Proceedings of the 6th International Conference on Biological Engineering and Medical Science
DOI: 10.54254/2753-8818/2026.39183

An "ON" Switch Mechanism Modulating Expression of IL-18 and Osr2 to Enhance the Efficacy of CAR-T Therapy in Pancreatic Ductal Adenocarcinoma

Henry Liu[1,†], Wanqi Luo[2,†], Yuhan Xu[3,†], Zihan Zhou[4,*,†]

[1]*Mater Dei High School, Santa Ana, United States*
[2]*Bashu Science City Senior High School, Chongqing, China*
[3]*Ulink College of Shanghai, Shanghai, China*
[4]*United World College of South East Asia (UWCSEA), East Campus, Singapore, Singapore*
[*]*Corresponding author: Zihan Zhou, sarahzhoumm@gmail.com*
[†]*These authors contributed equally to this work and should be considered as co-first authors*

Abstract. Pancreatic ductal adenocarcinoma (PDAC) remains difficult to treat, and its fibrotic, immunosuppressive tumor microenvironment are major obstacles to chimeric antigen receptor (CAR)-T cell therapy due to the T cell exhaustion and reduction in T cell activation. This proposal asks whether inducible interleukin-18 (IL-18) and biomechanical checkpoint Osr2 knockdown can improve the efficacy of mesothelin-targeted CAR-T cells in PDAC. The proposed CAR-T cell combined split CAR as a "ON" switch which can be integrated by lenalidomide, and inserted with inducible IL-18 cassette and Osr2 shRNA cassette. The drug-dependent CAR provides a method to control the T cell expression which can help reduce the toxicity. Furthermore, IL-18 is intended to support immune activation, while Osr2 shRNA is aimed to knockdown Osr2, in turn, reduce the exhaustion of CAR-T cells associated with the tumor environment. We proposed experiments in different treatments of CAR-T cells including combining a CAR-T cell control group without any modification, CAR-T cell with both modifications, CAR-T cell with only single modification lacking either cassettes, a T-cell group, a mesothelin-negative group, and a vehicle group. By comparing the tumor burden, survival, CAR-T infiltration, exhaustion markers, and toxicity, the results will prove whether "ON" switched CAR-T cells with inducible IL-18 and Osr2 shRNA cassettes can improve the tumor control.

Keywords: IL-18; Osr2 shRNA; CAR-T cell exhaustion; combination therapy; pancreatic ductal adenocarcinoma

1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the predominant subtype of pancreatic cancer, which accounts for over 90% of all pancreatic malignancies and remains among the most lethal human cancers, and has poor long-term survival [1]. PDAC develops through the staged accumulation of genetic alterations, most frequently activating mutations in KRAS together with inactivating mutations in

© 2026 The Authors. This is an open access article distributed under the terms of the Creative Commons Attribution License 4.0 (https://creativecommons.org/licenses/by/4.0/).

DOI: 10.54254/2753-8818/2026.39183

the tumor suppressor genes TP53, CDKN2A, and SMAD4 [2]. These oncogenic events drive malignant transformation and contribute to the fibrotic and immunosuppressive tumor microenvironment (TME).

A defining pathological feature of PDAC is its extensive desmoplastic stroma, which is dominated by cancer-associated fibroblasts (CAFs) that deposit excessive extracellular matrix (ECM) proteins, including collagen and fibronectin [3,4]. This dense stromal network frequently comprises up to 90% of the total tumor volume and acts as both a physical and an immunological barrier to therapy. The fibrotic stroma compresses blood vessels, limits drug delivery, restricts infiltration of cytotoxic immune cells, and generates biomechanical signals that promote immune dysfunction and T-cell exhaustion [5]. Together with the lack of reliable early diagnostic biomarkers and the largely asymptomatic nature of early disease, these characteristics contribute to delayed diagnosis and the poor clinical outcomes associated with PDAC.

Treatment for PDAC depends on disease stage. Surgical resection offers a potential route to remedy, however, many patients are diagnosed in a stage that surgery is no longer feasible. Therefore, systemic chemotherapy remains the central method for treatment, with radiation therapy in selected settings. Molecular profiling can identify feasible alternations for subsets of patients. Still, those methods do not address the needs for most patients in treating PDAC [6].

CAR-T cell therapy offers a different approach in the treatment of several cancers: T cells are engineered to recognize a selected antigen on the malignant cells. The success in treating hematological malignancies has encouraged the efforts to extend the approach to solid cancers [7]. Indeed, CAR-T cell therapy still shows limitations in solid tumours, including PDAC. Due to highly immunosuppressive solid tumor TME which will lead to T cell exhaustion, CAR-T cells are hard to infiltrate, persist, and perform cytotoxic function [8, 9]. Therefore, in PDAC, the engineered CAR-T cell should not only recognize the antigen, but also be able to enter the solid tumor while performing stubborn immunological activity.

1.1. Objective

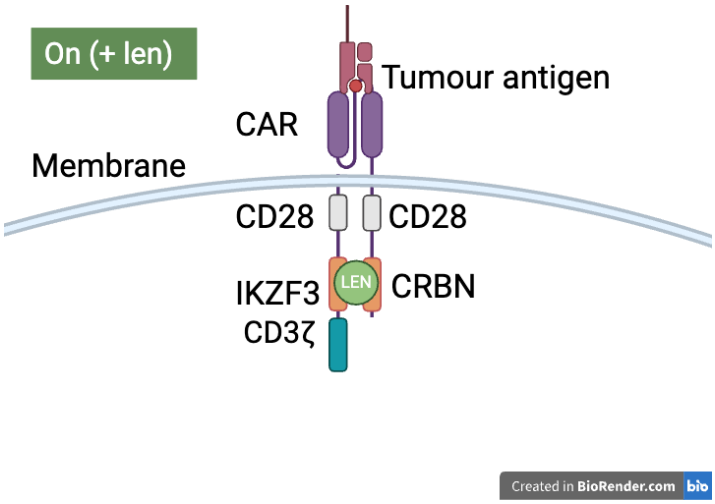


Figure1. Lenalidomide-driven ON-switch split CAR. The schematic demonstrates how lenalidomide (LEN) brings the two CAR modules together to activate the CAR, and how the CAR stays inactive without the drug. Based on Jan et al. [12]. Created in BioRender.

