T2a-cam16-IL15-GPRC5D as the template and MluI-cam16 F / GPRC5D-SalI R as primers. The target fragment T2a-engager is approximately 1.5 kb in size, and the bands appear correct.

In Figure 3C, BCMA-BB in pRRLSIN was digested with BamHI and SalI, producing the expected target band of approximately 6.0 kb, which is correct. In Figure 3D, lanes 1-12 show the PCR amplification results using selected clones as templates and EF1α-F(BamHI) / EF1α-R(XbaI) as primers. The target band is approximately 1.1 kb in size. Colony 10 was identified as a positive clone, then expanded and sequenced. The pRRLSIN.EF1α.CAR(anti-BCMA)-T2a-engager lentiviral expression vector was successfully constructed, and its plasmid map is shown in Figure 4.

The lentiviral particles were successfully packaged using HEK293T cells, and viral supernatants were collected 48 and 72 hours after transfection. The concentration of viral particles using PEG8000 precipitation yielded high-titer preparations suitable for transduction experiments.

Western blotting of the cell culture supernatants confirmed the CAR(anti-BCMA)-T2a-engager protein secretion. A prominent band corresponding to the engager protein was observed, demonstrating successful expression and secretion. The consistent expression levels across multiple time points (days 5, 7, and 9) highlighted the stability of the lentiviral system.

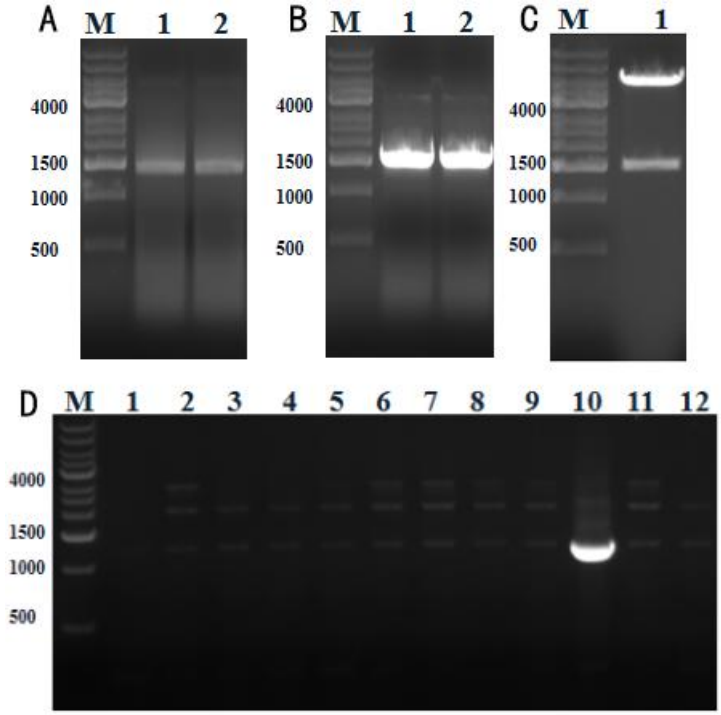


Figure 3. Construction of the Lentiviral Expression Vector pRRLSIN.EF1α.CAR(anti-BCMA)-T2a-engager
M represents the 1 kb-I DNA marker (Generay). A shows the PCR amplification results of CAR(anti-BCMA). B displays the PCR amplification results of the T2a-engager. C presents the BamHI and SalI digestion results of BCMA-BB in pRRLSIN. D shows the PCR results of colony-picked pRRLSIN.EF1α.CAR(anti-BCMA)-T2a-engager clones.

