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Abhijay Mudigonda March 20, 2023

Engineering Armored CAR T Cells to Secrete Functional VIPR Antagonist

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
of Emory University in partial fulfillment
of the requirements of the degree of
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Abstract

Engineering Armored CAR T Cells to Secrete Functional VIPR Antagonist

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While CAR T cells have proven to be an effective treatment option for cancers the therapy still faces many roadblocks, such as solid cancers. One such solid tumor is pancreatic ductal adenocarcinoma (PDAC), which is resistant to many treatments, and expected to increase by over two-fold in the next ten years.[25] The tumor microenvironment (TME) of PDAC is highly immunosuppressive and T cell infiltration is sparse. The VIP/VIPR axis has been studied as a targetable immune checkpoint pathway where antagonization of VIPR presents a possible strategy to increase T cell infiltration into the PDAC TME. This project presents the use of a type of armored CAR T cell that can secrete a VIP receptor (VIPR) antagonist to help retain T cell function in the immunosuppressive cancer microenvironment of PDAC. Here, we use an engineered CAR T cell that secretes the VIPR antagonist bound to Gaussia luciferase to monitor the ability of the armored CAR T cell to secrete the antagonist into the extracellular compartment as well as to validate its binding ability through measuring luminescence. Using these methods, we were able to detect Gaussia luciferase secreted into the extracellular space. We also confirmed that the binding of the VIPR antagonist to the VIPR was not disrupted by Gaussia. The peptide was also validated to be the correct size through a western blot. The results strongly support the hypothesis that our engineered armored CAR T cells can secrete the VIPR antagonist and that the antagonist can bind to T cells. With this confirmation, further studies could analyze if the secreting CAR T cells can help increase T cell infiltration in PDAC models, and Gluc can be used to monitor localization in mouse models.

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Acknowledgements

I would like to acknowledge and thank my advisor, Dr. Rafiq, for all her patience, guidance, and support throughout the honors thesis process. I would like to thank her for helping me navigate the thesis process and being not only an advisor for the thesis but for my life and career as well. I will forever be grateful for all that she has taught me. I would also like to thank my mentor, Heather Lin, who has dedicated many hours of her life to mentoring me throughout my research career in the lab. She was never too busy to help me when I needed it most, and she showed me what it means to be good scientist. I will never forget the lessons I learned under her mentorship. I would also like to thank all the members of the Rafiq lab for their collaboration, kindness, and unconditional support for me and for each other throughout our research and life.

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1. Introduction

1.1. CAR T Cells

Cancer therapy had long relied on three main pillars of effective treatment options: chemotherapy, radiation, and surgery. In the past two decades, the treatment landscape has been vastly changed by a fourth pillar: immunotherapy[1–5]. A crucial element of immunotherapy is the use of CAR T cells, which are immune cells taken out of a patient's body and engineered to express cancer-targeting chimeric antigen receptors (CAR). The CAR combines elements of antibody-like binding with T-cell-like signaling. A CAR typically consists of an antibody-like receptor that recognizes tumor antigens, a transmembrane domain, and an intracellular signaling domain[6,7]. The surface receptor of a CAR contains a binding portion that is typically a single-chain variable fragment (scFv), which is derived from the heavy and light chains of an antibody[8]. The intracellular domain includes CD3ζ, a part of a typical T-cell receptor complex[9] which in CAR T cells is fused to the CAR and a costimulatory domain such as CD28[10–12] or 4-1BB[13]. Historically, the first generation of CAR T cells did not contain a costimulatory domain and only had CD3ζ as the intracellular signaling domain[6]. However, having just CD3ζ for the intracellular domain proved problematic as it could not form a lasting T cell response[14]. The second generation of CAR T cells improved upon this design through the addition of a costimulatory domain, which amplifies the strength and duration of the signal, leading to far greater activation, proliferation, and persistence of CAR T cells[11,12]. Most CAR T cells that have shown clinical efficacy rely on the second-generation platform, but 3rd and 4th generations of T cells combining multiple costimulatory domains are currently under investigation[6] (Figure 1).

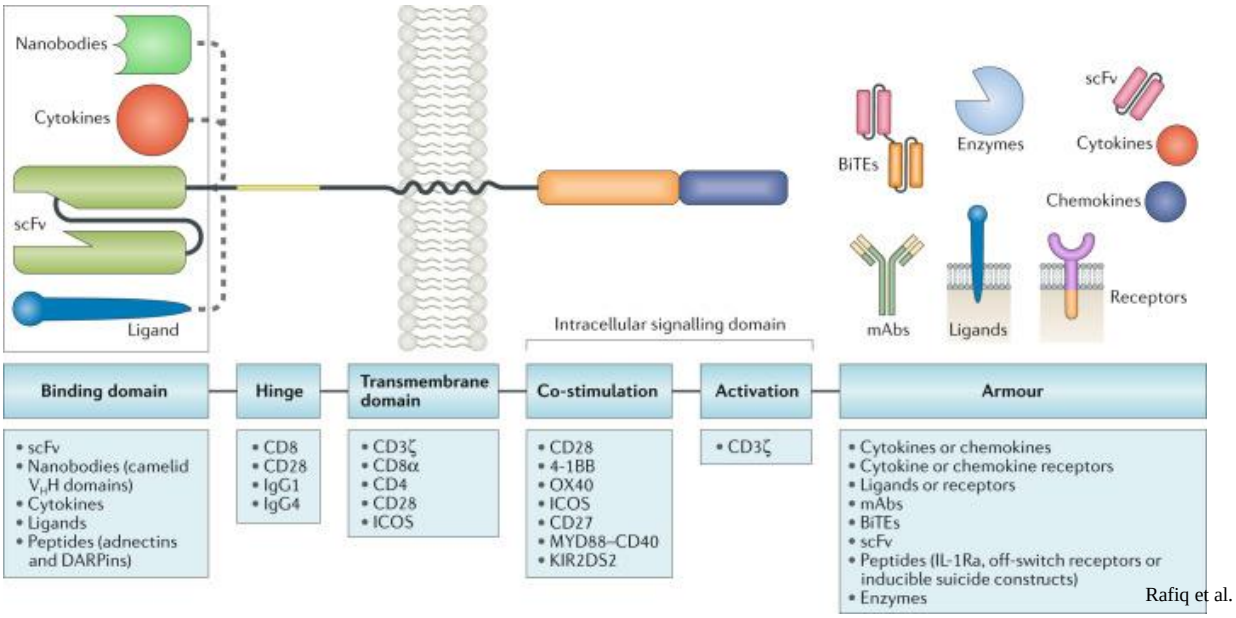


Figure 1: Outline of CAR Design

CARs are composed of multiple domains, including the binding domain, hinge, transmembrane, co-stim, activation, and armor. This figure illustrates engineering strategies used to enhance each domain.

CAR T cells have many advantages over endogenous T cells. While unmodified T cells depend on antigen-presenting cells[16], one of the advantages of CARs is their ability to recognize antigens without the need for the MHC-antigen-presenting cells[17]. Furthermore, they can recognize different types of antigens such as protein, glycolipid, or carbohydrate[18]. This allows CAR T cells to be highly specific for tumor cells[19].