DOI: 10.54254/2753-8818/2026.39183

The objective we proposed is to develop a next-generation CAR-T cell in treating PDAC that involves inducible IL-18 expression and Osr2 knockdown with the drug-related "ON" switch regulating the activation of CAR-T cells. The design is built upon three studies addressing cytokine support, regulation of T cell exhaustion, and signaling control [10-12]. We integrate three components into a single mechanistic model (Figure 1) that form the basis of combination CAR-T cell therapy for PDAC we designed.

1.2. Summaries of foundational papers

1.2.1. Paper 1: IL-18 metabolically reprograms CAR-expressing natural killer T cells and enhances their antitumor activity

Barragán Bravo et al. are investigating CAR-expressing natural killer T (CAR-NKT) cells with expression of IL-18 which infiltrate tumors more effectively and possess additional antitumor functions. IL-18, a potent activator of immune cells, is associated with increasing activity and reduction of exhaustion in CAR-T cells; however, its effects on CAR-NKT cells remained unknown. To testify the efficacy of IL-18 in improving CAR-NKT cells in the solid tumor therapy, the researcher conducts experiments and measures the results in two treatments: either using IL-18 alone or combined with IL-15, would enhance the therapeutic efficacy of CAR-NKT cell treatments. The study supports including IL-18 in engineered immune cells, but it also illustrates further investigation in the need of evaluating toxicity of proposed cytokines: combination groups of IL-18 and IL-15 showed severe toxicity while IL-18 alone did not show the same results. These findings confirm IL-18 as a promising strategy for improving CAR-NKT cell therapy against solid tumors and support the clinical development of IL-18-armored CAR-NKT cells [10].

1.2.2. Paper 2: Osr2 functions as a biomechanical checkpoint to aggravate CD8+ T cell exhaustion in tumors

Zhang et al. determine ECM stiffness is a hallmark of many solid tumors and participates in regulating responses to cancer therapy [11]. The authors examined whether the biomechanical properties of the ECM influence the function of tumor-reactive CD8+ T cells and whether mechanosensitive signaling pathways and downstream transcription factors promote T-cell exhaustion. The study identified Osr2, a biomechanical checkpoint, as associated with T cell exhaustion. Osr2 recruited HDAC3 and suppressed cytotoxic gene expression, and enhanced CD8+ T-cell exhaustion. In mouse subcutaneous tumor models, Osr2-deficient CAR-T cells exhibited improved persistence, proliferation, and antitumor activity. This work motivates the Osr2 knockdown as a potential strategy to enhance the durability and efficacy of CAR-T cell therapy in solid tumors. The future studies in tumor cell stiffness directly contribute to T-cell exhaustion and how collagen-rich and fibrotic stromal environments influence this process are needed.

1.2.3. Paper 3: Reversible ON- and OFF-switch chimeric antigen receptors controlled by lenalidomide

Jan et al. developed a controllable CAR-T cell using ON and OFF switches associated with lenalidomide in order to control severe toxicities after infusion, including excessive cytokine release syndrome (CRS).[12] By separating the binding side and signaling side of the receptor and using lenalidomide to form a trinary complex to control the combination of two separate parts, the researcher is able to form an "ON" switch to express CAR-T cells. Then, they design an "OFF" switch by enabling

DOI: 10.54254/2753-8818/2026.39183

CAR combined with CRL4CRBN polyubiquitinate using lenalidomide to form a ternary complex. Furthermore, the lower dose of drug is also available to activate switches by increasing their sensitivity. We proposed to connect this control principle with inducible IL-18 and Osr2 shRNA expression. Consequently, the toxicity of cytokines and knockdown factors can be better controlled.

1.3. Working model

IL-18 is a proinflammatory cytokine of the IL-1 superfamily that regulates innate and adaptive immunity. IL-18 acts on T helper 1 (Th1) cells, B cells, natural killer cells, dendritic cells, and macrophages to induce interferon-γ (IFN-γ) production in the presence of IL-12 [13]. Binding of IL-18 to the heterodimeric IL-18 receptor, composed of α and β chains, recruits the adaptor protein MYD88 to activate nuclear factor κB (NF-κB) and mitogen-activated protein kinase (MAPK) signaling, along with metabolic reprogramming, which leads to greater effector-cell differentiation and hence more IFN-γ production [14].

Among the approaches adopted to combat these challenges, IL-18 has drawn attention for its ability to enhance T-cell activation, stimulate IFN-γ production, and reprogram the TME [15,16]. IL-18-armored CAR models showed superior resilience, increased memory characteristics, decreased expression of exhaustion markers, and strong antitumor activity in studies of solid cancers [10,16]. In addition, controlled release of IL-18 can lead to stronger proliferation and killing activity of CAR-activated immune cells, indicating its potential for long-term immunotherapies [10].

The transcription factor Osr2 operates as a biomechanical checkpoint that is strongly influenced by ECM stiffness and drives exhaustion of tumor-reactive CD8+ T cells [11,17]. Osr2, induced by T-cell receptor (TCR) signaling and by ECM stiffness via Piezo1, recruits HDAC3, which suppresses effector genes, leading to higher expression of PD-1 and TOX and lower expression of Ifng and Gzmb [11]. Therefore, simultaneous expression of IL-18 and suppression of Osr2 were proposed to improve CAR-T cell persistence and antitumor efficacy. Based on this rationale, a drug-inducible dual-regulatory CAR-T cell system was designed.

The ON switch is applied to the CAR, in which the two parts of the CAR—the antigen-binding module and the signaling module—are separated (Figure 1). CAR-T cells can therefore be activated only when the two modules combine. Specifically, cereblon (CRBN) is fused to the signaling module, where it binds specifically to lenalidomide, and a zinc-finger degron, IKZF3, is fused to the antigen-binding module, which is also able to bind lenalidomide. Thus, lenalidomide functions as an initiator activating the CAR-T cells by bringing the two CAR modules together by forming a ternary complex with CRBN and IKZF3. Clinicians can then regulate CAR-T cell activity by infusing patients with the drug, which avoids the toxicity hazard associated with high cytokine levels [12].

Nevertheless, the side effects of cytokine overexpression like IL-18 are significant when treating solid cancers which may lead to severe inflammation and toxicity. Therefore, combining the “ON” switch in regulating expression of IL-18 and Osr2 shRNA can avoid uncontrolled expression and further ensure safety while treating solid tumors.

This poses the question: how can the “ON” switch mechanism be used to control the levels of IL-18 and Osr2 that affect the efficacy of CAR-T cell therapy?