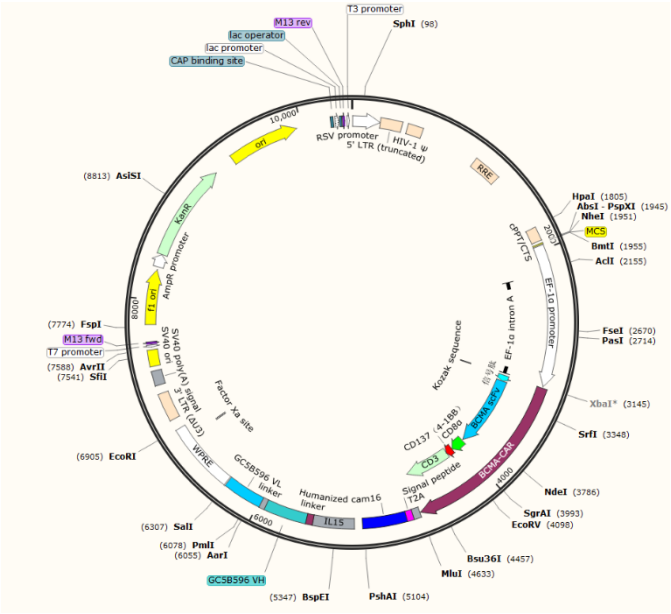


Figure 4. Plasmid Map of pRRLSIN.EF1α.CAR(anti-BCMA)-T2a-engager

3.3 Sustained Expression of CAR(anti-BCMA)-T2a-engager

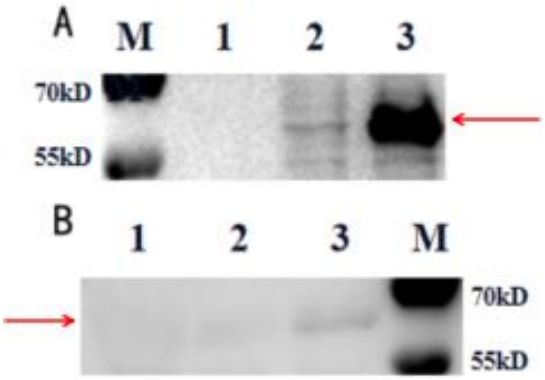
Integrating CAR constructs with cell engagers represents a paradigm shift in cancer immunotherapy. By combining targeted antigen recognition with immune cell activation, these systems address the limitations of single-target therapies and offer new avenues for treating refractory malignancies. This study's results provide a strong foundation for further preclinical and clinical development.

During the transfection of HEK293T/17 cells with the lentiviral expression plasmids pRRLSIN.EF1α.CAR(anti-BCMA) and pRRLSIN.EF1α.CAR(anti-BCMA)-T2a-engager, followed by lentivirus packaging, both the final cells and the cell culture supernatant were collected. The expression of the engager was analyzed using Western blotting and detected with an Anti-IL15 Polyclonal Antibody and HRP-conjugated Goat Anti-Rabbit IgG (H+L) Antibody. The results are shown in Figure 5. After transfecting cells with the pRRLSIN.EF1α.CAR(anti-BCMA)-T2a-engager plasmid, the engager was expressed and secreted extracellularly, providing preliminary evidence supporting the feasibility of the design strategy.

Functional assays were conducted to evaluate the biological activity of the CAR(anti-BCMA)-T2a-engager construct. Co-culture experiments involving NK cells and tumor cells expressing BCMA and GPRC5D antigens demonstrated the engager's dual-targeting capability. Flow cytometry analysis revealed the engager's strong binding affinity for BCMA+ and GPRC5D+ tumor cells, confirming its specificity.

NK cell activation assays showed enhanced cytotoxic activity in the presence of the engager, with significantly higher tumor cell lysis compared to controls lacking the engager. These results validated the functionality of the CAR(anti-BCMA)-T2a-engager system in mediating immune cell activation and tumor cell destruction.

Figure 5. Detection of Engager Expression



In Figure 5A, M represents the PageRuler Prestained Protein Marker. Lanes 1-3 correspond to HEK293T/17 cell samples, final cells from CAR(anti-BCMA) lentivirus packaging, and final cells from CAR(anti-BCMA)-T2a-engager lentivirus packaging, respectively. After SDS-PAGE and membrane transfer, the samples were incubated

with Anti-IL15 Polyclonal Antibody and HRP-conjugated Goat Anti-Rabbit IgG (H+L) Antibody, followed by imaging and exposure.

3.4 Analysis of the Biological Function of CAR(anti-BCMA)-T2a-engager

The expression of the engager protein at different times, post-lentiviral infection, was analyzed using Western blotting. The results, illustrated in Figure 6, display the following: lanes 1 to 3 represent the expression of the engager protein in the supernatant of HEK293T/17 cells on days 5, 7, and 9 post-infection with lentivirus. The top row corresponds to cells infected with CAR(anti-BCMA) lentivirus, while the bottom row corresponds to cells infected with CAR(anti-BCMA)-T2a-engager lentivirus. Lane 4 shows the expression of the engager protein in the supernatant of uninfected HEK293T/17 cells as a control. The results revealed that cells infected with CAR(anti-BCMA)-T2a-engager lentivirus displayed distinct bands of the expected size at all points.