CAR T cells work by binding to their target antigen that is presented on the surface of tumor cells. Binding will then result in the secretion of inflammatory cytokines which, along with the recruitment of apoptotic signaling molecules, leads to tumor cell death[5]. CAR affinity must also be carefully controlled, as too little binding will not reach the minimum threshold to induce activation, while too much binding can cause overwhelming cytokine release and immune system overactivation[20,21]. Currently CAR T cells have proven to be

clinically effective in treating hematological malignancies. An example is CD19-directed CAR T cells in the treatment of acute lymphoblastic leukemia (ALL) which target the malignant B cells[19,22]. The use of anti-CD19 CAR T cells also showed positive outcomes in the treatment of chronic lymphoid leukemia[23] and Non-Hodgkin's Lymphoma[24–26].

1.2. Challenges with Solid Tumors

Despite the success of CAR T cells in blood cancers, the therapy still faces roadblocks in usage for other types of malignancies, such as solid cancers, which account for 90% of cancer-related deaths[15]. There are a few properties of solid tumors that contribute to this difficulty. Solid cancers often lack tumor-associated antigens (TAAs) that can be targeted with tolerable toxicities; the antigens presented on these solid tumors are also presented on healthy tissue, which can lead to on-target, off-tumor toxicity (OTOT)[15,27]. The other issue with solid tumors lies in the tumor microenvironment (TME) that surrounds it. The TME is a unique environment that surrounds the proliferating tumor, altering its surroundings to facilitate tumor progression and reduce interference.[28] Among elements in the TME are immune cells, stromal cells, blood cells, and fibroblasts[29]. In some cancer types, these factors can create an environment around the tumor with extensive fibrosis and limited vascularization, making immune cell infiltration difficult[30]. When immune cells can infiltrate the TME, they are inactivated by immune checkpoint proteins expressed by the tumor[15,31]. Numerous techniques are being researched to overcome these roadblocks posed by solid tumors and the TME.

1.3. Armored CAR T Cells

One strategy to overcome the problems posed by the TME lies in armored CAR T cells (Figure 2). “Armored” refers to a CAR T cell that has been genetically engineered to express proteins that can protect it from the TME.[32] Compounds that could potentially be secreted are cytokines to promote inflammation, chemokines to direct migration, peptides or antibodies to act as antagonists, and many others.[33]

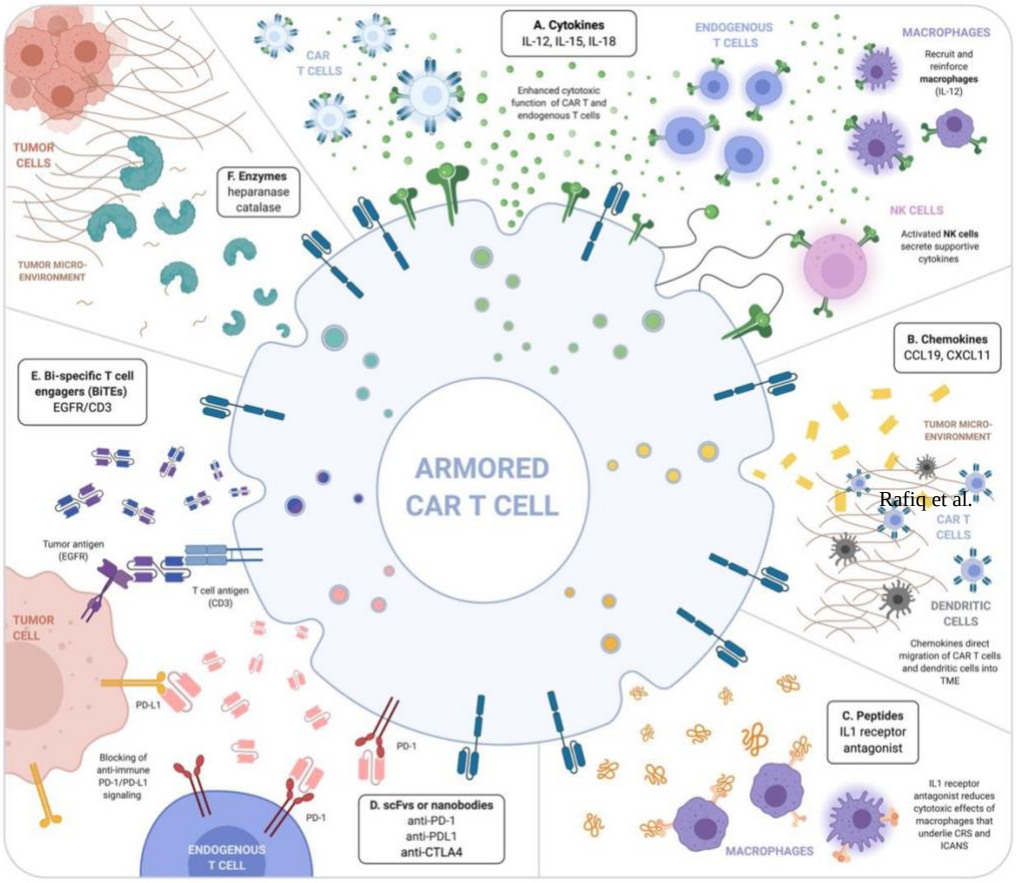


Figure 2: Armored CAR T cells can secrete compounds to modulate antitumor response.[33]

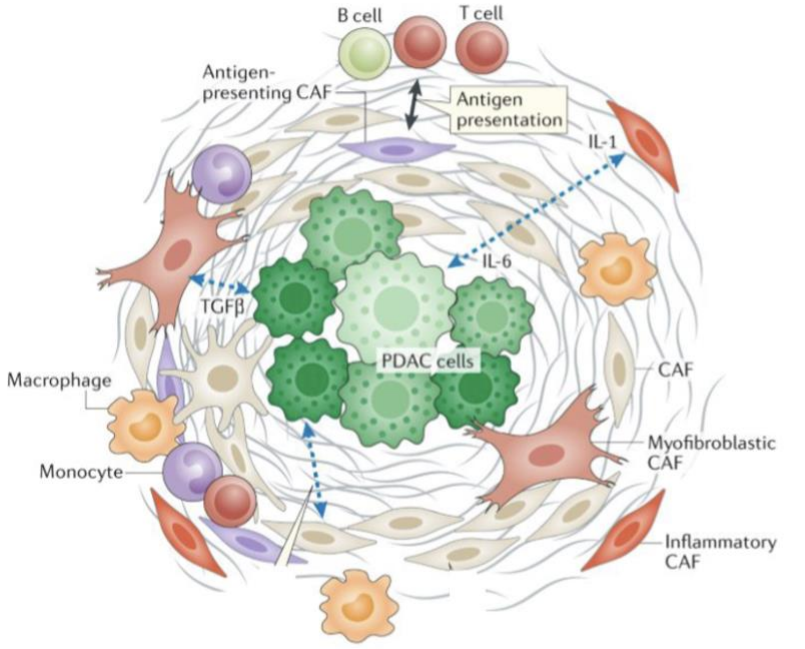
A well-studied focus in armored T cell research is the PD-1/PD-L1 system. This system is a common immune checkpoint system present in the TME of many cancer types[34]. Previous work has shown that an armored T cells can be designed to secrete an scFv

that binds to PD-L1 and consequently blocks the binding of PD-1, preventing the inactivation of the T cell by the TME[31]. PD-1 scFv-secreting armored CAR T cells provide encouraging evidence for the multifaceted uses of armored CARs that could potentially secrete peptide antagonists or antibodies to shield the cell from checkpoints pathways and significantly boost infiltration of the TME[33].

