

Reducing the immunosuppressive microenvironment in pancreatic adenocarcinomas through cell-type specific targeting of myeloid derived suppressor cells

Christopher Ren^{1,6,*}, Tina Li², Christina Liu³, Yana Yang⁴, Asher Zheng⁵

¹Shanghai High School International Division, Shanghai, 200231, China

²Chongqing Foreign Language School International Division, Chongqing, 400039, China

³The High School Affiliated to Yunnan Normal University, Kunming, 650106, China

⁴Shenghua Zizu Academy, Shanghai, 200241, China

⁵Shenzhen College of International Education, Shenzhen, 518043, China

⁶ren19921418702@gmail.com

*corresponding author

Abstract. Pancreatic adenocarcinoma is one of the leading causes of cancer related deaths worldwide and currently lacks effective treatments. The immune-suppressive microenvironment and high malignancy has made conventional targeted therapies and immunotherapies ineffective for most patients. Thus, there is an urgent need for alternative treatments to enhance therapeutic efficacy. Through a comprehensive literature review, we propose a novel CD300ld antigen-specific liposome cargo delivery system for targeting MDSC mediated immune-suppression in pancreatic adenocarcinoma. Hypothetically, this approach could exhibit enhanced immune engagement and significant anti-tumor effects through the artificial expression of CD47 inhibitor and suppression of STAT3-dependent immunosuppressive cytokines. Further validation in primate models, combined with conventional immune check-point inhibitor (ICI) treatments, could be conducted for a more comprehensive understanding of this model.

Keywords: Myeloid derived suppressor cells, Tumor immunosuppression, Combined liposome cargo delivery.

1. Introduction

1.1. Pancreatic adenocarcinoma is a profound risk factor for global health that lacks effective treatments

Pancreatic adenocarcinoma (PAAD) is one of the most common pancreatic malignancies, constituting about 90% of all primary pancreatic cancer[1]. With a 5-year average survival rate ranging from 2 to 10%, PAAD is the seventh leading cause of cancer-related deaths worldwide[2,3]. Prominent risk factors of PAAD include history of smoking and inherited genetic patterns[3], which often activates oncogenic pathways such as KRAS-PI3K-AKT[4], BRCA1/2[5], and CDKN2A[6]. Conventional cancer immunotherapies have shown limited efficacy against PAAD, primarily due to the

immunosuppressive tumor microenvironment (TME). This includes the recruitment of immunosuppressive cells, such as myeloid-derived suppressor cells (MDSCs), regulatory T cells (Treg), and tumor-associated macrophages (TAMs), enriched levels of suppressive cytokines (e.g. TGF- β , IL-10), downregulation of antigen presenting molecules (MHCs), and upregulation of immune checkpoints (including but not limited to PD-1, CTLA-4)[7–9]. Conventional immunotherapy treatments such as PD-1 and CTLA-4 inhibitors have limited efficacy in PAAD[10]. 5-fluorouridine (5-FU) is a common chemotherapy drug for pancreatic cancer; however, the dense stroma surrounding pancreatic tumors contributes to the 5-FU chemoresistance. Furthermore, the KRAS G12D inhibitor MRTX1133 is a recently discovered drug targeting KRAS interaction with downstream RAF1[11,12]. However, KRAS inhibitor efficacy has been limited by the compensatory activation of survival pathways[13]. Thus, novel strategies are required to target the immunosuppressive microenvironment of PAAD.

1.2. Myeloid derived suppressor cells contributes to PAAD by enhancing an immuno-suppressive TME

One profound factor contributing to the suppressive nature for PAAD TME is the abundance of myeloid derived suppressor cells (MDSC)[9]. MDSCs are a heterogeneous population of myeloid cells, primarily classified into two subsets: polymorphonuclear-MDSCs (characterized by CD11b+CD14-CD66b+ or CD11b+CD14-CD15+) and monocytic-MDSCs (characterized by CD11b+CD14+HLA-DR-/lo)[8]. Tumor cells secrete chemokines such as CCL2, CXCL8, CXCL12, CCL3, CCL5, CX3CL1 and CXCL1, that contribute to the recruitment of MDSCs to the tumor site[9]. MDSCs exert immunosuppressive effects/ through various mechanisms within the tumor microenvironment. Secreted immunosuppressive cytokines such as TGF- β , IL10 contributes to the inhibition of cytotoxic T lymphocyte function, induces T regulatory cell function and further recruits MDSCs[9]. Produced reactive oxygen and nitrogen species and the depleted cystine and cysteine created an unfavorable environment for functional T cells[14,15], reducing host immune surveillance and decreasing efficacy of conventional immunotherapies. These characteristics suggest that targeting myeloid lineages in TME to be a novel approach to increase immunotherapy responsiveness.

1.3. Neutrophils and macrophages contribute to T-cell suppression and tumor immune-evasion.

Neutrophils and macrophages could both obtain an immune suppressive phenotype of MDSCs via cytokine signaling (IL-10, TGF- β)[16,17]. Tumor cells and structural cells in the tumor microenvironment (TME) promote the migration of neutrophils into the TME by producing cytokines (G-CSF) and chemokines (CXCL5/CXCL8). Moreover, neutrophils transition into MDSCs by exposure to proteolytically (MMP-9)-activated TGF- β 1. Neutrophils express increased levels of PD-L1 and TGF- β , inhibiting cytotoxic T cell function and positively regulating MDSC infiltration[18]. In addition, activated neutrophils release Neutrophil extracellular traps (NET), which are web-like structures composed of DNA, histones, and various antimicrobial proteins. NETs could induce immune cell exhaustion and physically shield tumors from immune attacks[19]. Tumor associated macrophages (TAMs) were recruited to tumor site by cytokines and chemokines produced in the TME, such as GM-CSF, IL-1 β , SDF1 α , VEGF, CSF1, and CCL2. TAMs express inhibitory receptors and ligands such as PD-1, PD-L1, Tim3, Tim4, and B7-H4, which can inhibit macrophage functions including phagocytosis, inflammasome activation, and production of effector cytokines[20,21]. Thus, as predominant cell types contributing to immune-suppression, targeting neutrophils and macrophages could be therapeutically beneficial.

