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Jojo Liu April 10, 2024

Immunological Impacts of VIP and PDL1 Double Knockout in Mice

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a thesis submitted to the Faculty of Emory College of Arts and Sciences
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Abstract

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Vasoactive intestinal peptide (VIP) is an immunosuppressive protein overexpressed by pancreatic adenocarcinoma (PDAC) that suppresses T-cell responses. Combination treatment inhibiting both the VIP pathway and the protein death ligand-1 (PDL1) pathway has been shown to generate synergistic anticancer responses. To study the mechanistic basis for the synergy observed targeting VIP and PDL1 signaling, this study generates VIP PDL1 double knockout (DKO) mice and characterizes their immunology. Previous DKO mice targeting two immune checkpoints have resulted in compromised immune tolerance and led to autoimmune-related deaths. However, this study generated viable and healthy VIP PDL1 DKO by crossing VIP KO and PDL1 KO mice with a modulated immune system at ratios consistent with Mendelian frequencies and no autoimmune-related lethality. Under normal physiological conditions, we did not observe evidence of autoimmunity in the VIP PDL1 DKO mice. We then tested their ability to reject a transplantable PDAC cell line. The VIP PDL1 DKO had heightened anti-cancer responses with better survival and slower tumor growth rates when compared to single knockouts. In conclusion, these finding gives rationale to target both the VIP and PDL1 pathways as a potent therapeutic approach against PDAC with limited off-cancer toxicity.

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Introduction

A broad spectrum of genetic and epigenetic alterations characterizes cancers with diverse antigens that the immune system recognizes to differentiate tumor cells from normal cells. The costimulatory and inhibitory immune checkpoint signaling regulates the amplitude and quality of antigen-specific T-cell responses. Under normal physiological conditions, immune checkpoints preserve self-tolerance to prevent autoimmunity[1]. However, cancers upregulate the expression of immune checkpoint proteins to suppress and evade the immune system. We aim to understand better how T cells regulated by immune checkpoint pathways can be targeted by drugs that block signaling. Immune checkpoint blockade (ICB) uses monoclonal antibodies to inhibit immune checkpoint molecules. The clinical efficacy of ICB has demonstrated the power of reinvigorating cancer-exhausted immune systems and has revolutionized cancer therapy[2]. The Food and Drug Administration has already approved antibody-based drugs targeting the checkpoint molecules programmed cell death 1 (PD-1), programmed death ligand-1 (PDL1), and cytotoxic T lymphocyte-associated protein 4 (CTLA-4) for numerous malignancies[3].

Pancreatic ductal adenocarcinoma (PDAC) is the 3rd leading cause of cancer death in the United States[4]. The five-year survival rate of PDAC is 13%[5]. Although currently approved ICBs targeting the PDL1/PD-1 and CTLA-4 pathways bring hope to a multitude of cancers, PDAC has an immune desert tumor microenvironment with a paucity of effector T cells and consequently remains highly resistant to current ICBs. Thus, a novel treatment beyond current ICBs is urgently needed for PDAC. VIP, a 28-amino acid neuropeptide, is proposed to be a novel immune checkpoint molecule and is upregulated in PDAC tissue. Indeed, our lab found that PDAC patients have higher VIP levels in plasma than healthy volunteers and biopsies of PDAC show VIP expression in the cancer cells[6]. Previously, targeting PDL1, expressed by cancer cells, and its receptor PD-1, expressed by T cells, has shown low efficacy due to low PDL1 expression in PDAC. However, preclinical data from our lab revealed that PD-1 antibodies (anti-PD1), combined with VIP-R antagonist peptides, demonstrated synergistic T cell-dependent anti-cancer responses in murine models of PDAC[6]. The combination treatment of anti-PD-1 and VIP-R antagonists inhibited T cell exhaustion and tumor growth in PDAC murine models. Significantly, 30% of these mice became tumor-free and resisted tumor rechallenge, suggesting the development of immunological memory against PDAC.

Studies of knockout (KO) mice, in which a gene of interest is functionally deleted with genetic engineering, have revolutionized our understanding of immunology and were critical in immunotherapy development[7]. Immune checkpoints are key in regulation against autoimmunity and maintenance of self-tolerance. Knocking out a single immune checkpoint gene (i.e., either PD-1, PDL1. or VIP) did not lead to significant autoimmunity in KO mice[8–10]. However, when two immune checkpoint genes (e.g., PD-1 and lymphocyte-activation gene 3, LAG3) were knocked out, autoimmunity developed in double knockout (DKO) mice[11]. Up to now, no one has studied the immune response in PDL1 and VIP DKO mice.