1.4. Pancreatic Cancer

Pancreatic ductal adenocarcinoma (PDAC) is responsible for more than 90% of pancreatic cancers[35]. Furthermore, PDAC is the fourth most frequent cause of cancer-related deaths in the world; data from 2012-2018 suggest a 5-year survival rate of 11.5%[36]. PDAC incidence is predicted to have more than a two-fold increase in the number of cases within the next 10 years[35]. Treatment options for PDAC are limited. The only curative option available is surgical resection, which is only possible in 10-20% of patients[35]. The other 80-90% of patients present with PDAC that has either metastasized locally or, even worse, to distant tissues.[37] First-line treatment at this point consists mainly of adjuvant and neoadjuvant chemotherapy.[35,38]

PDAC also contains a highly immunosuppressive TME[39] (Figure 3).



(Zheng et al 2020, Nat Rev Clin Oncol)

Figure 3: Tumor Microenvironment of PDAC[30]

The PDAC TME is largely composed of desmoplastic stroma, which consists of extracellular matrix and stromal cells, manly cancer-associated fibroblasts (CAFs), endothelial cells, and immune cells[40]. In extreme cases, this stroma can comprise over 90% of the tumor mass[41]. This creates a solid environment with a high interstitial fluid pressure, making perfusion of therapies and invasion of CAR T cells difficult, as well as hindering checkpoint therapy[42]. The extracellular matrix also contains dysregulated integrin subunits and Hyaluronan, which both aid cancer cell survival and growth[40,43]. While the tumor does present neoantigens[44,45], it still fails to generate an effective immune response[39]. Due to these factors, the ability to treat the disease has not significantly progressed.[39,46]

1.5. Vasoactive Intestinal Peptide (VIP) in PDAC

VIP is a 28-amino acid long neuropeptide.[47] VIP is highly conserved in many organisms, including humans and mice.[48] The peptide is present throughout the central and peripheral nervous system, as well as the digestive, respiratory, reproductive, and cardiovascular systems.[46] The main VIP receptors are VPAC1, and VPAC2 are also expressed in immune cells such as activated T cells. VIP is also produced by T cells, B cells, other immune cells, and proinflammatory cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-6 and IL-1β.[48]

Activation of VIPR on immune cells appears regulatory, as it inhibits activation and proliferation while promoting the generation of Tregs and Th2 cells.[47] VIP has been shown to decrease the clonal expansion and cytokine production of CD3/CD28-activated primary human T cells in a dose-dependent manner. It does so via cell cycle arrest, preventing exit from G_0 and G_1 into the S phase.[48] This evidence supports that VIP has an immunoregulatory function.

The overexpression of VIP and VIPR has been shown in multiple cancers and is implicated in promoting the growth and metastases of tumors.[46] Research from Ravindranathan et al. (Figure 4) shows VIP overexpressed in both human and mouse pancreatic cancer, with human PDAC patients having significantly higher blood VIP levels than healthy volunteers. In this research, inhibiting VIP signaling using a novel and potent VIPR antagonist peptide promoted T cell activation, and resulting antitumor effects were suggested to be T cell dependent[46].

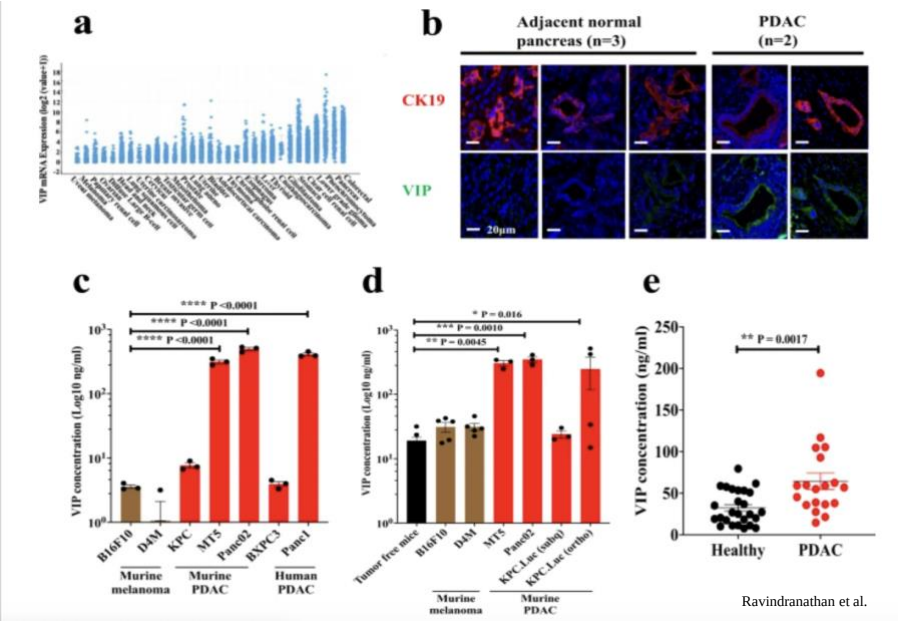


Figure 4: VIP is overexpressed in pancreatic cancer[46] .

Targeting VIP thus offers great potential to overcome the roadblocks in treatment for PDAC, as it may be able to increase immune infiltration of the tumor for further ICB treatment.

1.6. Engineering Armored CAR T Cells to Secrete VIPR Antagonist

1.6.1 Goals of the VIP Project

In this project, I investigate armored CAR T cells engineered by the Rafiq Lab that should secrete a novel and potent VIPR antagonist, which has the amino acid sequence KPRRPYTDNYTRLRKQMAVKKYLNLILN. The lab has shown that this engineered CAR T cell was functionally better than CAR T cells that do not secrete a VIPR antagonist. However, functional differences do not by themselves confirm that the peptide is secreted. Without other measures of validation, we cannot disprove the possibility that the peptide is not being secreted and instead acting on the cell from the inside. Therefore, I set out to confirm that the antagonist is secreted from CAR T cells. To achieve this goal, I used

a CAR construct that can secrete the VIPR antagonist attached to Gaussia Luciferase (Gluc). We chose Gaussia Luciferase because it does not depend on ATP for its luminescent properties. Instead, Gaussia Luciferase uses Coelenterazine as a substrate, oxidizing it into Coelenteramide and emitting luminescence at 475 nm (Figure 5). This feature allows us to detect the luciferase in the extracellular space[31].

