Design Brief

Boolean-logic gated engineered CAR T-Cells for pancreatic ductal adenocarcinoma therapy*

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Pancreatic Ductal Adenocarcinoma (PDAC) is a highly aggressive form of cancer, responsible for nearly half a million deaths annually worldwide. Despite advancements in treatments, PDAC remains difficult to treat with a poor survival rate, due to rapid disease progression, drug resistance, and late diagnosis. Chimeric Antigen Receptor (CAR) T-cell therapy has shown success and applicability in treating B-cell malignancies and blood tumors, which raised hopes for CAR T-cells to be used in solid tumors. However, its application in solid tumors is challenging due to antigen escape, heterogeneity observed and off-target effect. Therefore, in this study we demonstrate the use of Boolean logic to target multiple antigens for enhanced tumor discrimination and limited off-target effects. Mesothelin (MSLN) and Epidermal Growth Factor Receptor (EGFR) were the antigens chosen using the Antigen Explorer database (https://antigen.princeton.edu/). Gene delivery will be achieved using the piggyBac transposon system, an emerging non-viral method, using the pNB328-meso3 CAR plasmid and the pIRII-CAR.EGFR (EGFv1) CAR plasmid to transfect and engineer the T-cells. Furthermore, CAR T-cells were activated using co-stimulation signals that were split into two separately expressed CARs directed against the two selected antigens. In the split-dual CAR system, the first CAR (EGFR) provides the primary activation signal (CD3ζ) and the second CAR (MSLN) delivers the costimulatory CD28 signal. Full T-cell activation only occurs when both antigens are expressed, ultimately reducing the risk of off-tumor toxicity and increasing the specificity for solid tumors. This AND-Gated CAR T-cell approach may present a promising strategy to overcome PDAC heterogeneity and improve the precision of immunotherapy for PDAC.

Keywords: Pancreatic Ductal Adenocarcinoma; CAR T-Cell Therapy; Boolean-Logic Gated Cells

Pancreatic cancer is the 12th most common type of cancer and is the 7th leading cause of cancer-related death worldwide (Becker et al., 2014). In 2022, there were approximately 500,000 new cases of pancreatic cancer (World Cancer Research Fund, 2024). It has a poor prognosis and a low relative survival rate of 12% (Becker et al., 2014). A specific type of pancreatic cancer, pancreatic ductal adenocarcinoma (PDAC), is predicted to be the second leading cause of cancer-related death by 2030 (Becker et al., 2014). PDAC is a type of exocrine cancer (Yamada et al., 2023). It develops from the cells that secrete enzymes that facilitate the breakdown of carbohydrates, fats, and proteins in the gastrointestinal tract (Yamada et al., 2023). Symptoms of pancreatic cancer include pain in the abdomen or spine, new-onset diabetes,

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dark urine, jaundice, nausea, weight loss, and poor appetite (Yamada et al., 2023). However, since these symptoms are often indicators of other common clinical conditions, patients are often diagnosed in the later stages of disease, from symptoms of metastasis (Yamada et al., 2023). Due to its aggressive nature and late diagnosis in patients, it is often discovered late and leads to metastasis and lower life expectancy.

Current therapeutic options for systemic therapy are limited. Resistance (both intrinsic and acquired resistance) to chemotherapy has become a critical problem for treating PDAC (Abbott et al., 2022). Intrinsic resistance is due to genetic factors, drug transport mechanisms, metabolism, and apoptotic pathways (Abbott et al., 2022). Acquired resistance develops after treating the tumor for a period of time (Abbott et al., 2022). Exposure of the drug leads to genetic or epigenetic modifications within tumor cells leading to ineffective treatment (Abbott et al., 2022). Another treatment includes chimeric antigen receptor (CAR) T cell immunotherapy, which is a new class of therapies that redirects a patient's T cells to recognize and destroy malignant cells. In a CAR T-Cell, the chimeric receptor's specific design can be reshaped with ease. Several regions of different proteins can be put together in one fusion protein (De Marco et al., 2023). The receptor is created based on a specific, desired, predefined antigen that the modified T cells target (De Marco et al., 2023). CAR T cells are able to overcome the restraint of regular T cells as they do not rely on antigen presentation through MHC expression and can recognize all expressed surface antigens (De Marco et al., 2023). When CAR T cells recognize a specific receptor or ligand, an immune synapse is formed between the effector and target cell. A small and tight space where multiple receptor-ligand associations are formed and this synaptic clustering event leads to activation of CAR T cells (De Marco et al., 2023). Activated CAR T cells secrete cytokines that activate other immune cells like natural killer cells and macrophages, and together, all immune cells create a robust tumor-suppressing environment (De Marco et al., 2023). Although CAR T Cells work efficiently to treat hematological malignancies, its applicability is difficult in solid tumors like PDAC due to antigen escape, off-tumor effects, and heterogeneity.