In summary, our hypothesis is that two cassettes address different limitations of CAR-T cell therapy in treating PDAC. Expression of IL-18 is intended to support the activation, whereas Osr2 knockdown is to reduce the exhaustion related to fibrotic tumor environment. We will compare the combined modifications with each modification alone to determine the efficacy of our proposed method. Meanwhile, the “ON” switch is a means of controlling CAR-T cell signaling which avoids overexpression of cytokines.

DOI: 10.54254/2753-8818/2026.39183

2. Experimental plan

2.1. Experimental objective and model

To evaluate the therapeutic efficacy and immunomodulatory effects of engineered CAR-T cells, C57BL/6 mice bearing orthotopic KPC4662-Fluc tumors will be used. Because PDAC is characterized by a dense desmoplastic and highly immunosuppressive TME, immunocompetent mouse models are essential for studying tumor–immune interactions and treatment responses [18–20]. NSG mice will not be used because they lack functional T, B, and natural killer cells, which would prevent evaluation of IL-18-mediated modulation of endogenous immunity and of immune-related toxicity [21].

Orthotopic tumors will be established by implanting KPC4662-Fluc cells into the pancreas of C57BL/6 mice. Stable expression of firefly luciferase (Fluc) enables longitudinal monitoring of tumor burden by in vivo bioluminescence imaging (BLI) using the In Vivo Imaging System (IVIS) [22].

Primary T cells, enriched and isolated from the spleens of naïve C57BL/6J mice, will be engineered to express a murine mesothelin (MSLN)-specific CAR together with an inducible IL-18 cassette and an Osr2 miRNA-embedded shRNA knockdown cassette [23]. This dual-engineered CAR-T cell system will be evaluated for antitumor efficacy, immune activation, tumor infiltration, CAR-T cell persistence, and safety in a syngeneic orthotopic PDAC model [7–9].

By detecting CD4+ and CD8+ T-cell clusters, the study can further investigate ON-switched CAR-T cell infiltration. Separating the CD4+ and CD8+ CAR-T cell populations from other CD4+ and CD8+ T-cell populations is therefore important, as it ensures that the study focuses on infiltration of ON-switched CAR-T cells rather than of other populations. To that end, CD4- and CD8-specific fluorescent constitutive promoter constructs will be introduced. First, CD4+ and CD8+ ON-switched CAR-T cells will be selected and separated using fluorescence-activated cell sorting (FACS) [24]. The constitutive promoter constructs will then be introduced using a lentiviral vector [25,26]. Both constructs have the same structure: a constitutive promoter, an MSLN CAR coding sequence, a 2A ribosome-skipping site, a fluorescent protein coding sequence, and a poly(A) tail. The only difference is the color of the fluorescent protein expressed in CD4+ and CD8+ CAR-T cells. In this way, CD4+ and CD8+ CAR-T cells produce different fluorescence colors and can readily be separated into clusters by flow cytometry [27,28].

2.2. Experimental groups

To test the function of the combination therapy, mice will be randomly assigned to seven experimental groups (Table 1) according to their baseline tumor burden. All engineered CAR-T cells will express the same targeting moiety, MSLN; in addition, the ON switch is loaded on some of the CAR-T cells and is triggered by lenalidomide, allowing measurement of the control of cytokine and exhaustion factor expression. To activate the ON switch, every mouse will be injected with lenalidomide. ON-switched CAR-T cells that specifically express IL-18 carry an IL-18-inducible cassette delivered by a lentiviral vector. The IL-18-inducible cassette contains an NFAT response element for transcriptional activation, a minimal promoter defining the transcription start site, an IL-18 coding sequence, and a poly(A) tail for transcript stabilization (Figure 2). Similarly, ON-switched CAR-T cells that specifically express Osr2 shRNA carry an Osr2 shRNA-inducible cassette in the T-cell nucleus (Figure 3). These designs allow evaluation of the effects of IL-18 and Osr2 on immune cells under ON-switch control, separately and in combination.

DOI: 10.54254/2753-8818/2026.39183

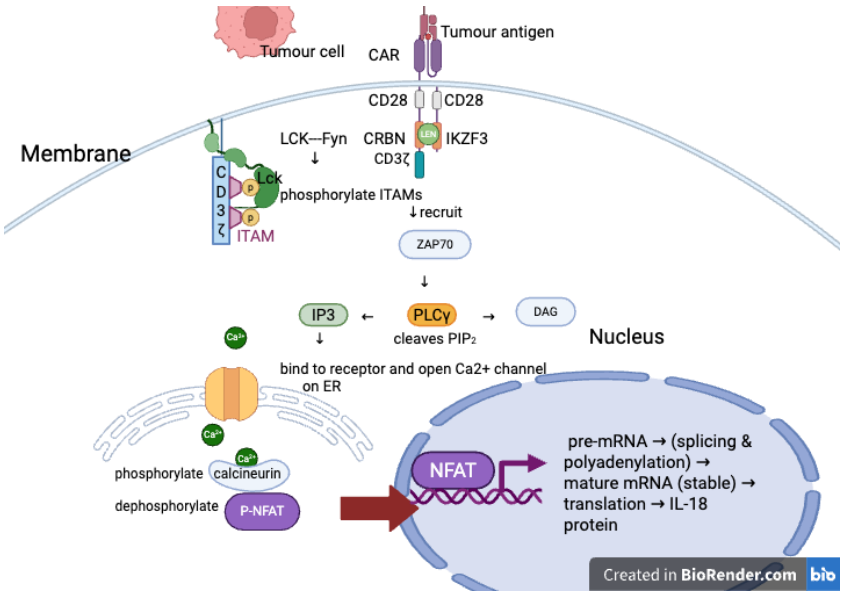


Figure2. IL-18-inducible cassette model. The schematic demonstrates the pathway following recognition of antigen by ON-switched CAR-T cells and activation of the IL-18-inducible cassette transcript. Created in BioRender.

