Maximizing Pancreatic Ductal Adenocarcinoma Immunotherapy Success: Assessing Murine B7-H3 CAR T Cells in Adjuvant, Neoadjuvant, and Monotherapy Application in Combination with GLUT1 Inhibitor

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Significance of Research: Pancreatic ductal adenocarcinoma (PDAC) presents a significant clinical challenge characterized by its aggressive nature and limited treatment options. Immunotherapy, particularly chimeric antigen receptor (CAR) T cell therapy, holds great promise for PDAC treatment. However, it is hindered by various factors, including the highly immunosuppressive tumor microenvironment (TME), lack of ideal tumor-specific targets, and fibrotic stroma that restricts immune cell infiltration and limits nutrients and oxygen access. To adapt and thrive, PDAC cells undergo metabolic reprogramming, transitioning from oxidative phosphorylation to aerobic glycolysis, a phenomenon referred to as the Warburg Effect. Therefore, our research focuses on addressing these challenges to enhance the efficacy of our CAR T cell-based immunotherapy, which targets B7-H3, an immune checkpoint molecule that is highly expressed in PDAC tumors and cancer-associated fibroblasts (CAFs). Furthermore, the incorporation of an inducible caspase 9 (iCas9) safety switch mitigates potential systemic toxicities, ensuring the safety of the treatment. We hypothesized that decreasing the desmoplastic tumor burden by surgical resection coupled with CAF glycolytic reprogramming using a GLUT1 inhibitor can enhance the infiltration of CAR T cells to effectively eradicate the metastatic tumor and provide a sustained response in PDAC. Therefore, we systematically evaluate the efficacy and immunological mechanisms of iCas9.B7-H3 CAR T cells in adjuvant, neoadjuvant, and monotherapy applications coupled with GLUT1 inhibitor for PDAC; we aim to provide valuable insights that could lead to the development of new combinatorial strategies for PDAC patients, ultimately enhancing clinical outcomes and prolonging survival rates.

Background Information: PDAC is a poor-prognosis cancer with an overall 5-year survival rate of 10%. Surgery combined with systemic chemotherapy remains the only treatment for resectable PDAC. Despite negative margin resections and subsequent systemic therapy, PDAC continues to have a poor overall survival rate, necessitating the exploration of novel therapeutic approaches. CAR T cells are engineered to recognize and target specific antigens expressed on cancer cells in an HLA class I antigen independence manner. Our research focuses on targeting B7-H3, a protein with an inhibitory role in T cell activation and proliferation, which is highly expressed in PDAC tumors, tumor-associated vasculature, and CAFs, making it an attractive target for CAR T cell therapy. Moreover, the incorporation of the iCas9 safety switch ensures the safety of the treatment. CAFs play a crucial role in impeding T-cell infiltration by constructing a dense extracellular matrix, providing aerobic glycolysis for PDAC cells, and fostering an immunosuppressive tumor microenvironment. This is also accomplished through the secretion of immunosuppressive cytokines such as CXCL12 and TGFβ, which pose additional challenges for immunotherapy. However, by decreasing this desmoplastic TME through GLUT1 inhibitor and surgical resection in different treatment settings, including adjuvant and neoadjuvant applications, we aim to overcome these obstacles and improve B7-H3 CAR T cell efficacy. This research builds upon our previous experiments on using iCas9.B7-H3 CAR T cell therapy in the

adjuvant setting in immunodeficient mice and will investigate mechanistic insights with murine B7-H3 CAR T in immunocompetent mice.

Preliminary Observations: In our preliminary investigations, we observed that B7-H3, the target antigen for our CAR T cells, was uniformly expressed across all patient-derived PDAC cell lines, including cancer-initiating cells (CICs), which are especially resistant to conventional therapies due to their stemness properties. Additionally, our iCas9.B7-H3 CAR T cells demonstrated potent in vitro antitumor activity against PDAC cells and CAFs, especially at favorable effector-to-target (E:T) ratios (Fig. 1-A). Interestingly, PDAC cells co-cultured with CAFs exhibited decreased susceptibility to CAR T cell-mediated killing, suggesting an immunosuppressive role of CAFs on CAR T cells (Fig. 1-B). To validate the therapeutic potential of iCas9.B7-H3 CAR T cells in vivo, NSG mice were orthotopically engrafted with patient-derived PDAC6 cells and hCAF1 cells at a ratio of 1:9, reflecting a clinically relevant model. After three weeks, the mice underwent surgical resection of the primary tumor (distal pancreatectomy and splenectomy) and were subsequently treated systemically with iCas9.B7-H3 CAR T cells. CD19 CAR T cells were used as the specificity control. iCas9.B7-H3 CAR T cells effectively and persistently eradicated both local and distant PDAC metastases throughout the experimental period (Fig. 2). Despite effectively controlling tumor growth, eliminating metastases, and significantly prolonging survival for over 180 days, iCas9.B7-H3 CAR T cells were unable to completely eradicate the tumor (Fig. 3). These results highlight the promise of adjuvant CAR T cell therapy for PDAC treatment.