To understand the mechanistic basis for VIP and PDL1 immunological synergy, this study generates VIP PDL1 double knockout (DKO) mice and characterizes their immunology, both under normal physiological conditions and in response to a transplantable PDAC cell line. We hypothesized that the complete genetic disruption of both the VIP and PDL1 pathways in DKO mice will lead to autoimmunity and increased intra utero death. We further hypothesized that the viable DKO mice would have clinical evidence for autoimmunity, with growth retardation, skin changes, and/or diarrhea. Finally, we predicted that DKO mice challenged with pancreatic cancer, will generate a greater anti-cancer response, in terms of survival and tumor growth rate, when compared to single knockouts or wild-type mice. Along with understanding the fundamental impact of disrupting both PDL1 and VIP pathways, this study examines the potential toxicity of genetically disrupting both pathways. The outcome of this project will assist the conduct of clinical trials of anti-PD-1/VIP-R antagonist combination therapy.

Methods

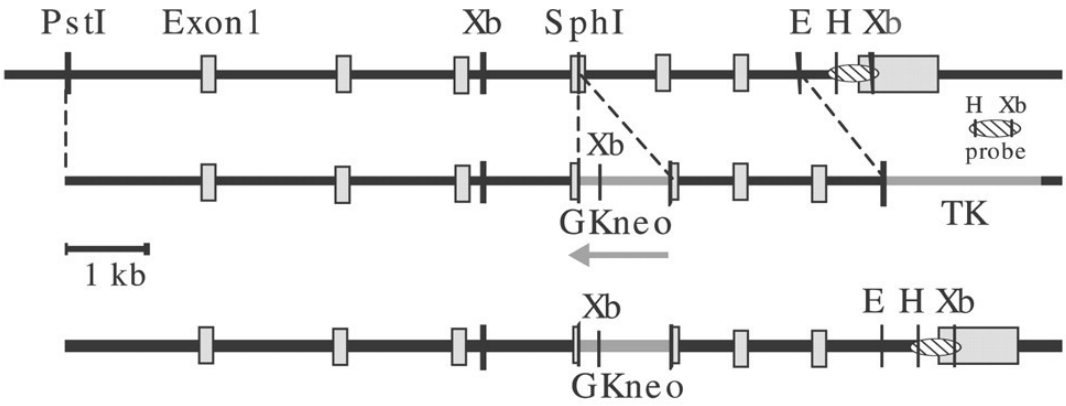


Figure 1. Gene-disruption strategy and generation of VIP KO mice. The upper portion depicts the normal VIP gene, with seven exons represented by boxes. In the modification construct (middle), the neomycin cassette was inserted in reverse orientation just before the PHI-encoding sequences on exon 4. Exon 5 contains the VIP encoding. The lower segment illustrates the intended gene structure after homologous recombination. The abbreviations used are Xb for XbaI, E for EcoRI, H for HindIII, GKneo for pPGK neo bpA, and TK for pIC19R/MC-1-TK. Adapted from Cowell et al. 2003, American Psychological Society. The original figure is entitled "Figure 1. Vasoactive intestinal peptide (VIP)/peptide histidine isoleucine (PHI) gene-disruption strategy and confirmation of the targeted mutation in mice"[12].