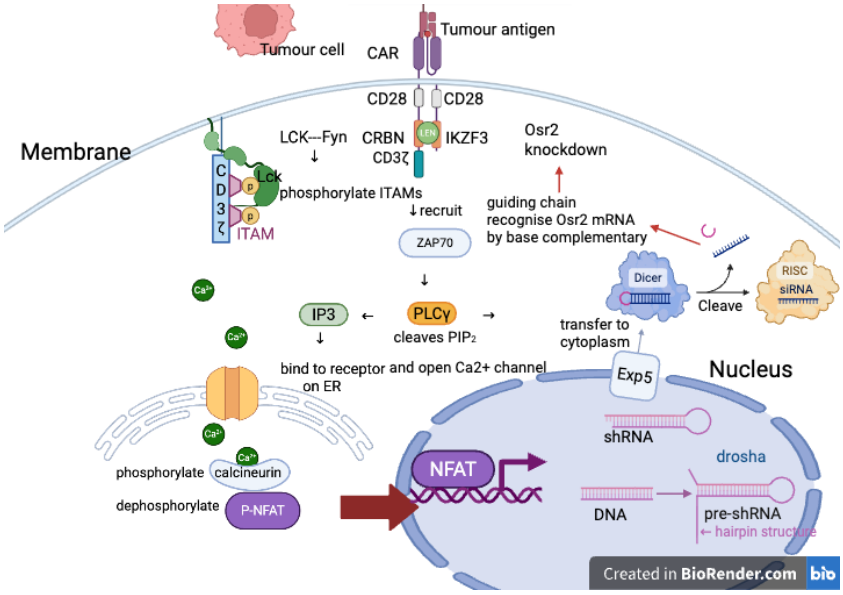


Figure3. Osr2 shRNA-inducible cassette model. The schematic demonstrates the pathway following recognition of antigen by ON-switched CAR-T cells and activation of the Osr2 miRNA-embedded shRNA cassette. Created in BioRender.

In the control group the mice will receive MSLN ON-switched CAR-T cells without inducible IL-18 and Osr-2 shRNA cassettes. This group provides the baseline of immune activity, infiltration, effects of cytokines, and exhaustion factors on immune cells. It also demonstrates the effects of fibrotic and immunosuppressive PDAC TME in leading to CAR-T cells dysfunction. By comparing the

DOI: 10.54254/2753-8818/2026.39183

control group with IL-18 only, Osr2 shRNA only, the combined group can determine the contribution of each modification to the efficacy of tumor control.

In experimental group one, mice will receive MSLN ON-switched CAR-T cells carrying an IL-18-inducible cassette. When these CAR-T cells recognize MSLN-positive PDAC tumors, they will release IL-18 to activate immune cells. This group will test whether IL-18 expression enhances activation of CAR-T cells and other immune cells, and will identify the efficacy of IL-18 in treating PDAC in comparison with the control group.

Conversely, the second experimental group involves mice receiving MSLN ON-switched CAR-T cells carrying an Osr2 shRNA-inducible cassette. After recognition of an MSLN-positive PDAC tumor, the Osr2 shRNA will be expressed and Osr2, an exhaustion factor, will be reduced. This group will investigate the effect of silencing Osr2 expression in a solid tumor environment and the relationship between T-cell exhaustion and Osr2. In experimental group three, mice will receive MSLN ON-switched CAR-T cells carrying both the IL-18-inducible and the Osr2 shRNA-inducible cassettes. After encountering an MSLN-positive tumor, both IL-18 and Osr2 shRNA will be expressed, so that immune cells can be activated and the exhaustion level of T cells will decrease. This combination group is designed primarily to test whether improving the TME through IL-18-mediated activation of immune cells together with shRNA-mediated silencing of Osr2 produces complementary or more efficient effects.

Furthermore, additional complementary groups are included to ensure the accuracy of the experiment. The first receives unmodified T cells, which identifies the specific effect of PDAC CAR-T cells on PDAC tumors. In the second, MSLN-negative tumors are implanted in the mouse pancreas and treated with PDAC CAR-T cells carrying both IL-18 and Osr2 shRNA cassettes, in order to determine the MSLN specificity of PDAC CAR-T cells. In the last group, a vehicle rather than lenalidomide will be injected, which will determine the effect of the drug in combination tumor therapy and whether the engineered circuit remains inactive in the absence of the drug.

Table1. Genetic and pharmacological modifications of each experimental group. LEN, lenalidomide; MSLN, mesothelin; +, present; −, absent.

Group	**MSLN CAR**	**IL-18**	**Osr2 shRNA**	**LEN**	**ON switch**
Control	+	−	−	+	+
1	+	+	−	+	+
2	+	−	+	+	+
3	+	+	+	+	+
T cell only	−	−	−	−	−
MSLN-negative tumor	+	+	+	+	+
Vehicle	+	+	+	−	+

DOI: 10.54254/2753-8818/2026.39183

2.3. Experimental readouts

The potential results enable us to evaluate tumor burden, CAR-T cell exhaustion, activation, and infiltration, which reveal immune mechanisms and treatment efficacy. BLI, flow cytometry, histology, enzyme-linked immunosorbent assay (ELISA), and quantitative polymerase chain reaction (qPCR) will provide complementary measurements of treatment activity and proposed mechanisms.

2.3.1. Tumor burden assessment

Tumor burden assessment is the core indicator of treatment efficacy. Tumor burden will be assessed by BLI using the IVIS as the primary detection method. BLI using Fluc enables non-invasive, real-time monitoring of tumor growth and of responses to therapy in orthotopic mouse models. After tail-vein injection of D-luciferin, the IVIS captures the Fluc signal expressed by KPC4662-Fluc cancer cells. Signal intensity correlates with viable cell number, allowing active and longitudinal assessment without sacrificing the animal. This measurement estimates tumor growth and metastasis, reflecting the therapeutic response in syngeneic mouse models [29–32].

2.3.2. Body weight

Disease progression and systemic toxicity will be monitored by mouse body weight. A body-weight loss of more than 10% is a validated indicator of tumor burden progression or of systemic toxicity arising from the immune mechanism [33].

2.3.3. Functional assessment

Flow-cytometric characterization of CAR-T cells will be performed using lineage-specific markers [34,35]. Lineage-specific markers are characteristic molecules, including surface proteins and transcription factors, that are used to identify specific cell types. T cells will first be identified as CD45+CD3+ cells and resolved into CD4+ and CD8+ subsets, followed by identification of CAR-positive (CAR+) T cells using a CAR-specific antibody. After sorting the CAR+ T-cell population, downstream functional analyses will be performed [34].

Detection of CAR-T cell exhaustion. Multiparameter flow cytometry will first be used to separate CD4+ and CD8+ MSLN ON-switched CAR-T cell clusters; flow cytometry will then be used to measure exhaustion factors in each cluster:

- TOX (thymocyte selection-associated high mobility group box protein)
- PD-1 (programmed cell death protein 1)
- TIM-3 (T-cell immunoglobulin and mucin domain-containing protein 3)
- LAG-3 (lymphocyte-activation gene 3)
- CD39 (ectonucleoside triphosphate diphosphohydrolase 1)

High expression of the exhaustion markers above indicates that T-cell function is exhausted, which is associated with poor response and outcome in therapy [11].