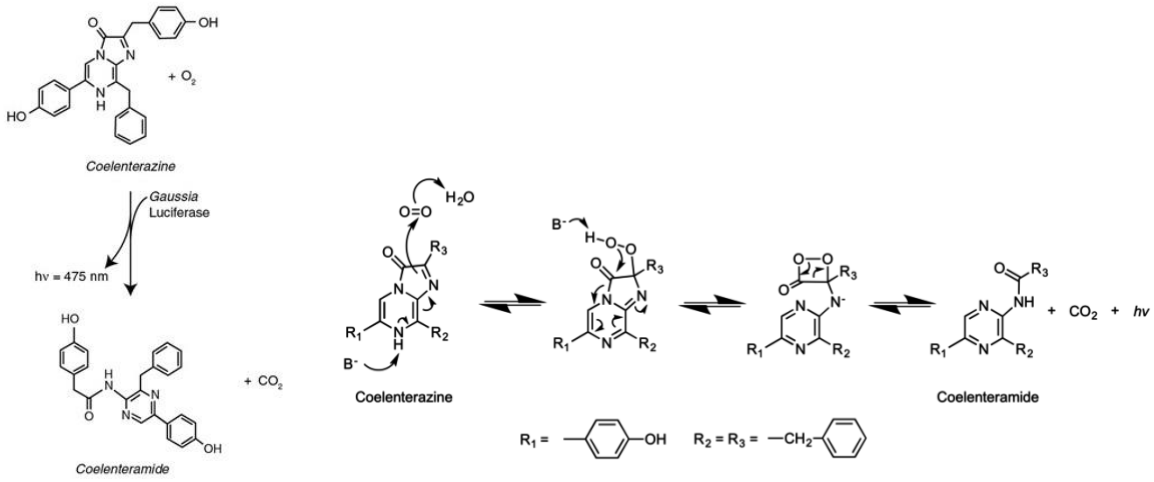


Figure 5: Reaction between Gaussia Luciferase and Coelenterazine to form Coelenteramide.[49,50]

Additionally, though the AntVIPR peptide alone, at 4kDa, is very small and challenging to visualize by western blot, Gaussia Luciferase is 18.2 kDa, allowing it to be more easily visualized (Figure 6). The antVIP-Gluc-secreting CAR construct therefore acts as a tool for the indirect confirmation of the secretion and characterization of the VIPR antagonist, as the Gluc and VIPR cell lines share all the same properties aside from genetic engineering for the addition of Gluc to the peptide.

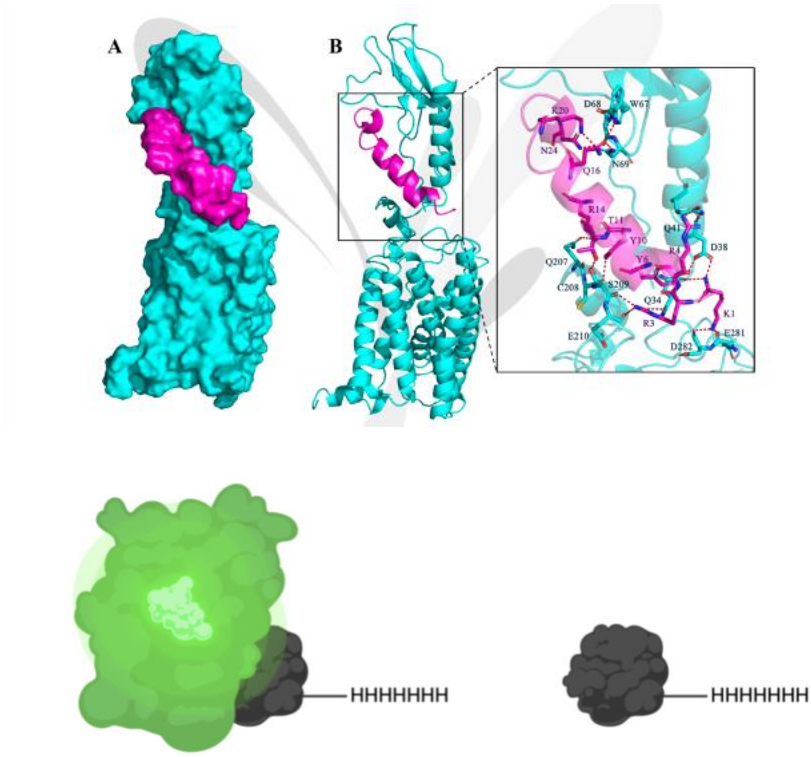


Figure 6: AntVIPR-08 structure and binding domain (top) (Waller Lab, unpublished data), AntVIPR-08-Gluc peptide size comparison (bottom) (illustrated through BioRender).

Therefore, we hypothesize that our AntVIPR-08-Gluc CAR construct secretes the functional antagonist bound to Gaussia Luciferase and that this antagonist can bind to VPAC1 or VPAC2 on T cells. The hypothesis is confirmed through two aims. The first aim is to validate through the Gaussia-expressing cell line that the antagonist is secreted. The second aim uses the same Gluc cell population to test whether the antagonist binds to T cells.

1.6.2 Designing the AntVIPR-08-Gluc Construct

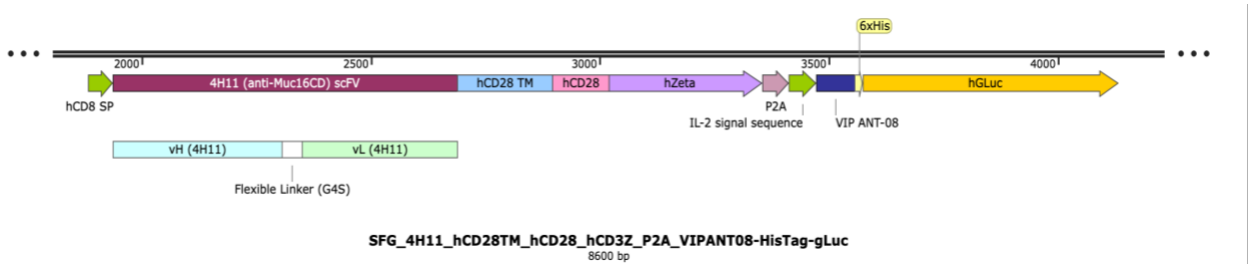


Figure 7: Map for AntVIPR-08-Gluc construct.

The AntVIPR-08-Gluc secreting CAR construct (Figure 7) was previously engineered in the Rafiq lab using techniques of molecular cloning. The construct map illustrates the DNA sequences that code for each part of the CAR. The hCD28 signal peptide (hCD8SP) sequence is derived from CD28, and it codes for the signal peptide that directs the CAR to the cell surface. Signal peptides direct the pathway of a nascent peptide through a cell, deciding the modification pathway, and, in part, the types of modifications it receives[51]. The next sequence codes for the 4H11 scFv heavy and light chains. 4H11 is specific to the antigen MUC-16, which is present on PDAC tumor cells[52,53]. The sequence is then followed by the CD28 transmembrane domain and activation domain. Finally, the sequence for the CAR codes for the hCD3z costimulatory domain and is then stopped with P2A, which is a ribosomal skipping sequence. The construct next codes for secretion using the IL-2 signal peptide sequence. IL-2 is a cytokine that is secreted from T cells that increases proliferation and activity of T and B cells[54]. Using the signal peptide sequence from IL-2 thus directs the following peptide to be secreted from the cell. This property is important, as the rest of the sequence codes for the AntVIPR peptide. The peptide sequence begins with coding for the AntVIPR peptide itself, followed by the Histidine tag

sequence, which is important for protein purification using Ni-NTA. It is finally followed by the Gaussia luciferase sequence.

To make this construct, SFG-4H1128z-antVIPR08 backbone was double digested and Gibson assembly was performed with an antVIPR08-Gluc-containing gBlock.