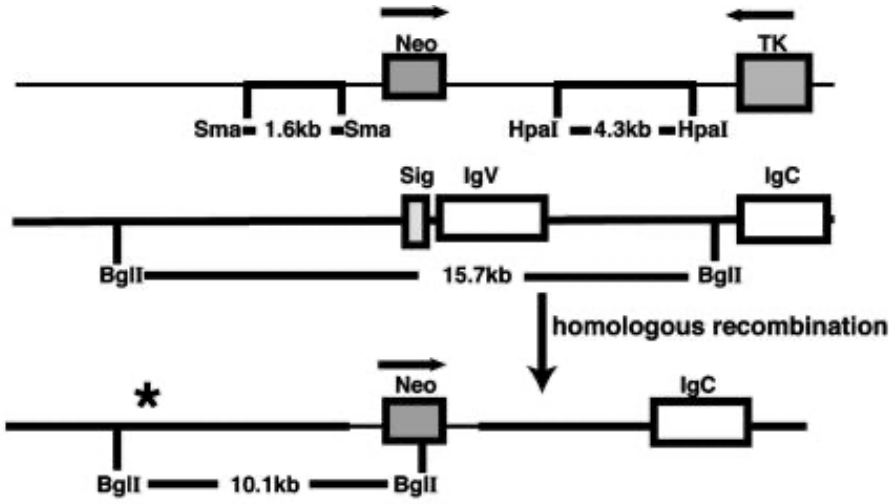


Figure 2. Gene disruption strategy and generation of PDL1 KO mice. The PDL1-targeting vector's arrangement is depicted above (Top), with Neo substituting the signal exon and the IgV region (C57BL/6 design). The PDL1 gene's genomic layout is displayed (Middle), with exons represented by open boxes (not to scale). The bottom section illustrates the homologous recombination of the PDL1 gene. The asterisk (*) marks the position of the probe. Adapted from Latchman et al. 2004, PNAS. The original figure is entitled “Fig 1. Generation of PD-L1 -/- mice”[9].

Mice. PDL1 KO mice were received as a generous gift from Dr. Sharp (Harvard Medical School, Boston, Massachusetts) and previously described[9]. Homologous recombination disrupted the PDL1 gene to generate KO mice (Figure 1). VIP KO mice were kept in the Waller Lab (Winship Cancer Institute, Atlanta, Georgia) and previously described[12]. Homologous recombination was used to disrupt the VIP gene to generate KO mice (Figure 2). VIP KO mice were bred with PDL1 KO mice to generate double heterozygous VIP PDL1 mice at an expected 100% frequency. Double heterozygous VIP PDL1 mice were then bred with double heterozygous VIP PDL1 mice to generate VIP PDL1 DKO mice at a 6.25% frequency[13].

Genotyping. DNA was first extracted from the tail sample with DNeasy Blood and Tissue Kit (Qiagen) to genotype the mice. VIP and PDL1 were then amplified with specific primers using PCR, and products were separated via gel electrophoresis. Primer pairs were used to amplify PDL1 and Neo. PDL1 primers to detect PDL1 were 5’-CTAACAG-GTGATCCGTTTCCTATG-3’ and 5’-GCCGTGATAGTAAACGCTGAA-3’. Sequences of the Neo primers were 5’-ATTGAACAAGATGGATTGCAC-3’ and 5’-CGTCCAGA-TCATCCTGATC-3’. PCR products were 305 bp for PDL1 and 474 bp for Neo[9]. A triple primer PCR was used for VIP genotyping. The primers were 5’-TTTCAAGGTGTGGG-GCTAGAGACATACA-3’, 5-TTACCTGATTCGTTTGCCAATGAGTGAC-3’, 5’-GCCCGGA GATGAGGAAGAGGAGAACAG-3’. PCR products for the mutant band was 708 bp, and the wildtype band was 318 bp[12].

Automatic Cell Counter. Peripheral blood was collected via cheek bleeding[14]. The tubes used for collection contained drops of heparin. White blood cell counts, red blood cell counts, hemoglobin counts, and platelet counts were analyzed with the DxH 500 Hematology Analyzer (Beckman Coulter).

Pancreatic Cancer Cell Line. KPC.Luc cells were used to model pancreatic cancer and were received as a generous gift from Dr. Logsdon (MD Anderson Cancer Center, Houston, Texas). KPC.Luc cells were cultured in 1X Dulbecco's modified eagle medium, 10% fetal bovine serum, and 1% penicillin-streptomycin in an incubator with 5% CO2 at 37°C[6].

In Vivo Tumor Burden and Survival Study. 0.5 million KPC.Luc in 50 µL of PBS was injected subcutaneously into the right flank of male C57BL/6, VIP KO, PDL1 KO, and VIP PDL1 DKO mice. 6 days after the initial injection, tumor volume, weight, and survival were monitored twice a week. Tumor dimensions were measured with Vernier calipers and tumor volume was calculated with the formula tumor volume – ½ (length x width x height). Mice with tumor volumes of $\geq$ 500 mm^3 were euthanized and recorded as deceased for the survival curve[6].