Detection of CAR-T cell activation. Flow cytometry will be used to detect expression of the following effector molecules:

- IFN-γ (interferon-gamma)
- TNF-α (tumor necrosis factor-alpha)

DOI: 10.54254/2753-8818/2026.39183

The effectors above are produced by CAR-T cells and provide a functional readout of effector activation and cytotoxic effect [36]. IL-18 levels in the TME and the spleen will also be compared to ensure that CAR-T cells are not overactivated.

Detection of infiltration. Multiparameter flow cytometry will be used to distinguish CD3+CD4+ and CD3+CD8+ CAR-T cell populations infiltrating the tumor model, which correlate with improved tumor control and with success of the immune mechanism. Histology will be used to observe where CAR-T cells are located in the TME. This will help distinguish CAR-T cells near the tumor margin from those located more deeply in the tissue[37].

2.3.4. Overall survival

Overall survival will be summarized by Kaplan-Meier curve for each group [38]. Median survival time for each experimental group will be reported using survival data recorded in the experiment. This approach allows clear comparisons between groups.

2.3.5. Mechanism validation

Osr2 transcript level will be measured by qPCR in the CAR-T cell population and compared with cells that lack the Osr2 shRNA cassette. The reduction in transcript magnitude greater than 50% will suggest that target mRNA perform efficient Osr2 silencing ability [39]. Furthermore, the reduction alone can't be evidence that the exhaustion has been reversed unless it is paired with interpretation of functional readouts.

ELISA will quantify IL-18 in the selected biological fluids and tissue sample [40]. These measurements will determine whether an inducible IL-18 cassette will increase IL-18 level and whether the increase of IL-18 level will lead to higher efficacy in restricting tumor development. Furthermore, immunofluorescence microscopy will provide complementary information about whether IL-18 correctly concentrated at the tumor site.

3. Results

3.1. Expected outcomes and interpretation

The expected results for each experimental group are summarized in Table 2.

MSLN ON-switched CAR-T cells control group without any modifications of cassettes is expected to be restricted by the PDAC immunosuppressive microenvironment. Thus, the T-cell exhaustion will be relatively high and immune activation will be low. This approach provides a baseline for the modifications group comparisons.

The central comparison is between the combined cassettes and single modification groups. We expect that the combined cassettes group will perform the greatest therapeutic efficacy: lowest tumor burden, longest survival time, highest T-cell infiltration, and lowest T-cell exhaustion. While two single-modification groups will demonstrate improved antitumor efficacy but higher exhaustion in IL-18 cassette group and lower activation of T-cell in Osr2 shRNA cassette group. Therefore, the combination of the findings will support the idea that by integrating inducible IL-18 cassette and Osr2-shRNA cassette in MSLN ON-switched CAR-T cell, the therapeutic efficacy will be improved by the high level of immune cell activation and low level of exhaustion.

If the combination therapy does not outperform the single-cassette group, several explanations would suggest this outcome. One modification may dominate response, one cassette may function

DOI: 10.54254/2753-8818/2026.39183

poorly in combination, or the proposed method may not provide additional benefit in this model. IL-18 expression and Osr2 knockdown measurements would help distinguish technical failure.

The unmodified-T-cell control group is expected to show no significant reduction in tumor burden, low T-cell infiltration, relatively high tumor growth rate compared to single-modification group and combined group, poor survival rate, and confining cytokine expression. This will demonstrate whether the ON-switched engineered receptor and modifications change treatment activity.

In the MSLN-negative tumor group treated with PDAC CAR-T cells carrying both IL-18 and Osr2 shRNA cassettes will test how strongly treatment depends on target antigen recognition. Significant decrease in the IVIS bioluminescence signal, minimal tumor cell killing, and reduced CAR-T cell activation and infiltration within the tumor will support the antigen dependency in CAR-T cell therapy.

The vehicle group will not receive lenalidomide. We expect little IL-18 secretion and no effective Osr2 knockdown, increased expression of exhaustion markers, reduced activation markers, and a higher tumor burden than in the other groups. These results will demonstrate that the engineered circuit remains inactive in the absence of lenalidomide, confirming drug dependence of this therapeutic method. In a group infused with significantly more lenalidomide, the therapeutic response and immune activation will increase, but decrease in mouse weight due to systemic inflammation.

Table2. Expected results for each experimental group. +, present or induced; −, absent or knocked down.

Group / Readout	**Tumor burden**	**IL-18**	**Osr2**	**T-cell exhaustion**	**T-cell infiltration**	**T-cell activation**
CAR-T cell only (control)	High	−	−	High	Low	Low
CAR-T cell + induced IL-18	Low	+	+	High	Improved	Improved
CAR-T cell + Osr2 knockdown	Low	−	−	Low	Improved	Improved
CAR-T cell + induced IL-18 + Osr2 knockdown	Lowest	+	−	Lowest	Highest	Highest
T cell only	Very high	−	−	Very high	Very low	Very low
MSLN-negative tumor	Very high	−	+	Very high	Very low	Very low
Vehicle	High	−	+	Very high	Very low	Very low

3.2. Alternative approaches and potential problems

Despite this, the limitations of the experiments are significant. The first limitation is insufficient MSLN expression in the selected PDAC tumor. If KPC4662 cells express a low level of MSLN, CAR-T cells will not sufficiently recognize MSLN presented on PDAC cells, which in turn will re-

DOI: 10.54254/2753-8818/2026.39183

duce CAR-T cell activation. Therefore, surface MSLN expression should be measured before the study begins. If expression is inadequate, another cell line with adequate MSLN expression should be selected.

The second limitation is that lenalidomide may produce direct immunomodulatory effects or unacceptable toxicity independent of ON-switch regulation. Furthermore, an excess of drug may cause potent activation of the ON switch, which could eventually lead to CRS. This problem may be addressed by a preliminary pharmacodynamic study comparing drug and vehicle under professional supervision. Other drugs, such as thalidomide and pomalidomide, are also capable of activating the ON switch; alternative drugs may therefore be selected if lenalidomide proves to be toxic. The body weight of mice should also be carefully monitored, since drug dosing is related to body weight.

The last limitation is that the experimental model is a mouse PDAC model, which may not be directly applicable to human PDAC. The experiments conducted in this study use murine immune cells, TME, and cytokines, whose relationships may differ in the human body. To that end, a human version of the experiment, using a human TME and human immune cells, should be conducted. Nevertheless, human experiments must be approved by an ethics committee. To save time, humanized mice are a better option for testing the efficacy of the combination therapy regulated by ON-switched CAR-T cells.