Methods & Designs: Experiment 1: Enhancing iCas9.B7-H3 CAR T Cell Infiltration and Efficacy in PDAC PDOTS through TME reprogramming using a GLUT1 inhibitor in vitro. Patient-derived organotypic tumor spheroids (PDOTS) in Group 1 will undergo pretreatment with the GLUT1 inhibitor to reprogram glycolytic cancer-associated fibroblast (glyCAF) metabolism within a three-dimensional microfluidic device for 48 hours. Meanwhile, Group 2 will serve as the untreated control. Both PDOTS groups will then be co-cultured with iCas9.B7-H3 CAR T cells, generated from peripheral blood mononuclear cells (PBMCs) of PDAC patients on day 5. After 24 hours, PDAC tumors will be assessed for various markers, including CAF and immune cell markers, and B7-H3 CAR T cell infiltration using multiplex immunofluorescence staining and flow cytometry. Experiment 2: Assessing mB7-H3 CAR T Cell Efficacy in Metastatic PDAC Eradication in a Desmoplastic Mouse Model of PDAC through Adjuvant, Neoadjuvant, and Monotherapy, Combined with TME Reprogramming by GLUT1 Inhibition. We will evaluate the efficacy of murine B7-H3 (mB7-H3) CAR T cell therapy in different treatment modalities following primary tumor resection in an orthotopic syngeneic desmoplastic mouse model of PDAC. Using murine GFP.Luc-labelled PDAC UN-KPC-961 cells and fibroblasts, we will establish the clinically relevant mouse model. Mice will receive systemic treatment with GLUT1 inhibitor BAY-876 and undergo various treatment protocols involving surgical resection of primary tumors and systemic administration of mB7-H3 CAR T cells (Table 1). Disease recurrence and immune cell infiltration and composition in resected pancreas tissue and peripheral blood will be assessed using Bioluminescence Imaging, flow cytometry, and multiplex immunofluorescence staining.

Data Analysis: The development of tumor among treatment groups in mice will be compared using Fisher's exact test. Continuous variables will be analyzed with the Kruskal-Wallis test or ANOVA, and the Mann-Whitney U test will be used for between-group comparisons. Categorical variables will be assessed with Fisher's exact test. Survival analysis will be conducted using the Kaplan-Meier method, and group comparisons will be made with the log-rank test. All tests will be two-tailed, with significance set at $p < 0.05$. We plan to include ten mice per group, providing >80% power to detect significant differences by Fisher's exact test at a two-sided a level of 0.05. This assumes treatment efficacy in >90% vs. < 20% of treated vs. untreated mice, respectively.

Adequate power (>80%) is also ensured for survival analysis, considering a median survival ratio of 40 days and a follow-up interval of 180 days, allowing for a 10% attrition rate. In the event of an insufficient sample size, we will conduct a power study based on observed effect estimates and adjust group sizes accordingly. Guidance will be sought from the Cedars-Sinai Medical Center Biostatistics Core if any statistical complexities arise.