In this study we aimed to develop the concept for a new treatment for PAAD. Our combined new approach is inspired by the following three studies each of which discusses different orthologous pharmacological and immunotherapeutic approaches:

2. Approach

2.1. Summaries of foundational studies

2.1.1. TAM family kinase inhibition reverses MDSC mediated immuno-suppression.

This study investigated the role of the TAM family receptor tyrosine kinases (TYRO3, AXL, and MERTK) in regulating the function of myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment. Holtzhausen and colleagues conclude that MDSCs upregulated TAM receptors and their ligands in tumor-bearing mice, and that genetic deletion or pharmacological inhibition of TAM receptors diminished MDSC suppressive capabilities, reduced tumor growth, and enhanced anti-PD-1 immunotherapy. Mechanistically, MERTK regulated MDSC suppression and differentiation through the STAT3 signaling pathway, and metastatic melanoma patients exhibited increased numbers of TAM+ MDSCs compared to healthy controls. These results suggest that targeting the TAM family receptors could be a promising approach to reverse MDSC-mediated immune suppression and improve cancer immunotherapy while highlighting the immunosuppressive function of STAT3 signaling pathway.[22]

2.1.2. Targeting SIRPA in tumor associated myeloid cells promotes anti-tumor immunity

This study investigates the role of myeloid cells, particularly tumor-associated macrophages (TAMs) and granulocytic myeloid-derived suppressor cells (gMDSCs), in the tumor immune microenvironment (TIME) of colorectal cancer (CRC). The researchers concluded that myeloid cells represented the most expanded and immunosuppressive populations in the colorectal cancer TME, exhibiting increased expression of immunoreceptor tyrosine-based inhibitory motif (ITIM)-bearing receptors, such as SIRPA. The SIRPA-CD47 interaction was known to prevent macrophage mediated phagocytosis. Notably, Sirp α (the mouse homolog of human SIRPA) deficiency suppressed tumor development and prolonged survival in mice by reprogramming the TIME, expanding myeloid subsets with strong antitumor activity, and promoting T cell infiltration and activation, independent of the CD47-SIRPA axis. These findings suggest that targeting SIRPA and the SIRPA-CD47 interaction could be promising strategies to improve the efficacy of cancer immunotherapy.[23]

2.1.3. CD300ld on neutrophils is required for tumor-driven immune suppression

This study identified CD300ld as a critical immune suppressor expressed on polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) in the tumor microenvironment. The researchers performed an in vivo CRISPR-Cas9 screen and found that CD300ld is specifically upregulated in PMN-MDSCs upon tumor development. Knockout of CD300ld inhibited tumor growth in a PMN-MDSC-dependent manner by impairing the recruitment and immunosuppressive function of PMN-MDSCs. CD300ld acted via the STAT3-S100A8/A9 axis, and its blockade reversed the tumor immune-suppressive microenvironment and had synergistic effects with anti-PD1 therapy. Importantly, CD300ld was upregulated in human cancers and associated with poor patient survival, making it a potential target for cancer immunotherapy. This study highlights the neutrophil-specific expression of CD300ld, and the significance of STAT3 signaling in tumor immunosuppression.[24]

2.1.4. Liposome drug delivery system is a well established method for therapeutic treatments

This paper provides a comprehensive review of liposomes as a drug delivery system, focusing on the current status of approved liposomal products, regulatory environments, and future perspectives. The paper highlights the major obstacles and challenges in the development and commercialization of liposomal products, including tissue specificity, the individual differences in the enhanced permeability and retention (EPR) effect, accelerated blood clearance (ABC) phenomenon of PEGylated liposomes, scale-up, and reproducibility/consistency. It also discusses the promising advancements in smart liposomal systems, such as active targeting and stimuli-sensitive liposomes, which are currently in the pre-clinical or clinical trial stages. The paper discussed examples of 14 liposomal drug products approved by the FDA and EMA, which cover various therapeutic areas, predominantly cancer treatment,

but also including infection, anesthesia, vaccine, lung disease, and photodynamic therapy. This study highlights that liposomes systems to be an effective drug delivery system, yet caveats such as specificity could still affect treatment efficacy[25].

2.2. Methods and rationale

2.2.1. General Approach

Based on the results of the previous foundational studies, we propose a novel model for targeting CD300ld specific neutrophils via an engineered liposome. This model directly inhibits receptor function of CD300ld and simultaneously targets immune-suppressive signaling pathways through siSTAT3. In addition, the liposome cargo contains engineered mRNA molecules encoding a soluble form of CD47, a high affinity ligand for SIRPa on macrophages, enhancing macrophage function locally in the TME for cancer cell elimination.

Liposome target specificity will be validated by Luciferase staining in the KPC mouse model, western blot and real-time quantitative PCR would be applied to assess the knockdown efficiency of siRNA and the translational enhancement of CD47 variant. We will apply a directed evolution method to generate a soluble CD47 variant lacking a transmembrane domain with enhanced binding affinity towards SIRPa, aiming to interfere with normal CD47-SIRPa mediated macrophage inhibition. Survival and tumor volume analysis will be conducted to evaluate the anti-tumor effects of our treatment. FACS analysis will quantify the modifications of TME immune cell composition, thereby validating the model's effect on reducing immunosuppression in PAAD.

The hypothesized antigen-specific liposome treatment is expected to decrease levels of immunosuppressive signals in the TME and increase tumor infiltrating T-cells via the inhibition of STAT3 signaling. Meanwhile, increase tumor associated macrophage phagocytosis tumor through the cytosolic translation of soluble CD47-inhibitor that competitively inhibits the SIRPa-CD47 axis.