Results

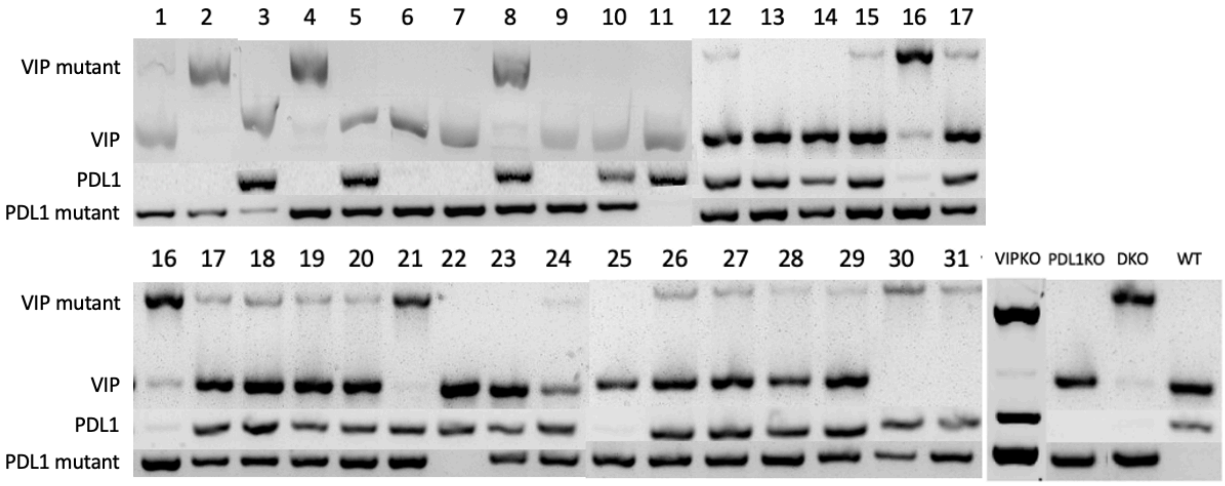


Figure 3. PCR gel electrophoresis for the progeny of VIP PDL1 double heterozygous breeding. Gels are rearranged for presentation. PDL1 band is at 305 bp and PDL1 mutant band is at 474 bp. VIP band is at 318 bp, and the VIP mutant is 708 bp. 31 mice were genotyped.

Genotype	Total	Actual %	Theoretical %
VIP -/- PDL1 -/-	**3**	**9.68**	**6.25**
VIP +/- PDL1 -/-	1	3.23	12.50
VIP +/+ PDL1 +/-	6	19.35	12.50
VIP +/+ PDL1 -/-	4	12.90	6.25
VIP +/+ PDL1 +/+	2	6.45	6.25
VIP +/- PDL1 +/- VIP +/- PDL1 +/+	11	35.48	37.50
VIP -/- PDL1 +/- VIP -/- PDL1 +/+	4	12.90	18.75

Figure 4. Summary of genotype frequency for progeny generated from crossing VIP PDL1 double heterozygous mice. VIP +/- PDL1 +/- and VIP +/- PDL1 +/+ frequencies are combined. VIP -/- PDL1 +/- VIP -/- and PDL1 +/+ are also combined.

Previous breeding showed that VIP KO and PDL1 KO mice produced viable and fertile offspring. When crossing VIP KO with PDL1 KO, double heterozygous progeny for both VIP and PDL1 were observed to be viable and at a Mendelian frequency of 100%. Double heterozygous VIP PDL1 mice produced progeny reflective of classical Mendelian rates. Among the initial 31 offspring, 2 healthy males and 1 healthy female DKO were generated, showing that the genetic deletion of VIP and PDL1 simultaneously does not impair the generation of progeny (Figures 3 and 4). Further genotyping is currently being done to show statistical significance.

Figure 5. Four out of five VIP PDL1 DKO show hair loss below neck at weaning age.

The DKO generated were placed into breeding cages post weaning. Of the first DKO mice litter generated, four of five mice were observed to have hair loss below the neck during weaning at 3 weeks (Figure 5). Hair was later fully regrown as the mouse matured. Hair loss was not recorded for all litters of DKO mice. Female and male mice were observed at a normal ratio. The average litter size for the DKO mice was 5.33 pups while wild-type mice have an average litter size of 7.5[15].