One method to help increase the applicability of CAR T Cell research is through logic gating (Zhao & Sadelain, 2023). Logic gating is a strategy for CAR T Cells to target tumor cells that lack antigens specific to tumors and also to reduce off-tumor toxicity (Zhao & Sadelain, 2023). CAR T cells may engage in 2 antigens in different ways: these cells may get activated upon binding to either one of the two antigens (OR gate), or they might get activated if both antigens are binded (AND-gating). AND-gating is especially useful to achieve tumor specificity by choosing two antigens that are not tumor specific (Zhao & Sadelain, 2023). In the case of PDAC, mesothelin and epidermal growth factor receptor (EGFR) are tumor-associated antigens that are expressed in a limited number of healthy tissues. The AND-Gating CAR T-Cell mechanism can be seen in Figure 1.

Therefore, this study focuses on using Boolean logic in AND-gated CAR T Cells to target the antigens mesothelin (MSLN) and epidermal growth factor receptor (EGFR) for enhanced tumor discrimination and limited off-target effects in PDAC. In order to choose the MLSN and EGFR receptor, the Antigen Explorer Database was utilized to screen for

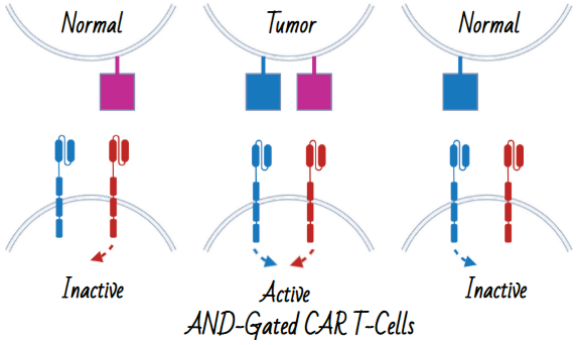

Figure 1. The AND-Gated CAR T-Cell Mechanism: in both normal cells, only one of the antigens (either MSLN or EGFR) is found. Since the AND-Gated CAR T-cell requires both antigens to be present, these CAR-T cells will not be activated against normal cells. However, since both antigens are found in a tumor cell, the CAR T-cell will activate and destroy the PDAC tumor cell. This ensures that the AND-Gated CAR T cells specifically target only tumor cells and reduces off-target toxicity.

the best combination of antigens to efficiently target PDAC cells. These 2 genes are then introduced in the CAR T cells using the piggyBac Transposon system and a designed plasmid. Then to activate the CAR T cell, EGFR will provide the primary activation signal (CD3ζ) and MSLN will provide the costimulatory signal. The hope is that there will be more specific and increased PDAC killing.

Device level

The scarcity of broadly expressed tumor-associated CAR-targets has led to the investigation of different combinations of antigen recognition patterns (Zhao & Sadelain, 2023). CAR T cells that require the presence are considered to be AND-gated and unless those two antigens are present, T cells are not activated enough to produce an effective tumor suppressive response (Zhao & Sadelain, 2023). One method to achieve this dual recognition pattern is to design a weakened CD3ζ response for one antigen, and rescue this response through the costimulatory (CD28) response for the second antigen (Zhao & Sadelain, 2023).

Parts level

Antigen Selection

Using the Antigen Explorer Database provided by Princeton University (Antigen Explorer), the combination of antigens that are known as clinical targets with the highest combined score were chosen. A normal CAR T Cell that only targeted one antigen (MSLN or EGFR) would lead to many off-target effects because normal cells also have those antigens, leading to lack of specificity.The EGFR and MSLN antigens have a predicted combined score of 0.58, as seen on the antigen explorer database.