Table 1. treatment protocols that will later be referred to. These four treatment groups would be applied to the majority of in vivo experiments unless stated otherwise.

Treatment	Description/composition	Function
Phosphate buffered saline	Phosphate buffered saline	Ctrl
Antigen specific liposome	Engineered liposome with CD300a specific surface antibody Cargo: siSTAT3, Cd47 inhibitor mRNA	Case
Antigen non-specific liposome	Engineered liposome with non-specific surface antibody (IgG1, IgG2) Cargo: siSTAT3, Cd47 inhibitor mRNA	Ctrl
Empty antigen-specific liposome	Engineered liposome with CD300a specific surface antibody Cargo: NA	Ctrl

2.2.2. Engineering High-Affinity CD47 Variants

We propose to engineer high-affinity variants of the extracellular domain (ECD) of CD47 using yeast surface display and directed evolution techniques. First, an initial library of CD47 ECD mutants will be generated via error-prone PCR to introduce random mutations. The mutant library will then be cloned into the pCTCON2 yeast display vector and transformed into *Saccharomyces cerevisiae* (EBY100 strain). Induced yeast cells displaying CD47 variants will be screened for enhanced binding to biotinylated recombinant SIRPa using fluorescence-activated cell sorting (FACS). Sorted populations will undergo several rounds of iterative selection to enrich high-affinity binders. Enriched high-affinity clones will be sequenced to identify beneficial mutations and subsequently expressed in a mammalian system (e.g., HEK293T cells) for further characterization. Binding affinities of the selected variants will be quantitatively assessed using biolayer interferometry (BLI), and functional inhibition of SIRPa signaling will be evaluated in phagocytosis assays utilizing RAW264.7 macrophage cells. These

experiments aim to yield CD47 variants with improved therapeutic potential for inhibiting SIRP α -mediated signaling in various disease models.

2.2.3. Validation Using the KPC Mouse Model

To validate the anti-tumor efficacy of our antigen-specific liposome model, we propose using the KPC mouse model for pancreatic adenocarcinoma, which is the most frequently employed genetically engineered model in pancreatic cancer research. The KPC model is generated by interbreeding LSL-Kras^{G12D/+} mice (mice with a lox-stop-lox [LSL] sequence preceding the Kras^{G12D} mutation) and LSL-Trp53^{R172H/+} mice (mice with an LSL sequence preceding the Trp53^{R172H} mutation). These offspring are then crossed with Pdx1-Cre or P48-Cre transgenic mice, which express Cre recombinase specifically in the pancreas. This leads to the activation of the oncogenic Kras^{G12D} and mutant Trp53^{R172H} alleles in the pancreatic tissue, resulting in the spontaneous development of pancreatic tumors that closely recapitulate human pancreatic ductal adenocarcinoma in terms of histopathology and genetic alterations. Tumor development will be monitored through palpation and ultrasonography. Mice will be randomized into control and experimental groups upon tumors reaching ~100 mm³. The KPC mice are similar to the parental primary tumors and possess a competent immune system, making them advantageous for understanding mechanisms of T cell exclusion, myeloid cell regulation of the tumor microenvironment, and tumor adaptation to immune pressure.

Essential proteins (CD300ld, SIRP α , CD47, STAT3) will be translated into their corresponding gene products in the *Mus musculus* ortholog (Cd300a, Sirpa, Cd47, Stat3) for responsible attribution of treatment effects.

2.2.4. Specificity Validation

To validate the cell-specificity of our proposed liposome model in KPC mice, we will exploit the expression specificity of CD300a on neutrophils for direct transportation of engineered liposomes.

Liposomes will be engineered to encapsulate firefly luciferase proteins (62-65 kDa) using standard thin-film hydration followed by extrusion techniques to achieve a uniform size distribution of 100-200 nm in diameter. Surface modification with CD300a-specific antibodies will be performed through covalent attachment to facilitate targeted delivery to neutrophils. Liposomes will be characterized for size, polydispersity index (PDI), and surface charge using dynamic light scattering (DLS) and zeta potential measurements.

The study comprises four experimental groups mentioned above. Liposome treatment groups will receive an intravenous injection of liposomes containing a specific concentration of luciferase protein (10 μ g/mouse), while the control group will receive an equivalent volume of PBS.

To quantify liposome distribution in vivo, D-luciferin substrate (150 mg/kg) will be intravenously injected into the mice at predetermined time points, ranging from 24 hours to 7 days post-treatment. Bioluminescence imaging will be conducted using an in vivo imaging system (IVIS), with images captured at optimal exposure settings to detect luciferase activity. Regions of interest (ROIs) will be drawn around tumors and other organs to quantify luminescent signals, providing insights into the biodistribution and accumulation of the liposomes. Image analysis software will be employed to quantify bioluminescence intensity, and statistical methods will be used to compare the results among the experimental groups.

2.2.5. Knockdown and Expression Efficiency

To validate the efficiency of siRNA knockdown and to quantify the expression levels of designated genes, we propose a fluorescence-activated cell sorting (FACS) protocol to isolate neutrophils engaged with liposome-attached cargos. The liposomal formulation will encapsulate siSTAT3, an mRNA coding for an inhibitor of CD47 with high affinity to SIRP α , and firefly luciferase. KPC mice will be administered this liposome solution. Following treatment, neutrophils will be identified using specific surface markers CD66a and Ly-6G (Gr-1). Neutrophils exhibiting high fluorescence and surface expression of these markers will be recognized as liposome-treated. Post-isolation, the expression levels

of STAT3, the CD47 inhibitor protein, and immunosuppressive cytokines (TGFB, IL10, S100A9/A8) will be quantified using Western blotting and RT-qPCR. A concurrent analysis will be performed on a control group of neutrophils, characterized by low fluorescence intensity but high expression of CD66a and Ly-6G, to benchmark against untreated cells. Comparative analysis between the treated and control groups in the mRNA and protein levels will assess the efficiency of siRNA knockdown and gene expression modification.