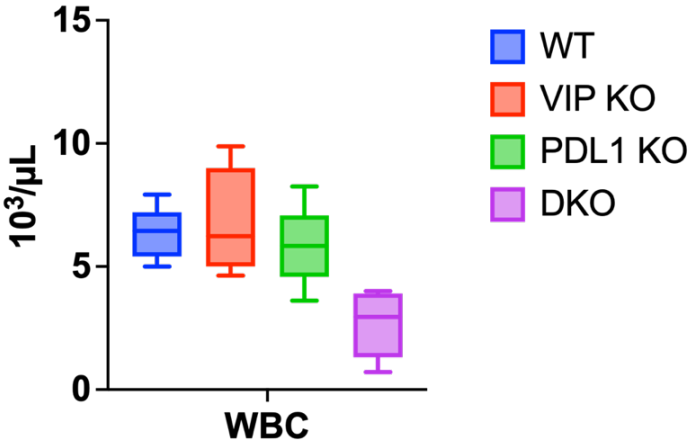
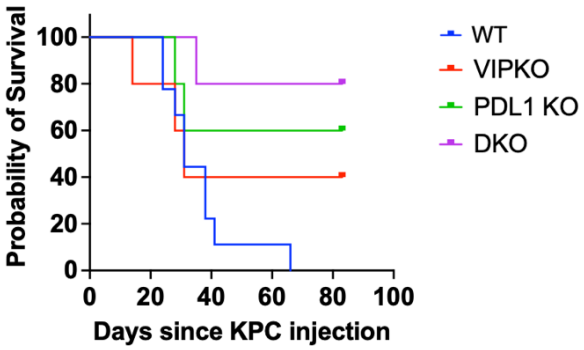


Figure 6. The basal white blood cell count of VIP PDL1 DKO mice is lower than VIP KO, PDL1 KO, and WT basal levels. Blood samples were taken from each experimental group (n = 5) and were analyzed with an automated cell counter to determine white blood cell count.

Basal-level blood was collected from WT, VIP KO, PDL1, and DKO mice. Cell counter analysis showed that overall white blood cell count was lower in DKO when compared to all groups (Figure 6). VIP KO and PDL1 KO mice white blood cell counts were not different from basal WT levels. Basal red blood cell, hemoglobin, and platelet count for VIP KO, PDL1 KO, DKO mice were not different from basal WT mice levels.

A)



B)

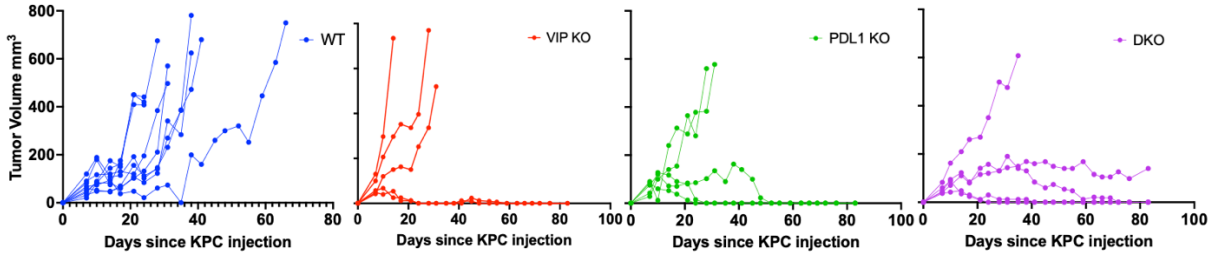


Figure 7. Greater survival of VIP PDL1 DKO mice when challenged with PDAC when compared to wildtype, VIP KO, PDL1 KO mice. (a) Kaplan-Meier survival plots for WT (n = 9), VIP KO (n = 5), PDL1 KO (n = 5), and VIP PDL1 DKO (n = 5) mice subcutaneously implanted with KPC.Luc. Male mice were used. (b) Spider plots showing tumor volume of WT, VIP KO, PDL1 KO, and VIP PDL1 DKO groups corresponding to the survival groups from panel (a).

Next, we challenged the VIP PDL1 DKO mice with the KPC.Luc PDAC cell line to characterize their immune response. 0.5 million KPC.Luc cells were injected subcutaneously into the right flank of male Wildtype (WT), VIP KO, PDL1 KO, and VIP PDL1 DKO mice. 6 days after the initial injection, tumor volume, weight, and survival were monitored twice a week. VIP PDL1 DKO had an 80% survival by day 80, which is greater when compared to all other groups including WT, VIP KO, and PDL1 KO (Figure 7a). 40% of VIP KO mice and 60% of PDL1 KO mice remained alive by day 80, while all WT mice were deceased by day 66. Furthermore, in the VIP PDL1 DKO group, three mice became tumor free, and one mouse had stagnated tumor growth. In comparison, three PDL1 KO mice became tumor free, and two VIP KO became tumor free. No wildtype mice became tumor free (Figure 7b).