4. Discussion

We propose testing whether IL-18 expression and Osr2 knockdown improve the activity of lenalidomate-controlled MSLN CAR-T cells in PDAC. In this design, the inducible IL-18 and Osr2 shRNA cassettes are intended to make different contributions, where IL-18 cassette is to provide immune activation and Osr2 shRNA aimed to knockdown Osr2 which targets on dysfunction of CAR-T cell in tumor immunosuppressive environment lead by exhaustion. Their combination is worth testing because stronger activation may not be sufficient if cells gradually lose function. The primary innovation of this study is the integration of combined modifications to improve the activation and reduce the exhaustion of CAR-T cells, while loading a controllable ON-switch into one CAT-T cell. IL-18 would activate CAR-T cells and other immune cells, and Osr2 knockdown would reduce the exhaustion of CAR-T cells in the TME. Supporting evidence would include infiltration of CD4+ and CD8+ MSLN CAR-T cells, reduction of exhaustion factors such as TOX, and improved cytokine levels. By comparing the experimental group combining both IL-18 and Osr2 shRNA with groups receiving single treatments, the results may directly show the efficacy of the combination therapy. In contrast with traditional immunotherapy, this system introduces drug-based regulation that provides a safer and more controllable platform, in turn reducing toxicity and improving efficacy. Furthermore, the IL-18 and Osr2 shRNA inducible cassettes ensure that CAR-T cells experience less exhaustion. Thus, the therapy addresses tumor killing, exhaustion, and toxicity together.

However, this research faces several potential challenges, of which experimental complexity and the associated high cost are the most notable.

The primary challenge of the experiment lies in the sophisticated engineering of the MSLN CAR-T cells. This experiment requires a CAR-T cell that combines a split CAR exclusively activatable by lenalidomide, an NFAT-driven inducible IL-18 expression cassette, and an inducible Osr2 shRNA cassette. Each module must be carefully engineered to prevent cross-interference, while IL-18 and Osr2 levels must also be titrated to ensure efficacy and eliminate toxicity. Moreover, as this research uses C57BL/6 mice and the KPC4662-Fluc model, in vivo work, especially tumor establishment, can face high technical attrition because of the slow-growing and heterogeneous characteristics of KPC cells as well as the anatomical location of the pancreas. The orthotopic injection process therefore

DOI: 10.54254/2753-8818/2026.39183

shows high variability in implantation site, tumor growth rate, and metastatic pattern [19]. In addition, there are seven experimental groups and multiple readouts, demanding extensive sample sizes for meaningful data. All of the complexity described above correlates with higher cost. Although C57BL/6 mice are cheaper than other mouse models, a large sample size increases animal cost, as the experimental readouts include longitudinal studies and surgical implantation of orthotopic PDAC models. Furthermore, the methodology used in the experiment, especially flow cytometry for quantifying exhaustion marker populations and FACS for sorting CD4+ and CD8+ cells, is labor-intensive and expensive [41–43].

CAR-T cell persistence will also be measured. Persistence is defined as the ability of infused CAR-T cells to survive, engraft, and remain detectable long-term within the host while retaining antitumor effector function, and it is widely recognized as a prerequisite for durable tumor control [44].

5. Conclusion

Clinical studies have demonstrated that prolonged CAR-T cell persistence is strongly associated with durable clinical responses and improved therapeutic outcomes [45,46]. Conversely, T-cell exhaustion progressively impairs proliferative capacity, survival, and effector function, ultimately limiting CAR-T cell persistence [47]. Therefore, persistence serves as an important functional indicator for determining whether Osr2 knockdown effectively alleviates exhaustion and enhances long-term CAR-T cell efficacy. CAR-T cell persistence will be assessed by quantifying the percentage of CAR-T cells in peripheral blood, the frequency of CAR+ T cells in spleen and tumor tissues by flow cytometry, and the CAR transgene copy number by qPCR.

Since traditional CAR-T cell therapy shows limitations in treating solid cancers, in which T cells readily become exhausted, ON-switched CAR-T cells combined with IL-18 and Osr2 shRNA cassettes may be a potential alternative. By substituting the antigen-binding module of the CAR with one suited to the target tumor antigen, while retaining the IL-18 and Osr2 shRNA cassettes in the nucleus of the CAR-T cells, ON-switched CAR-T cells could efficiently control tumor progression. Potential applications of this approach include tumors with immunosuppressive TMEs that restrict T-cell infiltration, activation, and persistence. Examples include small cell lung cancer (SCLC), which can be targeted via MSLN or prostate stem cell antigen (PSCA) [48,49]; ovarian cancer, which can be targeted via non-functional P2X7 (nfP2X7) [50]; and liver cancer, which can be targeted via glypican-3 (GPC3) [51]. However, further studies on Osr2 expression and its relationship with T-cell dysfunction in these TMEs should be conducted, since Osr2 should not be assumed to have the same function in every tumor.

Before clinical translation, the treatment should also be tested in a sequence of alternative models. Humanized patient-derived xenograft models would provide greater clinical relevance by allowing the efficacy of the therapy to be investigated in human-derived tumors within a human immune system. Progression to early clinical study requires stabilized CAR-T cell persistence, characterization of cytokine-related toxicity, and dose optimization. Together, these studies would determine whether the proposed combination treatment can be tested in human patients and would help ensure both generalizability and safety.

Acknowledgement

Henry Liu, Wanqi Luo, Yuhan Xu, and Zihan Zhou contributed equally to this work and should be considered co-first authors.

DOI: 10.54254/2753-8818/2026.39183

References

[1] Sarantis, Panagiotis, et al. "Pancreatic Ductal Adenocarcinoma: Treatment Hurdles, Tumor Microenvironment and Immunotherapy." World Journal of Gastrointestinal Oncology, vol. 12, no. 2, 2020, pp. 173–181,https://doi.org/10.4251/wjgo.v12.i2.173.

[2] Tsanov, Kaloyan M., et al. "SMAD4 Induces Opposite Effects on Metastatic Growth from Pancreatic Tumors Depending on the Organ of Residence." Nature Cancer, vol. 6, no. 11, 2025, pp. 1839–1856,https://doi.org/10.1038/s43018-025-01047-5.

[3] Erkan, Mert, et al. "The Role of Stroma in Pancreatic Cancer: Diagnostic and Therapeutic Implications." Nature Reviews Gastroenterology & Hepatology, vol. 9, no. 8, 2012, pp. 454–467,https://doi.org/10.1038/nrgastro.2012.115.