2.2.6. *Anti-Tumor Effects*

Anti-tumor effects would be tested on KPC mice strains by two separate experiments.

First, mice would be divided into 4 groups (n=5), each treated with phosphate buffered saline, antigen non-specific liposome, empty antigen-specific liposome or antigen-specific-liposome (treatment every 7 days starting day 0). Tumor growth and volume would be measured daily for 60 days. Tumor length and width would be measured, tumor volume calculated by the formula: $V = \frac{1}{2}(L \times W)$. Second, mice would be divided into 4 groups (n=20), each treated with phosphate buffered saline, antigen non-specific liposome, empty antigen-specific liposome or antigen-specific liposomes. (treatment every 7 days starting day 0). Mice survival would be monitored, and survival outcomes would be compared at day 60.

2.2.7. *Macrophage Activation*

KPC mice will be divided into 4 groups, each treated with phosphate buffered saline, antigen non-specific liposome, empty antigen specific liposome, and antigen specific liposome. Tumor samples will be collected after 12 and 24 hours post-treatment (2 experiments, select results with better outcome). Due to the limited pancreatic cancer specific tumor antigen, we alternatively use multiple assays to analyze macrophage mediated phagocytosis.

First, we propose to perform flow cytometric analysis of isolated macrophages from tumor tissue, and compare cell composition between 4 groups. (Macrophage markers: CD11b+ F4/80+, Phagocytosis markers: CD64 high, CD32 high, CD16 high)

For the second round of experiment, after tumor tissue is extracted, we apply a pH-sensitive fluorescent dye pHrodo™[26]. That emits light at low pH levels, indicating lysed cellular components.

2.2.8. *Immune Cell Infiltration*

We aim to examine the magnitude of immune responses treatment of antigen-specific liposomes.

Mice will be divided into 4 groups, each treated with phosphate buffered saline, antigen non-specific liposome, empty antigen-specific liposome, or antigen-specific liposome separately. Tumor microenvironment tissue will be isolated, cell composition will be analyzed by flow cytometry. T cell, Tregs, NK, M1/M2 macrophages, and neutrophil subtypes will be documented. Specifically, CD8 effector T cells (CD8+ KLRG1+ CD122+ TIM3+), CD8 naive T cells (CD8+ CCR7+ IL7Ra+ CD62L), Treg (CD304+ FR4+ CD121a+ CD121b+), NK (NK1.1+ TCR Vα14+ CD314+), M1 macrophage (CD86+ CD80+ Ly-6C+), M2 macrophage (Ly-6C+ MerTK+ CD369+), neutrophil (CD66a+ Ly-6G+ CD181+)

2.3. *Materials*

2.3.1. *FACS Analysis*

We propose to prepare a single-cell suspension for flow cytometry, tumor tissues will be excised from euthanized mice and minced into small pieces using sterile scissors. The minced tissue will be then digested into a single-cell suspension using a collagenase/DNase I enzyme cocktail at 37°C for 45 minutes with intermittent shaking. Following digestion, the suspension will pass through a 40 μm cell strainer to remove debris and clumps. The single-cell suspensions will be collected by centrifugation at 300g for 10 minutes at 4°C and resuspended in FACS buffer (PBS containing 2% FBS and 1 mM EDTA). For staining, cells will be first counted, and 1×10^6 cells were aliquoted per sample. Cells will then be

incubated with an Fc receptor blocking agent for 10 minutes at 4°C to prevent non-specific antibody binding. Subsequently, cells will be incubated with fluorophore-conjugated antibodies specific to designated cells. at 4°C for 30 minutes in the dark. After incubation, the cells will be washed twice with FACS buffer, resuspended in 300 µL of FACS buffer, and kept on ice until analysis. Stained cells will be analyzed on a flow cytometer (e.g., BD LSRFortessa™). Gated populations will be identified, and comparative analyses were performed across treatment groups.

2.3.2. Flow cytometry

For immune cell profiling, single-cell suspensions will be prepared from tumor and spleen tissues using the methods described for FACS. Once the single-cell suspensions are obtained, cells will be stained for both surface and intracellular markers. Cells will be incubated with Live/Dead fixable dye to exclude dead cells, followed by staining with fluorophore-conjugated antibodies specific to the immune cell markers at 4°C for 30 minutes. For intracellular staining, cells will undergo fixation and permeabilization using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) and then stained with the intracellular marker Foxp3. Stained cells will be analyzed on a BD LSRFortessa™ flow cytometer (or any suitable flow cytometer), with gates set according to isotype controls and fluorescence-minus-one (FMO) controls.

2.3.3. RT-qPCR

To extract total RNA from tumor tissues, the RNeasy Mini Kit (or any RNA extraction kits) will be used according to the manufacturer's protocol. Tumor tissues were homogenized in a lysis buffer and centrifuged to clear debris. The clarified lysate will be applied to the RNA binding column, washed, and the RNA was eluted in RNase-free water. For cDNA synthesis, 1 µg of extracted RNA will be reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad) under the following conditions: 25°C for 5 minutes, 42°C for 30 minutes, and 85°C for 5 minutes. Quantitative PCR will then be performed using the iQ™ SYBR® Green Supermix (Bio-Rad) on a CFX Connect Real-Time PCR Detection System (Bio-Rad). Each reaction mixture contains 10 µL of SYBR Green Supermix, 0.5 µM of each primer, and 1 µL of cDNA in a total volume of 20 µL. The cycling conditions are as follows: initial denaturation at 95°C for 3 minutes, followed by 40 cycles of 95°C for 10 seconds, and 60°C for 30 seconds. All expression values will be normalized to gene GAPDH or B-actin.