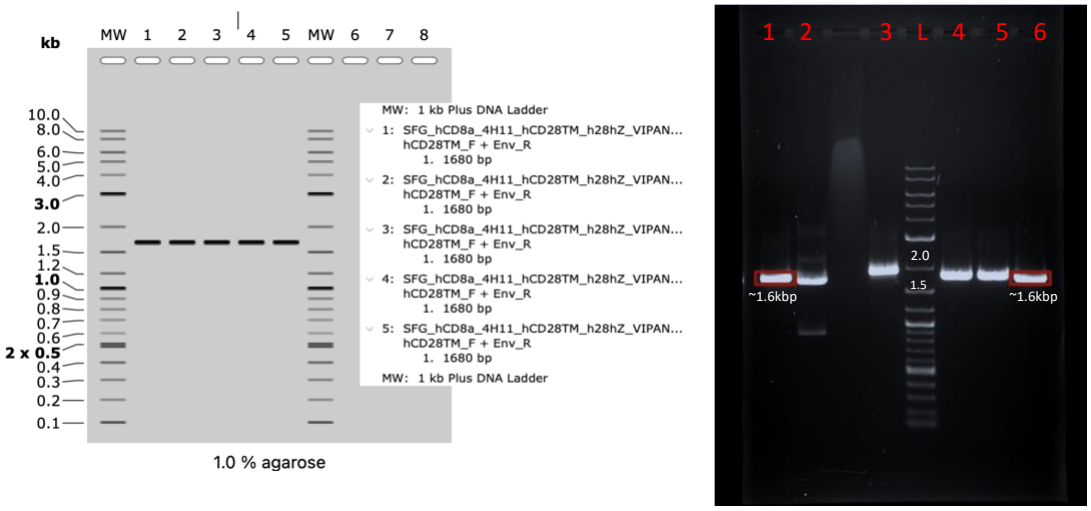


Figure 8: Gel electrophoresis results for colony PCR of the AntVIPR-08-Gluc construct, expected bands(left) and actual bands(right). Two chosen colonies for MidiPrep are indicated in red. (Data produced by Naeman Mahmood)

The ligated product was then transformed into E coli and DNA was isolated with a MidiPrep Kit. Digestion, ligation, and transformation were validated through gel electrophoresis (Figure 8). Sanger sequencing confirmed the results of which indicated that we engineered the correct sequence. With this construct, I was able to perform the following experiments.

2. Materials and Methods

2.1. Experimental Conditions

All experiments were carried out with four conditions of cell populations, generally managed with long-term 293galv9 packaging cells. The cells were split biweekly at either 1:5 or 1:10 to maintain a healthy growth phase.

- 293galv9-Empty: Construct does not encode a CAR and does not secrete compounds.
- 293galv9-4H1128z: Construct encodes a second-generation CAR. Does not secrete compounds.
- 293galv9-4H1128z-AntVIPR08: Construct encodes a second-generation CAR and secretes AntVIPR peptide.
- 293galv9-4H1128z-AntVIPR08-Gluc: Construct encodes a second-generation CAR and secretes AntVIPR peptide bound to Gaussia Luciferase.

The empty and 4H1128z conditions are intended as negative controls in the following experiments.

2.2. Transfection into H29 and Transduction into 293galv9

In the Rafiq Lab, H29 cells are used to take up a desired construct and transiently express it. For safety, constructs used contain two out of three domains necessary for retroviral production, and H29 cells contain the third domain. With a $CaCl_2$ kit, H29 cells can be transiently transfected with the plasmid to produce retrovirus that can then be used to transduce long-term 293galv9 cells[31,55].

To transfect the H29 short-term packaging cells, DNA was added to ddH_2O and vortexed with $CaCl_2$. Addition of 500uL of 2X HEPES-Buffered Saline (HBS) was added dropwise while vortexing. The mixture was incubated at room temperature for 20 minutes. Media of H29 cells was aspirated, then the prepared transfection mixture was added dropwise. 8mL DMEM+10%FBS was added on the side of the plate, and the plate was placed in an incubator overnight.

To transduce 293galv9 cells, the 293galv9s were plated on a 10cm dish. Overnight, 24 hours after plating 293galv9 cells, supernatant was collected with a 10cc syringe from the H29 plate and filtered using a 0.45μm filter into a 15mL tube. Polybrene was added at 800μg/μL. Supernatant was aspirated from the 293galv9 cells, and the filtered H29 supernatant with polybrene was added to the 293galv9s. Media was replaced on H29s. The process of filtering H29 supernatant, adding polybrene, and adding the mixture to 293galv9s was repeated after another 24 hours (t=48h). The H29s were then discarded. At t=72h, the supernatant on the 293galv9 cells was changed to DMEM+10%FBS.

2.3. Generation of CAR T Cells

To generate CAR T cells, T cells from different donors were retrieved from liquid nitrogen storage and resuspended in RPMI media at 3e6 cells/mL in a flask and activated with 2ug/ml PHA and 100IU/mL IL-2 for two days. Media on the 293galv9 packaging cells was changed from DMEM to RPMI. RPMI was used because it is standard media for T cells[56], to which the 293galv9 supernatant will be added. Days 3-5 consist of transduction of the T cells with the viral supernatant of each 293galv9 condition. 293galv9-4H1128z, 293galv9-AntVIPR-08, and 293galv9-AntVIPR-08-Gluc viral supernatants were passed

through a 0.45μm filter onto a retronectin-coated plate. Retronectin is a fragment of recombinant human fibronectin that binds T cells and increases the efficiency of retroviral transduction[57]. T cells were then collected from the flask, counted, and resuspended at 3E6cells/mL with 100IU/ml IL-2 supplementation. Then 1mL of the cells was added to each of the three supernatants on retronectin-coated plates. The plate was then "spinoculated" in a centrifuge at 3000rpm for one hour at 30C. RPMI was replaced on the 293galv9 cells, and this process was repeated on days 4 and 5. The CAR T cells were stored at 37C overnight for two more days to rest and transduction was measured on day 7. Transductions of 293galv9-4H1128z, 293galv9-AntVIPR-08, and 293galv9-AntVIPR-08-Gluc were all stained with an idiotype CAR antibody conjugated to Fluorescein Isothiocyanate (226G-FITC)[58], which targets the single chain variable fragments in our anti-Muc16 CAR. The cells were stained for one hour and compared with an untransduced condition as the negative control. Transduction was then measured on a Cytek Aurora flow cytometer.

2.4. Validating Peptide Secretion Through Luminescence Assay

Transduced T cells were generated in the following conditions: 4H1128z CAR, 4H1128z-AntVIPR-08 CAR, and 4H11-AntVIPR-08-Gluc CAR. Two replicates were run of the experiment. Cells were resuspended in RPMI in a 6-well plate at 1E6cells/well in 2mL. The plate was allowed to incubate overnight. In order to prevent cell uptake from sampling of the supernatant, the plates were then centrifuged at 1800rpm for 3 minutes. A sample of 20uL was collected and plated in 3 wells each for each condition. Condition samples

were mixed with 50uL each of 1:100 coelenterazine solution according to manufacturer's protocol. Luminescence was measured on a Biotek Synergy plate reader at 475nm.

The 4H11 CAR and AntVIPR-08 CAR are both negative controls in this assay due to their inability to luminesce in the presence of Coelentarazine. Only Gaussia Luciferase should be able to emit luminescence, so the AntVIPR-08-Gluc CAR is the experimental condition here.