Table 1

Group 1-Adjuvant	Primary tumor resection on day 25, systemic $5x10^5$ mB7-H3 CAR T cells on day 30
Group 2-Adjuvant+ GLUT1	Pretreatment with 5 mg/kg BAY-876 on day 10-20, primary tumor resection on day 25, systemic $5x10^6$ mB7-H3 CAR T cells on day 30
Group 3-Neoadjuvant	Systemic $5x10^6$ mB7-H3 CAR T cells on day 25, primary tumor resection on day 30
Group 4-Neoadjuvant+ GLUT1	Pretreatment with 5 mg/kg BAY-876 on day 10-20, systemic $5x10^6$ mB7-H3 CAR T cells on day 25, primary tumor resection on day 30
Group 5-Monotherapy	Systemic $5x10^6$ mB7-H3 CAR T cells on day 25
Group 6-Monotherapy+ GLUT1	Pretreatment with 5 mg/kg BAY-876 on day 10-20, systemic $5x10^6$ mB7-H3 CAR T cells on day 25
Group 7-Control	Primary tumor resection on day 25, systemic $5x10^6$ mCD19 T cells on day 30
Group 8-Control	Pretreatment with 5 mg/kg BAY-876 on day 10-20, primary tumor resection on day 25, systemic $5x10^6$ mCD19 CAR T cells on day 30

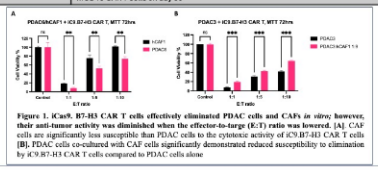
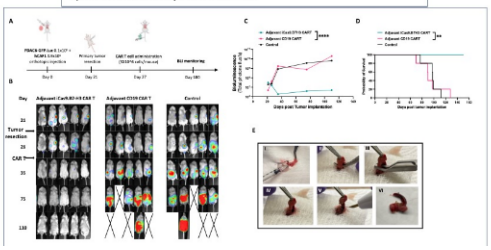


Figure 1. iCas9. B7-H3 CAR T cells effectively eliminated PDAC cells and CAFs *in vitro;* however, their anti-tumor activity was diminished when the effector-to-turge (E:T) ratio was lowered. [A]. CAF cells are significantly less susceptible than PDAC cells to the cytotoxic activity of iC9.B7-H3 CAR T cells **[B].** PDAC cells co-cultured with CAF cells significantly demonstrated reduced susceptibility to elimination by iC9.B7-H3 CAR T cells compared to PDAC cells alone



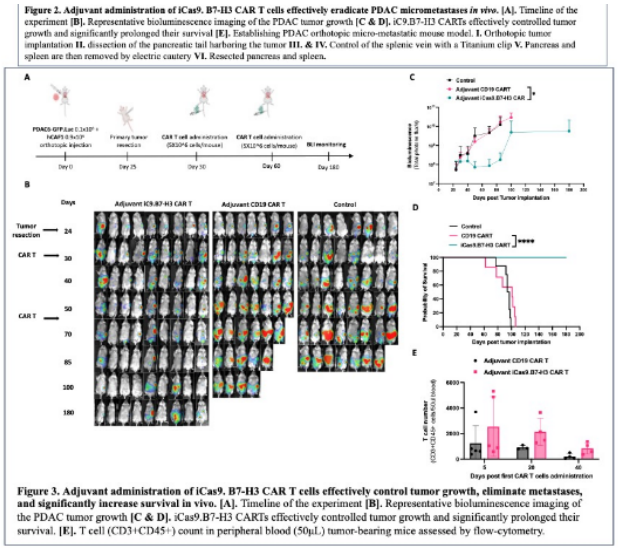


Figure 2. Adjuvant administration of iCas9. B7-H3 CAR T cells effectively eradicate PDAC micrometastases *in vivo*. [A]. Timeline of the experiment **[B].** Representative bioluminescence imaging of the PDAC tumor growth **[C & D].** iC9.B7-H3 CARTs effectively controlled tumor growth and significantly prolonged their survival **[E].** Establishing PDAC orthotopic micro-metastatic mouse model. **I.** Orthotopic tumor implantation **II.** dissection of the pancreatic tail harboring the tumor **III. & IV.** Control of the splenic vein with a Titanium clip **V.** Pancreas and spleen are then removed by electric cautery **VI.** Resected pancreas and spleen.

Figure 3. Adjuvant administration of iCas9. B7-H3 CAR T cells effectively control tumor growth, eliminate metastases, and significantly increase survival in vivo. [A]. Timeline of the experiment **[B].** Representative bioluminescence imaging of the PDAC tumor growth **[C & D].** iCas9.B7-H3 CARTs effectively controlled tumor growth and significantly prolonged their survival. **[E].** T cell (CD3+CD45+) count in peripheral blood (50µL) tumor-bearing mice assessed by flow-cytometry.