2.3.4. pHrodo Staining

The pH-sensitive staining with pHrodo™ will be performed on freshly excised tumor tissues from mice. Tumor tissues will be cut into small pieces and incubated in RPMI-1640 medium. Tumor pieces will then be incubated with the pH-sensitive dye pHrodo™ Red AM (Thermo Fisher) at a concentration of 0.5 µM for 30 minutes at 37°C according to the manufacturer's instructions. After incubation, the tissues will be washed with PBS to remove any excess dye. The labeled tissues will then be visualized using a fluorescence microscope (e.g., Zeiss Axio Observer). Images will be captured and analyzed to determine fluorescence intensity, indicating acidic intracellular environments characteristic of cellular degradation.

2.3.5. Firefly Luciferase Staining

For Firefly Luciferase staining, tumor tissues will be excised from treated mice, minced, and enzymatically digested to obtain a single-cell suspension as previously described. These cells will then be resuspended in PBS and aliquoted into 1×10^6 cells per sample. Cells will be fixed and permeabilized using the Fixation/Permeabilization Solution Kit (BD Biosciences) according to the manufacturer's instructions. The permeabilized cells will be incubated with anti-luciferase antibodies conjugated to a fluorophore at 4°C for 30 minutes in the dark. Following staining, cells will be washed three times with PBS and resuspended in FACS buffer. Stained cells will then be analyzed using a flow cytometer (e.g., BD LSRFortessa™). The percentage of luciferase-positive cells will be determined, and the mean fluorescence intensity (MFI) will be quantified to assess the level of luciferase expression

within the tumor cells. Data analysis will be performed using FlowJo software, facilitating comparisons between different treatment groups.

2.3.6. Mouse model

Female KPC (Kras^{LSL-G12D/+}; Trp53^{fl/fl}; Pdx1-Cre) mice, aged 6-8 weeks old, will be housed under specific-pathogen-free (SPF) conditions with regulated temperature and humidity, and a 12-hour light-dark cycle. The experimental procedures conducted in this study will receive approval from the Institutional Animal Care and Use Committee (IACUC) and will comply with the ARRIVE guidelines. For tumor induction, mice will be monitored for spontaneous pancreatic tumor development inherent to the KPC model. Tumor growth will be assessed regularly via calipers or ultrasonography, and mice will be assigned to treatment groups when tumors reach an appropriate size, typically around 100 mm³.

3. Anticipated results

This section provides anticipated results of the experimental procedures proposed, which would support our hypothesis.

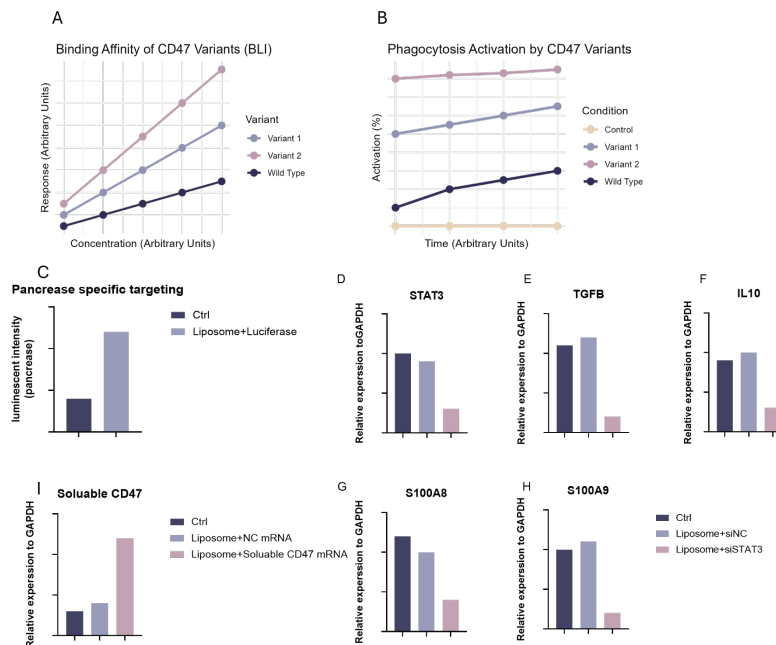


Figure 1. Engineered CD47 variants should exhibit increased binding affinity towards SIRPa (A) and increased activation of macrophage mediated phagocytosis (B). Meanwhile, the CD300a decorated liposome should exhibit high tissue specificity to the pancreas (C). Moreover, Liposome delivered STAT3 siRNA should decrease expression of immuno-suppressive cytokines (D,E,F,G,H).