2.5. Using Luminescence to Detect Binding

293galv9 packaging cells for the 4H, AntVIPR-08, and AntVIPR-08-Gluc condition were counted, resuspended in RPMI at 1E6cells/mL, and added to a 12-well plate overnight. Meanwhile, PBMCs were thawed and resuspended in RPMI, FBS, and IL-2 overnight. At t=24, supernatant from each condition was added respectively to each well of T cells and allowed to incubate for 1 hour. The cells were then collected in FACS tubes and washed 3x with 2mL PBS through centrifugation at 1800rpm for 3 minutes. T-cell pellets were then resuspended in 60µL RPMI. Afterward, each condition was added to 3 wells of a 96-well plate, then mixed with the coelenterazine working solution to be measured for luminescence on a Biotek Synergy plate reader. Luminescence was measured at t=5d.

This experiment is designed to test binding through luminescence. Since the Gaussia Luciferase is attached to the AntVIPR-08 peptide, if the peptide is able to bind to the VPAC receptors on T cells, then it should luminesce through the reduction of coelenterazine. The triple washing step ensures that any Gaussia Luciferase in the supernatant is completely washed off so that the only luminescence measured must come from AntVIPR-08-Gluc bound to T cells in the media. The AntVIPR-08 and 4H cells should

not luminesce as they do not have Gaussia. The experimental population is then the AntVIPR-08-Gluc, which will only luminesce if it is bound to T cells through the attached AntVIPR-08.

2.6. Nickel Purification and Western Blot

Supernatant was collected from empty, AntVIPR-08, and AntVIPR-08-Gluc 293galv9 cells. Both AntVIPR-08 and AntVIPR-08-Gluc proteins are fused to a His tag, allowing for binding to Nickel. Nickel bead slurry was washed on a in three tubes on a magnetic stand through rinses with equilibration buffer, containing $NaPO_4$, NaCl, Tween-20, and 30mM Imidazole. Each supernatant was then added to each of the three tubes of nickel bead slurry and diluted with equilibration buffer. The solution was then vortexed and incubated with the bead slurry for one hour. Following the incubation, the protein-bead solution was washed two times with wash buffer, containing the same ingredients as equilibration buffer but instead with 50mM Imidazole. Protein was then eluted twice using elution buffer, which contains $NaPO_4$, NaCl, and 250mM Imidazole.

Purification was followed by western blot. For each population, 30uL of eluted protein solution was loaded in the gel, flanked by 15uL of Kaleidoscope ladder on each side, and Posi-tag opposite of one ladder. The gel was run for 1 hour at 100 volts. After electrophoresis, the gel was transferred to a PVDF membrane over 1 hour at 50 volts at 4C and blocked overnight in 5% blocking buffer. After blocking, the blot was incubated in 1:10000 rabbit anti-His antibody overnight, then incubated with 1:5000 HRP-conjugated goat anti-rabbit antibody. The antibody was detected using Pierce ECL western blot substrate[59]. A solution was made using 2mL of 1:1 peroxide solution and luminol

enhancer, in which the blot was incubated for two minutes. The blot was then removed, placed in an Xray cassette, and imaged manually for 5 minutes.

The western blot contained 293galv9-AntVIPR-08 supernatant as a negative control. While the VIPR line should have the antagonist in the supernatant, which contains a His tag and can be purified through the nickel beads and magnetic stand, the protein is expected to be too small to appear on a western blot, weighing about 4kDa. Gaussia Luciferase is larger, at 18.3kDa, so a band should be expected at around 26kDa with the added weight of AntVIPR-08 and His-tag. Thus, the 293galv9-AntVIPR-08-Gluc supernatant acts as the experimental condition in this experiment, with a correctly sized band confirming the correct compound is being secreted.

3. RESULTS

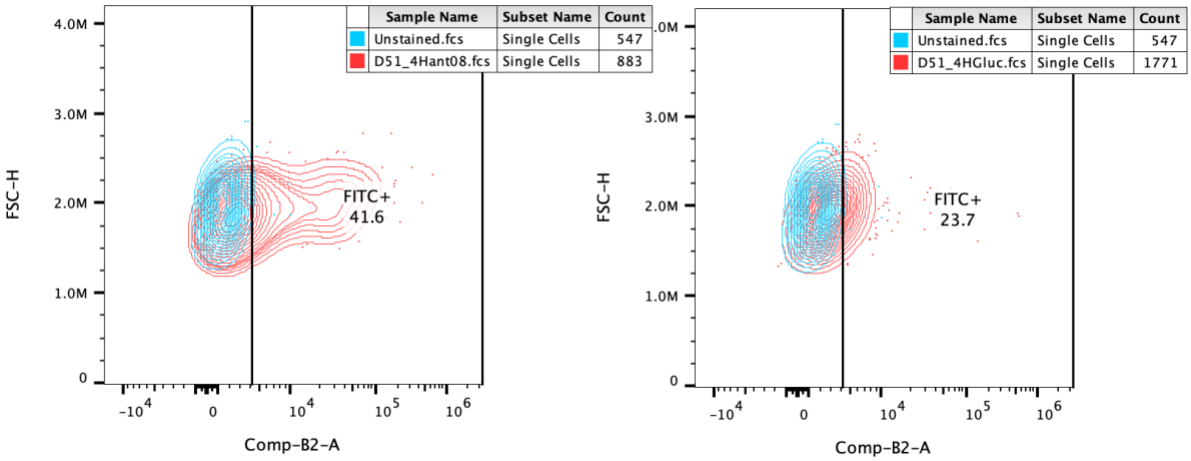


Figure 9: AntVIPR-08 (left) and AntVIPR-08-Gluc (right) constructs transduce T cells.

The transduction of 4H1128z-AntVIPR-08 and 4H1128z-AntVIPR-08-Gluc conditions (red; Figure 9), overlaid on an untransduced population (blue; Figure 9). Both conditions

showed FITC+ cells compared to the negative control. This data supports that 293galv9 constructs were successful in transducing human primary T cells.

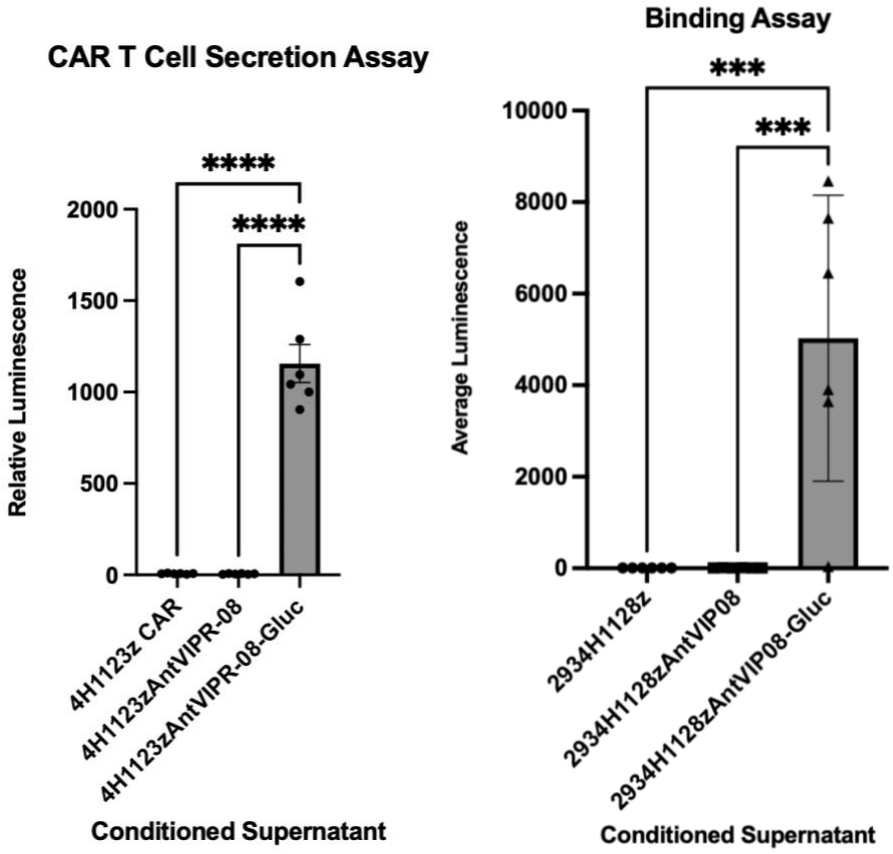


Figure 10: AntVIPR-Gluc antagonist is secreted by 4H1128z-AntVIPR-08-Gluc CAR T cells and binds to other T cells.