Introduction of MSLN and EGFR genes in CAR T Cells

A single bicistronic vector would be designed containing the genes for MSLN and EGFR, as seen in Figure 2. This ensures that both CARs are expressed in all transduced cells at the same density level. The pNB328-meso3 CAR plasmid and pIRII-CAR.EGFR(EGFv1) CAR plasmid would be inserted into the vector. DNA encoding the

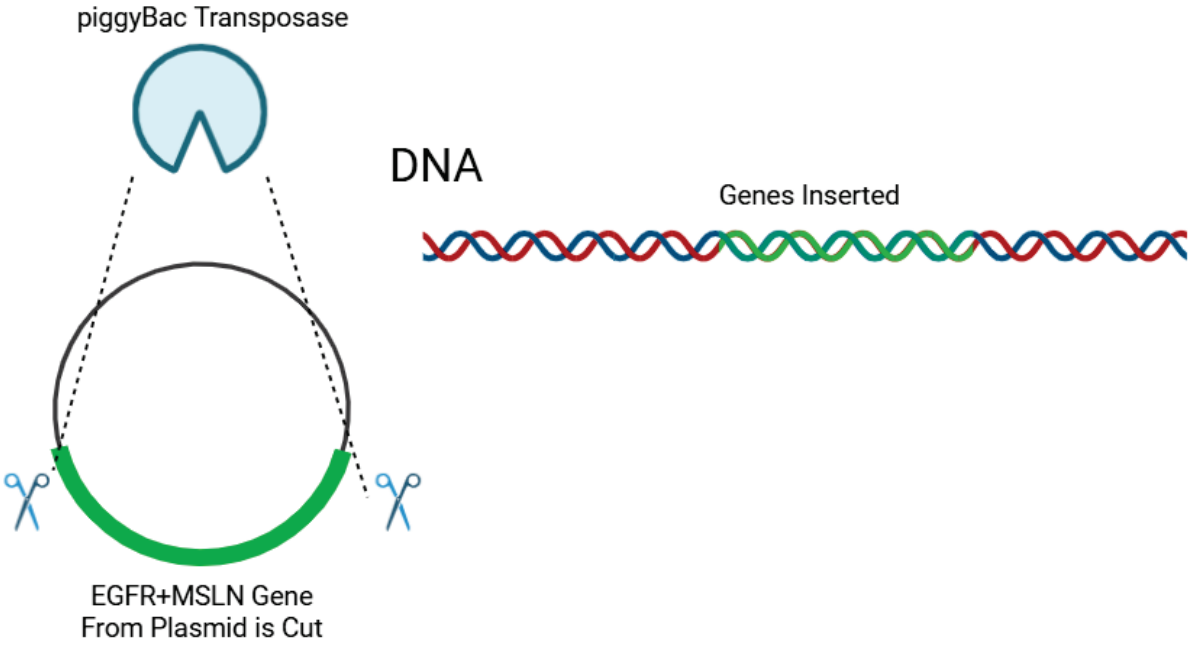

Figure 2. he transposon contains the genes of interest (EGFR and MSLN). Transposase cuts the transposon out of the donor plasmid and integrates it into the CAR T-Cell genome. Once the gene from the plasmid is integrated into the CAR T-cell DNA, the gene of interest is expressed.

piggyBac transposase is cloned separately from the MSLN and EGFR plasmids. This vector is then transfected into the CAR T cells. Transfected cells are phenotyped at weekly intervals: cell staining, flow cytometry, and gating can be done to check whether transfection was successful.

T Cell activation and culture

Human peripheral blood mononuclear cells (PBMC) would need to be obtained from healthy donors provided with written informed consent (Kim et. al., 2023). Cryopreserved PBMCs would be quickly thawed in a 37 degrees Celsius water bath and cultured in RPMI-1640 medium (Kim et. al., 2023). They would be activated using human-T-activator CD3/CD28 dynabeads (Kim et. al., 2023). T-cells would be cultured for 10 days by adding fresh medium and human IL-2 every 2 days (Kim et. al., 2023). EGFR provides the primary activation signal (CD3ζ) and MSLN provides the costimulatory CD28 signal. This is important because the first signal (CD3ζ) is not enough to sustain robust T cell activity. Costimulatory signals are important to enhance cytokine release and prevent T cell exhaustion. Without the CD28 signal, T cells may become inactive and undergo apoptosis.