[4] Provenzano, Paolo P., et al. "Enzymatic Targeting of the Stroma Ablates Physical Barriers to Treatment of Pancreatic Ductal Adenocarcinoma." Cancer Cell, vol. 21, no. 3, 2012, pp. 418–429, https://doi.org/10.1016/j.ccr.2012.03.007.

[5] Blank, Christian U., et al. "Defining 'T Cell Exhaustion.'" Nature Reviews Immunology, vol. 19, no. 11, 2019, pp. 665–674,https://doi.org/10.1038/s41577-019-0221-9.

[6] Ryan, David P., Theodore S. Hong, and Nabeel Bardeesy. "Pancreatic Adenocarcinoma." The New England Journal of Medicine, vol. 371, no. 11, 2014, pp. 1039–1049,https://doi.org/10.1056/NEJMra1404198.

[7] June, Carl H., and Michel Sadelain. "Chimeric Antigen Receptor Therapy." The New England Journal of Medicine, vol. 379, no. 1, 2018, pp. 64–73, https://doi.org/10.1056/NEJMra1706169.

[8] Newick, Kheng, et al. "CAR T Cell Therapy for Solid Tumors." Annual Review of Medicine, vol. 68, 2017, pp. 139–152, https://doi.org/10.1146/annurev-med-062315-120245.

[9] Fesnak, Andrew D., Carl H. June, and Bruce L. Levine. "Engineered T Cells: The Promise and Challenges of Cancer Immunotherapy." Nature Reviews Cancer, vol. 16, no. 9, 2016, pp. 566–581, https://doi.org/10.1038/nrc.2016.97.

[10] Barragán Bravo, Gabriel A., et al. "IL-18 Metabolically Reprograms CAR-Expressing Natural Killer T Cells and Enhances Their Antitumor Activity." Molecular Therapy, vol. 34, no. 4, 2026, pp. 2154–2173, https://doi.org/10.1016/j.ymthe.2026.01.001.

[11] Zhang, Jinjia, et al. "Osr2 Functions as a Biomechanical Checkpoint to Aggravate CD8+ T Cell Exhaustion in Tumor." Cell, vol. 187, no. 13, 2024, pp. 3409–3426,https://doi.org/10.1016/j.cell.2024.04.023.

[12] Jan, Max, et al. "Reversible ON- and OFF-Switch Chimeric Antigen Receptors Controlled by Lenalidomide." Science Translational Medicine, vol. 13, no. 575, 2021, article eabb6295,https://doi.org/10.1126/scitranslmed.abb6295.

[13] "Interleukin-18." ScienceDirect Topics, Elsevier, www.sciencedirect.com/topics/agricultural-and-biological-sciences/interleukin-18. Accessed 20 July 2026.

[14] Hu, Bin, et al. "Augmentation of Antitumor Immunity by Human and Mouse CAR T Cells Secreting IL-18." Cell Reports, vol. 20, no. 13, 2017, pp. 3025–3033,https://doi.org/10.1016/j.celrep.2017.09.002.

[15] Zhou, Tiancheng, et al. "IL-18BP Is a Secreted Immune Checkpoint and Barrier to IL-18 Immunotherapy." Nature, vol. 583, no. 7817, 2020, pp. 609–614, https://doi.org/10.1038/s41586-020-2422-6.

[16] Jaspers, Janneke E., et al. "IL-18-Secreting CAR T Cells Targeting DLL3 Are Highly Effective in Small Cell Lung Cancer Models." The Journal of Clinical Investigation, vol. 133, no. 9, 2023, article e166028, https://doi.org/10.1172/JCI166028.

[17] Nicolas, Morgane, et al. "Mechanical Regulation of T Cell Function." Nature Reviews Immunology, vol. 22, no. 12, 2022, pp. 743–757, https://doi.org/10.1038/s41577-022-00750-9.

[18] Ho, Won Jin, Elizabeth M. Jaffee, and Lei Zheng. "The Tumour Microenvironment in Pancreatic Cancer: Clinical Challenges and Opportunities." Nature Reviews Clinical Oncology, vol. 17, no. 9, 2020, pp. 527–540, https://doi.org/10.1038/s41571-020-0363-5.

[19] Hingorani, Sunil R. "Epithelial and Stromal Co-Evolution and Complicity in Pancreatic Cancer." Nature Reviews Cancer, vol. 23, no. 2, 2023, pp. 57–77, https://doi.org/10.1038/s41568-022-00530-w.

[20] Hosein, Ali N., Rolf A. Brekken, and Anirban Maitra. "Pancreatic Cancer Stroma: An Update on Therapeutic Targeting Strategies." Nature Reviews Gastroenterology & Hepatology, vol. 17, no. 8, 2020, pp. 487–505, https://doi.org/10.1038/s41575-020-0300-1.

[21] Shultz, Leonard D., et al. "Humanized Mice for Immune System Investigation: Progress, Promise and Challenges." Nature Reviews Immunology, vol. 12, no. 11, 2012, pp. 786–798, https://doi.org/10.1038/nri3311.

[22] Contag, Christopher H., et al. "Visualizing Gene Expression in Living Mammals Using a Bioluminescent Reporter." Photochemistry and Photobiology, vol. 66, no. 4, 1997, pp. 523–531, https://doi.org/10.1111/j.1751-1097.1997.tb03188.x.

[23] Stegmeier, Frank, et al. "A Lentiviral microRNA-Based System for Single-Copy Polymerase II-Regulated RNA Interference in Mammalian Cells." Proceedings of the National Academy of Sciences of the United States of America, vol. 102, no. 37, 2005, pp. 13212–13217, https://doi.org/10.1073/pnas.0506306102.

[24] Liao, Xiaofeng, et al. "Fluorescence-Activated Cell Sorting for Purification of Plasmacytoid Dendritic Cells from the Mouse Bone Marrow." Journal of Visualized Experiments, no. 117, 2016, article 54641, https://doi.org/10.3791/54641.

[25] van Hooijdonk, Lenneke W. A., et al. "Lentivirus-Mediated Transgene Delivery to the Hippocampus Reveals Sub-Field Specific Differences in Expression." BMC Neuroscience, vol. 10, 2009, article 2, https://doi.org/10.1186/1471-2202-10-2.

[26] Gill, Katherine P., and Mark Denham. "Optimized Transgene Delivery Using Third-Generation Lentiviruses." Current Protocols in Molecular Biology, vol. 133, no. 1, 2020, article e125, https://doi.org/10.1002/cpmb.125.