For the secretion assay (Figure 10, left), each sample was plated in three technical replicates. Two CAR T cell biological donors were run (n=2) for the experiment. Therefore, a total of 6 data points is plotted. The luminescence of 4H1128z-AntVIPR-08-Gluc is statistically significantly higher than both 4H1128z and 4H1128z-AntVIPR-08, suggesting that there is a larger amount of Gaussia Luciferase present in the extracellular matrix.

The binding assay (Figure 10, right) was also plated in 3 technical replicates each, for two biological replicates. 4H1128z-AntVIPR-08-Gluc binding condition had greater

luminescence than the both 4H1128z and 4H1128z-AntVIPR-08 supernatant, suggesting Gluc was bound to T cells in the sample.

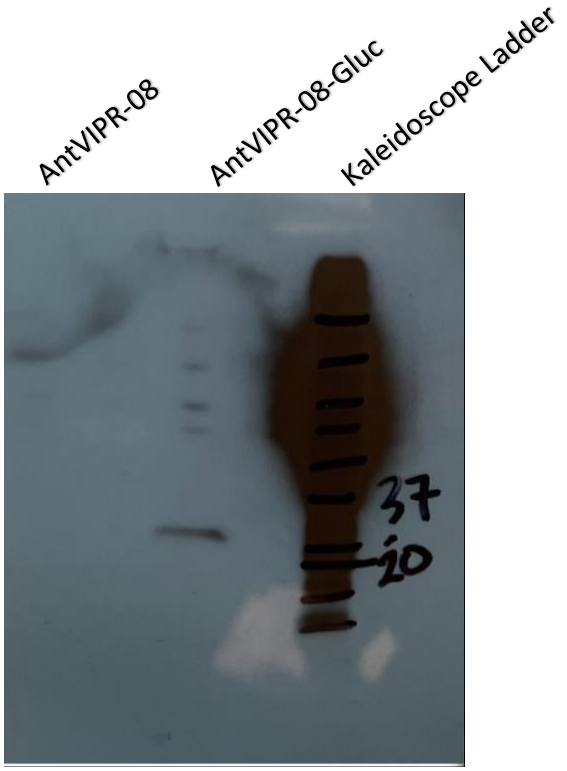


Figure 11: Western blot bands indicate peptide of the same expected weight as AntVIPR-08-Gluc.

Samples for western blot was electrophoresed on a precast gradient gel from 4-25% to optimize separation of proteins, as increasing polyacrylamide concentrations in a gel allow for more precise detection of small proteins[60]. The expected size of Gaussia Luciferase is 18.3kDa, AntVIPR-08 is 4kDa, and AntVIPR-08-Gluc is 24kDa.

4. Discussion

4.1. Transduction of CAR T cells

The transduction results showed a positive population for FITC on both AntVIPR-08 and AntVIPR-08-Gluc conditions. For a transduction, we use an untransduced negative control to observe the baseline reading of fluorescence. Furthermore, one can quantify transduction by taking a percentage of cells that exceed the negative control's reading over the cells that do not. Any fluorescence reading exceeding the negative control's measurement suggests a binding of 226G-FITC to CARs on transduced cell surfaces. If no reading is observed that exceeds that of the unstained, it would mean that transduction did not occur. Here, both AntVIPR-08 and AntVIPR-08-Gluc show higher measurements of FITC than the untransduced control. Therefore, the data suggests that the construct can transduce T cells, and the binding domain is detectable on the surface of the CAR. While the transduction was slightly lower for the AntVIPR-08-Gluc condition, this is likely the result of the AntVIPR-08-Gluc cell population being a newer line that was not single-cell cloned, which initially demonstrated difficulties in adhering and proliferating during the time of CAR T cell production. This issue was likely coupled with the inherently lower capabilities of newer cell lines to transduce CAR T cells. Future measurements will be performed over time to check for improved transduction in the AntVIPR-08-Gluc cell line. Furthermore, the transduction was only run on one donor. Primary T cells can vary in their ability to be transduced by retroviruses from 293galv9 cells due to biological differences.

Running transductions of 293galv9-AntVIPR-08-Gluc supernatant with more than one donor at once may provide a better idea of the ability of the construct to transduce cells.

4.2. Luminescence Assays

The luminescence assay measurements found higher luminescence in the AntVIPR-08-Gluc condition for the secretion assay. The luminescence assays served as a first step to indirectly validate the secretion and binding of the AntVIPR peptide. In measuring luminescence of the supernatant of CAR T cells, luminescence is not expected in 293galv9-AntVIPR-08 or 293galv9-4H1128z supernatants. Presence of luminescence in these conditions would most likely be related to experimenter error: either the negative control would have been transduced with the AntVIPR-08-Gluc construct, or supernatant from the AntVIPR-08-Gluc condition could have contaminated the negative control supernatant. In the inverse scenario, if no luminescence is detected in the AntVIPR-08-Gluc supernatant, it would suggest that the cells do not secrete Gaussia luciferase conjugated to AntVIPR-08 peptide. In the secretion assay, there was a significant difference in luminescence between the Gluc condition and the two other conditions. This difference indicates that the CAR T cells that we engineered successfully secrete the AntVIPR-08 peptide attached to Gaussia Luciferase. A possible limitation exists where Gluc-containing cells could be taken up along with the supernatant, which would provide a false positive for the presence of Gluc in the supernatant. However, the possibility of this circumstance occurring was reduced through the centrifugation step prior to sampling the supernatant. All cells should be collected at the bottom of the plate post-centrifugation,

meaning sampled supernatant would contain only AntVIPR-08-Gluc peptide. Only two donors were used for the luminescence assay, so a future goal is to run this experiment with more donors to increase the number of replicates. Furthermore, in addition to centrifugation step prior to sampling the supernatant, we can optimize this experiment by using a 0.45 µm filter to further ensure the removal of any possible cells after sampling the supernatant.