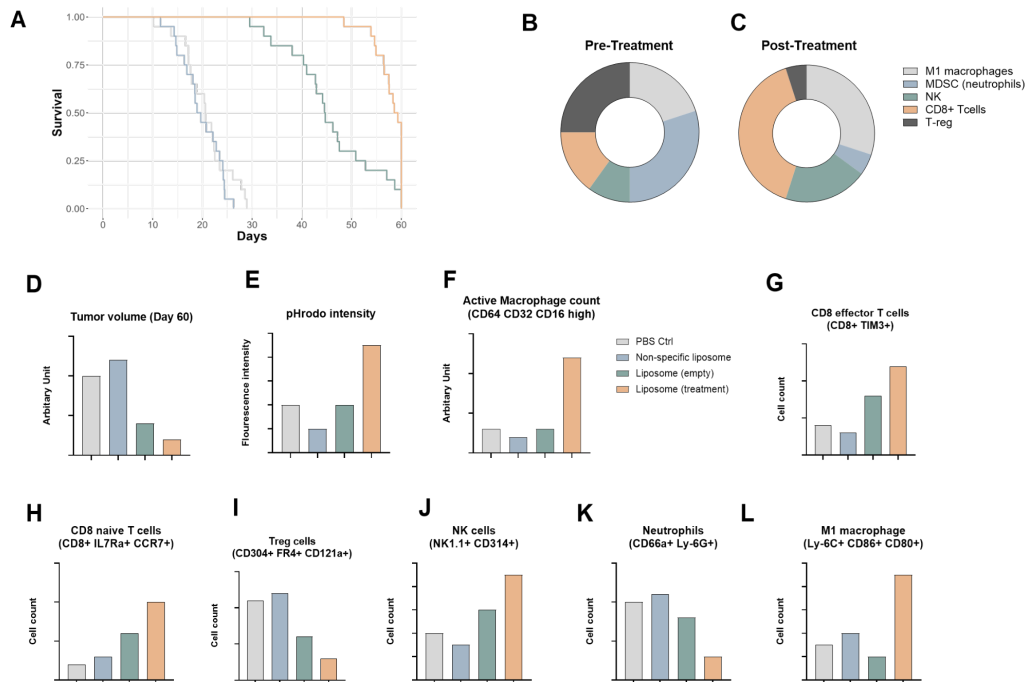


Figure 2. Liposome treatment significantly increased survival rates for tumor bearing mice (A), and tumor growth of treated mice were inhibited (D). Meanwhile, post treatment, TME immune cell composition was modified (B-C), with increased pro-inflammatory cells and decreased immune-suppressive cells (G-L). In addition, macrophages exhibit increased phagocytosis activity (E,F).

3.1. Soluble CD47 binding affinity

Biolayer interferometry analysis should indicate that engineered soluble CD47 variants exhibit increased binding affinities to SIRPα compared to wild type CD47 (Fig1A), suggesting its success in competitive docking with wild type CD47. Moreover, interfering with the CD47-SIRPα axis shows increased macrophage activity (Fig1B).

3.2. Model efficacy

Bioluminescent concentrates in mice pancreatic cancer, indicating that the engineered liposomes effectively deliver cargo to designated tissue (Fig1C). Moreover, RT-qPCR and western blot analysis reflects decreased levels of immune-suppressive cytokines TGF-β, S100A8/A9, IL10 as well as STAT3 mRNA and protein in liposome-attached neutrophil cytosol (Fig1D,E,F,G,H). In addition, considerable amounts of CD47 inhibitor mRNA and protein were also detected (Fig1I), indicating that the antigen-specific liposome treatment model is effective in siRNA delivery and exhibits regulatory effects against immunosuppressive neutrophils.

3.3. Anti-tumor effects

Mice treated with antigen-specific liposomes would exhibit significant decrease in tumor size compared to all other groups (Fig2D). In addition, survival data is expected to indicate that the antigen-specific liposome has a positive effect on mice survival, indicating anti-tumor effects (Fig2A).

3.4. Macrophage activation

Flow cytometry analysis revealed a marked increase in the proportion of active macrophages in the tumor microenvironment following treatment with antigen-specific liposomes (increased percentage of CD11b, F4/80, CD64, CD32, CD16). In addition, pH-dependent staining indicates increased lysosomal

activity in macrophages from the antigen-specific liposome treatment group. This suggests that macrophages were activated by antigen-specific liposome treatment and exhibited increased phagocytosis function (Fig2E, F).

3.5. Immune cell infiltration

Flow cytometry analysis demonstrates a significant increase in the infiltration of effector CD8⁺ T cells, naive CD8⁺ T cells, and active NK cells in the tumor microenvironment following antigen-specific liposome treatment. Meanwhile, abundance of MDSCs significantly decreased or remained unchanged in most samples (Fig2G-L). Suggesting that treatment increased immune engagement with tumor microenvironment.

4. Discussion

Our primary anticipated results demonstrate that the antigen-specific liposome treatment effectively downregulated immune-suppressive factors and enhanced immune cell infiltration in the TME, suggesting promising antitumor effects. Moreover, it is anticipated that treatment remodeled TME, decreasing. As a novel approach to target hyper-suppressive TME in pancreatic adenocarcinoma, antigen-specific liposome mediated regulation of MDSCs remains hypothetical. Here we dissect this study's applications, limitations and potential improvements.

4.1. Foundational Insights and Mechanistic Considerations

Foundational studies have highlighted therapeutic potential of targeting the interactions of the proposed conceptual model consisting of CD47-SIRPA axis, CD300ld inhibition and STAT3 knockout. CD47 is highly expressed in several cancer types including PAAD, with its elevated expression correlating with immune checkpoint gene expression and poor prognosis[27]. Moreover, CD47 suppresses IgG- and phosphatidylserine- mediated phagocytosis by repositioning SIRPA to the phagocytic synapse, inhibiting integrin activation and macrophage function[28]. In addition, studies suggest the disruption of SIRPA-CD47 axis enhances macrophage mediated phagocytosis of tumor cells, macrophage polarization towards an anti-tumor state, as well as promotion of adaptive immune responses mediated by CD8⁺T cells[29]. Suggesting that successful interference of SIRPA-CD47 interaction prompts therapeutic benefits in anti-tumor therapies. Moreover, studies have shown inhibition of STAT3 signaling with the selective inhibitor S31-201 decreased the expression of the "don't eat me" signal molecule CD47 and its phagocytic receptor SIRPα in head and neck cancer[30]. Moreover, STAT3 inhibition led to a significant reduction in tumor burden and MDSC frequencies, and was associated with enhanced antitumor activity of chimeric antigen receptor T (CAR-T) cells, suggesting that targeting STAT3 in MDSCs can improve the efficacy of immunotherapy[31]. Studies have also suggested CD300ld as a specific surface marker and critical immune suppressor on polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs) that promotes tumor progression. Blocking CD300ld remodels the immunosuppressive tumor microenvironment, exhibits anti-tumor efficacy, and shows synergistic effects with immune checkpoint inhibitors, providing a promising target for cancer immunotherapy[32]. In addition, CD300ld is suggested to be implicated in norovirus, the ectopic expression of CD300lf or CD300ld can render non-susceptible cells permissive to murine norovirus (MNV) infection[33].