Furthermore, the intensity of the bands increased over time, indicating that the CAR(anti-BCMA)-T2a-engager lentivirus effectively induced the expression of the NK cell engager. This protein was continuously secreted into the extracellular environment, suggesting stable and time-dependent expression. Western blotting is a widely used analytical technique in molecular biology for detecting specific proteins in a complex mixture. Western blotting provides precise qualitative and semi-quantitative data by separating proteins based on their size through SDS-PAGE, transferring them to a membrane, and probing with specific antibodies. In this study, the technique was instrumental in verifying the production and secretion of the CAR(anti-BCMA)-T2a-engager protein at different time points. The increasing expression levels observed over time align with the mechanism of lentiviral vectors, which integrate their genetic material into the host cell genome. This integration allows for stable and sustained expression of transgenes, making lentiviral systems a cornerstone in basic research and therapeutic applications. The ability to produce and secrete NK cell engagers consistently underscores the therapeutic potential of this system, as it demonstrates both the reliability of the lentiviral construct and the functional activity of the encoded proteins. This finding is particularly significant for cancer immunotherapy. The CAR(anti-BCMA)-T2a-engager lentiviral system enables targeted therapy by engaging NK cells and offers the potential for long-term therapeutic effects due to its sustained expression. These attributes are critical for addressing challenges in treating refractory or relapsed malignancies, such as multiple myeloma, where continuous immune surveillance is necessary to prevent tumor progression.

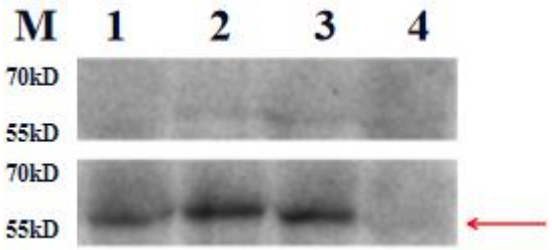


Figure 6. Detection of Engager Expression in the Culture Supernatant of CAR(anti-BCMA)-T2a-engager Gene-Modified Cells

M represents the marker (10-180 kDa). Lanes 1-3 correspond to the detection results of the culture supernatant collected at 5, 7, and 9 days post-lentiviral infection. Lane 4 serves as the culture supernatant control. The upper row (samples 1-3) represents cells infected with CAR(anti-BCMA) lentivirus, while the lower row (samples 1-3) represents cells infected with CAR(anti-BCMA)-T2a-engager lentivirus.

IV. Conclusion

The CAR(anti-BCMA)-T2a-engager construct enables lentiviral-transduced cells to target BCMA+ tumor cells and facilitates NK cell activation and targeting of GPRC5D+ tumor cells through the secreted engager. These results provide preliminary evidence supporting the biological functionality of the CAR(anti-BCMA)-T2a-engager system.

Immunotherapy for cancer cells has become the fourth recognized treatment method for tumors worldwide. Recent clinical trials in Europe and the United States demonstrate its promising application prospects. Major international pharmaceutical companies have also prioritized immunotherapy as a critical area of development for the future. Among various immunotherapies, CAR-T therapy has emerged as a groundbreaking method. Several approved CAR-T products have shown excellent treatment results. However, current CAR-T therapies are primarily single-targeted, which may not fully address the complex mechanisms of malignant tumors. To address this, dual-target and multi-target CAR-T therapies are being actively developed.

Cell engagers can link immune cells (mainly T cells and NK cells) to tumor cells, activating the immune system to target and selectively destroy tumor cells precisely. Several cell engagers have already entered clinical

research, yielding impressive therapeutic outcomes. Combining CAR-T/CAR-NK therapies with cell engagers makes the potential to streamline production costs and reduce patients' financial burden achievable.

This study successfully constructed a molecular framework for co-expressed NK cell engagers and validated its expression through Western blotting. The research was based on the hypothesis illustrated in Figure 7, where the CAR(anti-BCMA) sequence and NK cell engager sequence are connected using a 2A peptide. This strategy enables three key effects with a single administration: (1) conferring the ability of lentivirus-infected adoptive cells to target BCMA+ tumors; (2) allowing the adoptive cells to secrete cell engagers that, through bispecific antibodies, empower NK cells to target GPRC5D+ tumor cells; and (3) utilizing the bispecific antibody crosslinker IL-15 further to promote the proliferation and survival of NK cells. This innovative strategy opens up new possibilities for tumor immunotherapy.

This research offers a novel approach to tumor cell immunotherapy. However, the current study only verifies the feasibility of this idea and includes initial validations. Further in vitro and in vivo studies are required to assess the effectiveness of this method fully.

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