Though the experiment provides evidence about the ability of the cells to secrete AntVIPR-08-Gluc, further information that will help characterize the cells and the peptide for future therapeutic use is still needed. Answers are still required for the rate at which the peptide is secreted, as well as structural stability and rate of degradation. Both values are important to understand how to maintain specific concentrations of antagonist in the extracellular matrix. In a secretion assay, cells could be plated in fresh media and measured at intervals for concentration of AntVIPR-08-Gluc at each time point. For a degradation assay, supernatant containing AntVIPR-08-Gluc can be checked at specific time intervals to measure at what rate the concentration of protein in the supernatant decreases. One way to find these answers is to sample the supernatant at the time points and run them through luminescence assays to check for increasing/decreasing luminescence. However, using a luminescence assay can make it difficult to accurately measure concentration based off luminescence, unless one were to create a standard curve to correlate concentration from luminescence. Furthermore, in measuring degradation, there is a possibility that AntVIPR-08 could be cleaved from Gluc or degrade earlier, which would cause luminescence to appear in the absence of functional peptide. This possibility is due to the likely presence of proteases in tissue culture media[61]. A

western blot will also allow for visualization of increases or decreases in protein concentration through the strength of the bands. However, a more direct measurement could be made by liquid chromatography-mass spectrometry (LCMS). LCMS allows for separation of multiple compounds from a sample through a mobile phase and stationary phase which are opposite in polarity.[62] The separation of the molecules is based on their differing strength of adherence to the stationary phase versus the mobile phase. As the molecules are separated, the machine also runs them through a mass spectrometer.[62] Using LCMS allows for more accurately measuring concentrations through the intensity of the measured peak associated with AntVIPR-08-Gluc. Secretion can be measured through the increase in peak size at each time point, while degradation can be measured through the decrease of the peak size. The mass measurement for the peak allows monitoring for whether the peptide remains intact or separates from Gluc, leading to a more accurate rate of degradation measurement.

4.3. Binding Assay

The luminescence measurements for the binding assay showed significantly higher luminescence for the AntVIPR-08-Gluc condition versus the negative controls. The experiment performed here incubated 293galv9 supernatants with T cells for binding of secreted AntVIPR. The reason 293galv9 cells were chosen over CAR T cells was because of the high volume of cells and secreted compounds in the supernatant. CAR T cells were lower in number due to lower transduction and would not have likely secreted as large of a quantity of VIPR antagonist as the much more proliferated 293galv9 cells. However, inherent to the 293galv9 cells is their ability to also secrete retrovirus to

transduce T cells. While the incubation time was relatively short at 1 hour and did not formally follow the process for manufacturing CAR T cells, there is the possibility that some T cells could have been transduced through retrovirus in the supernatants. The hypothetical transduction would then cause the T cells themselves to manufacture AntVIPR-Gluc, which could cause luminescence leading to a false positive. The magnitude of luminescence in the experiment was large enough that this is unlikely. However, to fully control for any false-positive reading, future experiments should incorporate a reverse transcriptase inhibitor during the incubation step to ensure that no transduction is occurring during the binding step. Further checks to prevent false positive measurements can be taken by centrifuging the cell plates post-incubation to ensure no Gluc-producing cells are taken up with the supernatant. Additionally using a 0.45μm filter with a syringe can most significantly reduce the likelihood of any cells present in measuring of supernatant. Future experiments will also measure luminescence immediately after binding rather than at t=5d.

The binding assay allowed T cells to incubate with the supernatants of 293galv9-4H1128z, 293galv9-AntVIPR-08, and 293galv9-AntVIPR-08-Gluc. By washing away the supernatant with PBS after the incubation period, it was ensured that the only Gaussia species that remain should be bound through the attached AntVIPR to the VIP receptors on the T cells. This experiment accomplishes two goals. If luminescence were detected, it would suggest that AntVIPR-08 can bind to VIPR on T cells, and it also means that Gluc does not significantly hinder the binding interaction between AntVIPR and VIPR. Otherwise, if the Gluc condition does not show luminescence, it could mean that either the antagonist does not bind to T cells or Gaussia luciferase could be hindering the

binding interaction between the antagonist and VIPR on T cells. Structural assays through programs like Alphafold could help to investigate any such complications related to lack of binding. The AntVIPR-08 and 4H11 supernatants both should not lead to luminescence because 293galv9-4H1128z cells do not secrete any compounds, and 293galv9-AntVIPR-08 secretes AntVIPR without Gluc. If there were to be any luminescence on these controls, it is likely contributed to experimenter error that would have let AntVIPR-08 Gluc supernatant contact the negative controls. Because a significantly higher luminescence was observed on the Gaussia condition over the other two conditions, it suggests that binding occurred between secreted AntVIPR and VIPR on the T cells. However, binding of the AntVIPR-08 peptide to the VIPR does not necessarily mean that the peptide can still functionally antagonize the VIPR. The functionality can be tested through a CD69 assay to view T cell activation in the presence of the VIPR antagonists.

4.4. Western Blot

Using western blot is a supplemental method to confirm that the compound being secreted is AntVIPR-Gluc. While it can be indirectly detected in the supernatant using luminescence, visualizing Gaussia luciferase does not equate to visualizing the entire peptide. However, using western blot can help to validate that it is specifically Gaussia Luciferase bound to AntVIPR that is present in the supernatant and is secreted by the cells. Furthermore, using Ni-NTA coated magnetic beads for purification allowed for maximizing concentration of all AntVIPR-Gluc peptides in the supernatant. The total weight of the peptide is approximately 24kDa, which can be visualized on the lower end of the gradient gel used for the western blot. If no AntVIPR-08-Gluc is visualized on the

gel, it may suggest that it is secreted in very small amounts or very slowly. It could also point to experimenter error in the purification step. If bands other than 24kDa are visualized either in the experimental condition or the two negative controls, it may suggest contamination with other proteins, or errors during the magnetic bead purification protocol. We would also be able to visualize whether the AntVIPR-08-Gluc is separated from the antagonist, as the band would appear below 20 kDa. This western blot showed a strong band present at ~24kDa, which is the expected size of the AntVIPR-Gluc peptide. More replicates of the western blot will be needed to demonstrate reproducibility that the correct protein is being secreted and is present in the supernatant. The combination of evidence from the western blot results alongside the luminescence assays is a strong indicator that the AntVIPR-08-Gluc CAR T cells and 293galv9 cells both secrete the VIPR antagonist bound to Gaussia luciferase, and that this antagonist stays conjugated with Gaussia post-secretion and during binding.

4.5. Future Directions

The experiments provide positive support for the hypothesis that AntVIPR-Gluc can be detected through luminescence assays in both the extracellular matrix and bound to receptors. An even more promising finding is the observation that AntVIPR can bind to the receptors even while conjugated to Gaussia luciferase. This property opens new doors of versatility for the usage of our AntVIPR-Gluc peptide. In future experiments, CAR T cells can be tested not only for functionality in mice, but now also for localization. Using luminescence from Gaussia luciferase, we can visualize where in the body AntVIPR-08-Gluc CAR T cells are acting through measuring luminescence intensities of secreted

peptide antagonist. These experiments can allow us to check for potential on-target off-tumor toxicities, as we want targeting only of tumor wmass. The ability to monitor localization is a useful tool to fine-tune the design of our secreting CAR T cell to more accurately target the PDAC TME.

5. Conclusion

The transduction data suggests that CAR T cells can indeed be transduced with the AntVIPR-08-Gluc construct, and the luminescence assays suggest that the AntVIPR-08-Gluc peptide is both secreted by CAR T cells and binds to the VIPR on T cells. The western blot further helped to characterize the peptide. The two goals of this experiment were to validate through the AntVIPR-08-Gluc condition that the antagonist is secreted by engineered cells and that it is capable of binding to the target receptors. The experimental data strongly supports the hypothesis that both events occur, and that the correct peptide is being secreted from the engineered cells. Future experiments may take these results to the next stage and use Gaussia luciferase to better understand in-vivo targeting of the AntVIPR-08-Gluc CAR T cells.

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