4.2. Addressing MDSC-Mediated Immunosuppression and Broader Applicability

Myeloid-derived suppressor cells (MDSCs) pose a significant challenge to the efficacy of conventional immune checkpoint inhibitor (ICI) treatments, as they contribute to an immunosuppressive tumor microenvironment (TME) that inhibits T cell functionality and promotes tumor progression[34]. Consequently, ICI treatments remain effective for only a subset of patients diagnosed with certain cancer subtypes[35]. Although this study focuses on an antigen-specific liposome treatment in pancreatic adenocarcinoma (PAAD), the high efficacy observed suggests broader applicability across various other cancer types wherein MDSCs are abundant in the TME. The combination of antigen-specific liposome treatment with conventional ICIs, such as CTLA-4 and PD-1 inhibitors, have the potential to produce

synergistic anti-tumor effects by simultaneously tackling multiple immunosuppressive pathways[36,37]. However, in vivo validation of antigen-specific liposome treatment preliminary translating the proposed therapeutic strategy into clinical applications. Further validation using primate models and humanized organoids are essential to ensure the treatment's efficacy and safety in more complex biological systems. Moreover, the complexity of the mammalian immune system demands sophisticated regulations when depleting a specific cell type such as MDSCs. There is a risk that artificial depletion could impair immune homeostasis, making patients more susceptible to minor infections and other immune-related complications[38,39]. To mitigate these risks, future studies should focus on refining the model through the identification of novel MDSC-specific surface biomarkers.

4.3. Future Directions and Considerations

Moreover, targeting multiple immunosuppressive pathways via specific siRNA could provide a more comprehensive approach to target the immunosuppressive TME. Future studies could investigate the genetic and molecular characteristics of different MDSC subtypes, aiming to identify novel pathways that might be co-regulated with known immunosuppressive signals. Such an approach could lead to more effective strategies for targeting various myeloid lineages, thereby enhancing the efficacy of tumor-suppressive microenvironment modulation[40]. A more sophisticated regulatory mechanism is also required to localize the inflammatory effects induced by antigen-specific liposome treatment. While upregulation of CD47 inhibitors and downregulation of immunosuppressive pathways are beneficial, they could lead to chronic dysregulation of inflammatory responses, potentially causing pro-tumorigenic effects if not adequately controlled[41,42]. Developing mechanisms to tightly regulate these responses, possibly through engineering feedback-controlled systems, will be crucial in achieving sustained therapeutic benefits without *exacerbating underlying conditions*.

5. Conclusion

In summary, this study proposes a promising therapeutic strategy to address the immunosuppressive TME in pancreatic adenocarcinoma by an antigen specific liposome cargo delivery system, with potential applicability to other cancer types. Further validation and refinement of the proposed approach, including identification of novel biomarkers and development of advanced regulatory mechanisms, are necessary to maximize its clinical potential and ensure patient safety.

Author contributions

CR, TL, CL, YY, AZ wrote and reviewed the manuscript as well as provided conceptual aggregation for foundational studies. TL, CR are responsible for graphical design. CR is responsible for overall conceptual, methodological design and administration of the project. All authors read and approved the final manuscript.

References

- [1] Pancreatic Cancer: Pathogenesis and Diagnosis. *Asian Pac. J. Cancer Prev.* 16, 5619–5624 (2015).
- [2] Afghani, E. & Klein, A. P. Pancreatic Adenocarcinoma. *Hematol. Oncol. Clin. North Am.* 36, 879–895 (2022).
- [3] Gupta, N. & Yelamanchi, R. Pancreatic adenocarcinoma: A review of recent paradigms and advances in epidemiology, clinical diagnosis and management. *World J. Gastroenterol.* 27, 3158–3181 (2021).
- [4] Kent, O. A. Increased mutant KRAS gene dosage drives pancreatic cancer progression: evidence for wild-type KRAS as a tumor suppressor? *Hepatobiliary Surg. Nutr.* 7, 403–405 (2018).
- [5] Lucas, A. L. *et al.* High Prevalence of BRCA1 and BRCA2 Germline Mutations with Loss of Heterozygosity in a Series of Resected Pancreatic Adenocarcinoma and Other Neoplastic Lesions. *Clin. Cancer Res.* 19, 3396–3403 (2013).