[27] Cazaux, Marine, et al. "Single-Cell Imaging of CAR T Cell Activity In Vivo Reveals Extensive Functional and Anatomical Heterogeneity." The Journal of Experimental Medicine, vol. 216, no. 5, 2019, pp. 1038–1049, https://doi.org/10.1084/jem.20182375.

[28] Diem, Kurt, et al. "Image Analysis for Accurately Counting CD4+ and CD8+ T Cells in Human Tissue." Journal of Virological Methods, vol. 222, 2015, pp. 117–121, https://doi.org/10.1016/j.jviromet.2015.06.004.

[29] Agarwal, Anubhav, et al. "Bioluminescence Imaging in Mouse Models." Cold Spring Harbor Protocols, 2009. PubMed Central, https://pmc.ncbi.nlm.nih.gov/articles/PMC2713342/.

[30] "Bioluminescence Imaging." ScienceDirect Topics, Elsevier, www.sciencedirect.com/topics/agricultural-and-biological-sciences/bioluminescence-imaging. Accessed 21 July 2026.

[31] "Firefly Luciferase." ScienceDirect Topics, Elsevier, www.sciencedirect.com/topics/medicine-and-dentistry/firefly-luciferase. Accessed 21 July 2026.

[32] Cao, Y., et al. "Bioluminescence Imaging in Cancer Immunotherapy Research." 2024. PubMed Central, https://pmc.ncbi.nlm.nih.gov/articles/PMC11115821/.

[33] Morton, David B., et al. "Refinement and Reduction in Animal Experiments: Body Weight as a Humane Endpoint." Laboratory Animals, vol. 35, no. 4, 2001, pp. 323–329.

[34] "Lineage Markers." ScienceDirect Topics, Elsevier, www.sciencedirect.com/topics/medicine-and-dentistry/lineage-markers. Accessed 21 July 2026.

[35] Cossarizza, Andrea, et al. "Guidelines for the Use of Flow Cytometry and Cell Sorting in Immunological Studies." European Journal of Immunology, vol. 49, no. 10, 2019, pp. 1457–1973, https://doi.org/10.1002/eji.201970107.

[36] "CAR T Cell Therapy." Molecular Therapy, vol. 34, 2026, www.cell.com/molecular-therapy-family/molecular-therapy/fulltext/S1525-0016(26)00001-8.

[37] Prasad, Vinay, et al. "Histology." StatPearls, StatPearls Publishing, 2024. NCBI Bookshelf, www.ncbi.nlm.nih.gov/books/NBK557663/.

[38] Rich, Jason T., et al. "A Practical Guide to Understanding Kaplan–Meier Curves." Otolaryngology–Head and Neck Surgery, vol. 143, no. 3, 2010, pp. 331–336, https://doi.org/10.1016/j.otohns.2010.05.007.

[39] Bustin, Stephen A., et al. "The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments." Clinical Chemistry, vol. 55, no. 4, 2009, pp. 611–622, https://doi.org/10.1373/clinchem.2008.112797.

[40] Alhajj, Mandy, Muhammad Zubair, and Aisha Farhana. "Enzyme Linked Immunosorbent Assay." StatPearls, StatPearls Publishing, 2023. NCBI Bookshelf, www.ncbi.nlm.nih.gov/books/NBK555922/.

[41] National University of Singapore, Yong Loo Lin School of Medicine. "Flow Cytometry Unit: Services and Charges." NUS Medicine Core Facilities, medicine.nus.edu.sg/core-facilities/flow-cytometry-unit/services-and-charges/. Accessed 22 July 2026.

[42] Akadeum Life Sciences. "Cost Comparison of Cell Separation Methods." Akadeum Life Sciences, www.akadeum.com/technology/cost-comparison-of-cell-separation-methods/. Accessed 22 July 2026.

[43] Li, Y., et al. "Recent Advances in Cell Separation Technologies for Immunotherapy Applications." Cells, vol. 13, no. 3, 2024, article 245. PubMed Central, https://pmc.ncbi.nlm.nih.gov/articles/PMC10806555/.

[44] Zhang, X., et al. "Strategies for Improving CAR T Cell Persistence in Solid Tumors." Current Oncology Reports, vol. 26, 2024, https://doi.org/10.1007/s11912-024-01568-3.

DOI: 10.54254/2753-8818/2026.39183

[45] Louis, Catherine U., et al. "Antitumor Activity and Long-Term Fate of Chimeric Antigen Receptor-Positive T Cells in Patients with Neuroblastoma." Blood, vol. 118, no. 23, 2011, pp. 6050–6056, https://doi.org/10.1182/blood-2011-05-354449.

[46] Fraietta, Joseph A., et al. "Determinants of Response and Resistance to CD19 Chimeric Antigen Receptor (CAR) T Cell Therapy of Chronic Lymphocytic Leukemia." Nature Medicine, vol. 24, no. 5, 2018, pp. 563–571, https://doi.org/10.1038/s41591-018-0010-1.

[47] Seo, Hyungseok, et al. "TOX and TOX2 Transcription Factors Cooperate with NR4A Transcription Factors to Impose CD8+ T Cell Exhaustion." Proceedings of the National Academy of Sciences of the United States of America, vol. 116, no. 25, 2019, pp. 12410–12415, https://doi.org/10.1073/pnas.1905675116.

[48] Zheng, Song, et al. "CAR Immune Cell Therapy Strategies and Advances for Lung Cancer." Discover Oncology, vol. 17, no. 1, 2026, article 873, https://doi.org/10.1007/s12672-026-04981-7.

[49] Kandra, Prameela, et al. "Utility and Drawbacks of Chimeric Antigen Receptor T Cell (CAR-T) Therapy in Lung Cancer." Frontiers in Immunology, vol. 13, 2022, article 903562, https://doi.org/10.3389/fimmu.2022.903562.

[50] Bandara, Veronika, et al. "Engineered CAR-T Cells Targeting the Non-functional P2X Purinoceptor 7 (P2X7) Receptor as a Novel Treatment for Ovarian Cancer." Clinical & Translational Immunology, vol. 13, no. 5, 2024, article e1512, https://doi.org/10.1002/cti2.1512.

[51] Yang, Ze, et al. "Advancing Liver Cancer Treatment with Dual-Targeting CAR-T Therapy." Journal of Nanobiotechnology, vol. 23, no. 1, 2025, article 462, https://doi.org/10.1186/s12951-025-03512-w.