Safety

To ensure the safe construction of the AND-gated CAR T cells targeting the two antigens, MSLN and EGFR, gene transfer will be conducted in a biosafety level 2 laboratory setting using the piggyBac transposon system. This is a non-viral integration method that reduces the risk of viral vectors. Additionally, T cells will be isolated and engineered under sterile conditions and preclinical testing will begin *in vitro* to assess the specificity and cytotoxicity of the CAR T Cells. Additionally, the isolation of T-cells from PBMC will be ethically approved and will gain the consent of the patient.

Discussions

The proposed AND-gated CAR T-cell system offers several key benefits over the traditional single-antigen CAR T-cell approach. By requiring the expression of both EGFR and MSLN for full T-cell activation, the dual nature of the system enhances tumor specificity, reduces off-target toxicity, and therefore leads to higher tumor specific killing. However, the tumor microenvironment is immunosuppressive, with inhibitory cytokines, and this may affect the CAR T-cell efficacy. There is also potential for antigen downregulation in tumor cells and tumor escape, leading to lower CAR-T efficiency in killing PDAC cells. Future improvements could look at more antigen combinations to further improve PDAC targeting accuracy and could also look at other logic-gating (OR, NOT, AND-NOT, OR-NOT). Additionally, further killing assays with the MSLN-EGFR AND-gated Car T cells could reveal the percent of tumor specific killing compared to a single-antigen CAR T-cell system.

Next Steps

One future study that is crucial to conduct after designing the dual AND-gated CAR T-cell therapy is conducting killing assays. A viability assay to conduct is flow cytometry-based cytotoxicity assay. This would determine live vs. dead tumor cells. The setup would include co-culturing CAR T-Cells with target cells expressing EGFR only, MSLN only, both EGFR and MSLN, or neither antigen. The expected result would be to see significant killing in the EGFR-MSLN-target group. Another study could include other antigens targeting PDAC and the development of a logic-gated CAR T cell in order to improve the targeting of those antigens. Furthermore, it is crucial to elucidate the mechanisms by which EGFR-MSLN AND-gated CAR T cells can persist and remain functional within the immunosuppressive microenvironment of PDAC patients, thereby sustaining high levels of T-cell activation over extended periods. Ultimately, these engineered CAR T cells should also be explored for their therapeutic potential in other solid tumor types.

Author contributions

Both AA and JJ worked on the project idea and proposal. AA wrote the paper and JJ proofread it.

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References

Abbott, R. C., Hughes-Parry, H. E., & Jenkins, M. R. (2022). To go or not to go? Biological logic gating engineered T cells. Journal for ImmunoTherapy of Cancer, 10(4), e004185. https://doi.org/10.1136/jitc-2021-004185

Becker, A. E., Hernandez, Y., Frucht, H., & Lucas, A. (2014). Pancreatic ductal adenocarcinoma: Risk factors, screening, and early detection. World Journal of Gastroenterology, 20(32), 11182–11198. https://doi.org/10.3748/wjg.v20.i32.11182

De Marco, R. C., Monzo, H. J., & Ojala, P. M. (2023). CAR T Cell Therapy: A Versatile Living Drug. International Journal of Molecular Sciences, 24(7), 6300. https://doi.org/10.3390/ijms24076300

Kim, Y., Da Yeon Lee, Choi, J., Jin Song Park, So Myoung Lee, Chung Hyo Kang, & Chi Hoon Park. (2023). Optimized conditions for gene transduction into primary immune cells using viral vectors. Scientific Reports, 13(1). https://doi.org/10.1038/s41598-023-39597-2

World Cancer Research Fund. (2024, October 4). Pancreatic cancer statistics | World Cancer Research Fund. World Cancer Research Fund. https://www.wcrf.org/preventing-cancer/cancer-statistics/pancreatic-cancer-statistics/

Yamada, R., Tsuboi, J., Yumi Murashima, Tanaka, T., Nose, K., & Nakagawa, H. (2023). Advances in the Early Diagnosis of Pancreatic Ductal Adenocarcinoma and Premalignant Pancreatic Lesions. Biomedicines, 11(6), 1687–1687. https://doi.org/10.3390/biomedicines11061687

Zhao, Z., & Sadelain, M. (2023). CAR T cell design: approaching the elusive AND-gate. Cell Research, 33(10). https://doi.org/10.1038/s41422-023-00828-w