- [6] Prevalence of CDKN2A mutations in pancreatic cancer patients: implications for genetic counseling | European Journal of Human Genetics. <https://www.nature.com/articles/ejhg2010198>.
- [7] Hegde, S., Leader, A. M. & Merad, M. MDSC: Markers, development, states, and unaddressed complexity. *Immunity* 54, 875–884 (2021).
- [8] Bronte, V. *et al.* Recommendations for myeloid-derived suppressor cell nomenclature and characterization standards. *Nat. Commun.* 7, 12150 (2016).
- [9] Umansky, V., Blattner, C., Gebhardt, C. & Utikal, J. The Role of Myeloid-Derived Suppressor Cells (MDSC) in Cancer Progression. *Vaccines* 4, 36 (2016).
- [10] Li, H.-B., Yang, Z.-H. & Guo, Q.-Q. Immune checkpoint inhibition for pancreatic ductal adenocarcinoma: limitations and prospects: a systematic review. *Cell Commun. Signal.* 19, 117 (2021).
- [11] Bannoura, S. F., Khan, H. Y. & Azmi, A. S. KRAS G12D targeted therapies for pancreatic cancer: Has the fortress been conquered? *Front. Oncol.* 12, (2022).
- [12] Frontiers | KRAS G12D targeted therapies for pancreatic cancer: Has the fortress been conquered? <https://www.frontiersin.org/journals/oncology/articles/10.3389/fonc.2022.1013902/full>.
- [13] Concurrent inhibition of pBAD599 synergistically improves MEK inhibitor efficacy in KRASG12D-mutant pancreatic ductal adenocarcinoma | Cell Death & Disease. <https://www.nature.com/articles/s41419-024-06551-7>.
- [14] Kemp, S. B., Pasca di Magliano, M. & Crawford, H. C. Myeloid Cell Mediated Immune Suppression in Pancreatic Cancer. *Cell. Mol. Gastroenterol. Hepatol.* 12, 1531–1542 (2021).
- [15] Siret, C. *et al.* Deciphering the Crosstalk Between Myeloid-Derived Suppressor Cells and Regulatory T Cells in Pancreatic Ductal Adenocarcinoma. *Front. Immunol.* 10, (2020).
- [16] Yang, L., Pang, Y. & Moses, H. L. TGF- β and immune cells: an important regulatory axis in the tumor microenvironment and progression. *Trends Immunol.* 31, 220–227 (2010).
- [17] Mechanisms of immune suppression by myeloid-derived suppressor cells: the role of interleukin-10 as a key immunoregulatory cytokine - PMC. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7536076/>.
- [18] Molecules | Free Full-Text | Role of the Neutrophil in the Pathogenesis of Advanced Cancer and Impaired Responsiveness to Therapy. <https://www.mdpi.com/1420-3049/25/7/1618>.
- [19] Kaltenmeier, C. *et al.* Neutrophil Extracellular Traps Promote T Cell Exhaustion in the Tumor Microenvironment. *Front. Immunol.* 12, (2021).
- [20] Christofides, A. *et al.* The complex role of tumor-infiltrating macrophages. *Nat. Immunol.* 23, 1148–1156 (2022).
- [21] Mehta, A. K., Kadel, S., Townsend, M. G., Oliwa, M. & Guerriero, J. L. Macrophage Biology and Mechanisms of Immune Suppression in Breast Cancer. *Front. Immunol.* 12, (2021).
- [22] Holtzhausen, A. *et al.* TAM Family Receptor Kinase Inhibition Reverses MDSC-Mediated Suppression and Augments Anti-PD-1 Therapy in Melanoma. *Cancer Immunol. Res.* 7, 1672–1686 (2019).
- [23] Targeting the CD47-SIRP α Innate Immune Checkpoint to Potentiate Antibody Therapy in Cancer by Neutrophils - PMC. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC9319280/>.
- [24] Wang, C. *et al.* CD300ld on neutrophils is required for tumour-driven immune suppression. *Nature* 621, 830–839 (2023).
- [25] Liu, P., Chen, G. & Zhang, J. A Review of Liposomes as a Drug Delivery System: Current Status of Approved Products, Regulatory Environments, and Future Perspectives. *Mol. Basel Switz.* 27, 1372 (2022).
- [26] Miksa, M., Komura, H., Wu, R., Shah, K. G. & Wang, P. A novel method to determine the engulfment of apoptotic cells by macrophages using pHrodo succinimidyl ester. *J. Immunol. Methods* 342, 71–77 (2009).
- [27] Huang, J. *et al.* Role of CD47 in tumor immunity: a potential target for combination therapy. *Sci. Rep.* 12, 1–11 (2022).

- [28] Morrissey, M. A., Kern, N. & Vale, R. D. CD47 Ligation Repositions the Inhibitory Receptor SIRPA to Suppress Integrin Activation and Phagocytosis. *Immunity* 53, 290-302.e6 (2020).
- [29] Zhang, W. *et al.* Advances in Anti-Tumor Treatments Targeting the CD47/SIRP α Axis. *Front. Immunol.* 11, 18 (2020).
- [30] Targeting STAT3 signaling reduces immunosuppressive myeloid cells in head and neck squamous cell carcinoma - PMC. <https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4910719/>.
- [31] Guha, P. *et al.* STAT3 inhibition induces Bax-dependent apoptosis in liver tumor myeloid-derived suppressor cells. *Oncogene* 38, 533–548 (2019).
- [32] Li, Y., Wang, C., Lu, Z. & Luo, M. Targeting PMN-MDSCs via CD300ld receptor for cancer immunotherapy. *Clin. Transl. Med.* 14, e1534 (2024).
- [33] Haga, K. *et al.* Functional receptor molecules CD300lf and CD300ld within the CD300 family enable murine noroviruses to infect cells. *Proc. Natl. Acad. Sci. U. S. A.* 113, E6248–E6255 (2016).
- [34] Next generation of immune checkpoint therapy in cancer: new developments and challenges | Journal of Hematology & Oncology | Full Text. <https://jhoonline.biomedcentral.com/articles/10.1186/s13045-018-0582-8>.
- [35] A decade of immune-checkpoint inhibitors in cancer therapy | Nature Communications. <https://www.nature.com/articles/s41467-020-17670-y>.
- [36] Ma, G.-L. & Lin, W.-F. Immune checkpoint inhibition mediated with liposomal nanomedicine for cancer therapy. *Mil. Med. Res.* 10, 20 (2023).
- [37] Wu, K. *et al.* The efficacy and safety of combination of PD-1 and CTLA-4 inhibitors: a meta-analysis. *Exp. Hematol. Oncol.* 8, 26 (2019).
- [38] Lee, M., Lee, S. Y. & Bae, Y.-S. Emerging roles of neutrophils in immune homeostasis. *BMB Rep.* 55, 473–480 (2022).
- [39] Johansson, C. & Kirsebom, F. C. M. Neutrophils in respiratory viral infections. *Mucosal Immunol.* 14, 815–827 (2021).
- [40] Myeloid cell-targeted therapies for solid tumours | Nature Reviews Immunology. <https://www.nature.com/articles/s41577-022-00737-w>.
- [41] Tumor initiation and early tumorigenesis: molecular mechanisms and interventional targets | Signal Transduction and Targeted Therapy. <https://www.nature.com/articles/s41392-024-01848-7>.
- [42] Fishbein, A., Hammock, B. D., Serhan, C. N. & Panigrahy, D. Carcinogenesis: Failure of resolution of inflammation? *Pharmacol. Ther.* 218, 107670 (2021).