Discussion

This study offers evidence to support the efficacy of anti-VIP-R and anti-PD-1 combination treatment against PDAC. The tumor study suggests that VIP PDL1 DKO in mice produces a greater anti-cancer response in terms of survival and tumor growth rate when compared to single knockout and wild-type mice. The decreased growth of the tumors is a marker for adaptive immunity. Indeed, T cells can recognize antigens specific to the cancer cells. If properly equipped and activated, these T cells can infiltrate into the tumor microenvironment to mount an attack and eradicate the cancer cells[16]. These results mirror the effects of pharmacological anti-VIP-R and anti-PD-1 treatment in PDAC-bearing mice, in which combination treatment generated greater anti-cancer responses than single treatments[6]. Future studies with a greater sample size will be conducted to statistically confirm additive or synergistic anti-cancer effects in VIP PDL1 DKO mice. Ultimately, these results suggest that VIP/VIP-R and PDL1/PD-1 pathways can be targeted together for enhanced anti-cancer treatment.

One risk of knocking out two immune checkpoints is autoimmune related lethality. Indeed, a substantial 25% of knockout mice generated are embryonically lethal due to the removal of genes that are indispensable for life[17]. For example, PD-1 and Lag3 DKO compromised self-tolerance, resulting in immune cells infiltrating multiple organs and ultimately leading to fatal outcomes[11]. Generation of VIP PDL1 DKO from double heterozygous breeding appears to be consistent with expected Mendelian frequencies thus far, suggesting that DKO mice may not be affected by embryonic lethality. Although, future genotyping needs to be done to show statistical significance. On the other hand, the average litter size observed for the DKO was less than the expected for wild-type mice[15]. This may suggest embryonic lethality may affect the generation of DKO mice. Nevertheless, VIP PDL1 DKO mice were healthy and fertile, and no observable late autoimmune-related death was observed through adulthood. However, autoimmune-like phenotypes such as hair loss and lowered WBC count were observed[18]. Because the DKO model mimics a full blockade of the VIP and PDL1 pathways, these results suggest the anti-VIP-R and anti-PD-1 combination treatment may have limited toxicity.

The heightened anti-cancer response of the DKO mice to PDAC may be explained by white blood cell (WBC) modulations. WBC levels in DKO mice were lower than in single KOs and WT. One hypothesis of this immune phenotype is activation-induced cell death within the T cell population. Because there may be a decrease in myeloid cells exhibiting suppressor activity, T cells experience prolonged and excessive activation. In turn, these T cells undergo programmed cell death as a mechanism to prevent overactive immune responses and maintain immune homeostasis[19]. This reduced number of WBC counts in DKO, not observed in VIP KO or PDL1 KO, may suggest that the VIP and PDL1 pathways interact to develop heightened immune activity.

The heightened anti-cancer response of VIP PDL1 DKO mice may be the result of distinct immunological modulations from both VIP KO and PDL1 KO. VIP KO alone in mice leads to increased anti-cancer response in terms of survival and tumor growth rate. One explanation of this immune response may be that the lack of VIP paracrine signaling

from dendritic cells to VIP-R on T cells[20] and the elimination of VIP autocrine signaling (unpublished data) on T cells. In turn, the lack of VIP signaling to VIP-R on T cells prevents T cell exhaustion and may downregulate VIP-R on T cells, leading to a T cell dependent anti-cancer response.

Similarly, disruption of the PDL1 pathway in PDL1 KO mice leads to increased anti-cancer responses with higher survival and slower tumor growth rates. Normally, myeloid cells, such as dendritic cells and macrophages, express PDL1 and confer immunosuppressive signals to T cells[21,22]. Thus, mice genetically deficient in PDL1 may have stronger T cell activity due to the absence of such signaling. Furthermore, PDL1 expression by macrophages limits their phagocytic and immune function. Previous studies have shown that the blockade of PDL1 on macrophages increases phagocytosis, reduces tumor growth, increases survival, and produces macrophage-dependent anti-cancer responses in mice[22]. Thus, PDL1 KO mice may also utilize a macrophage-dependent anti-cancer response.

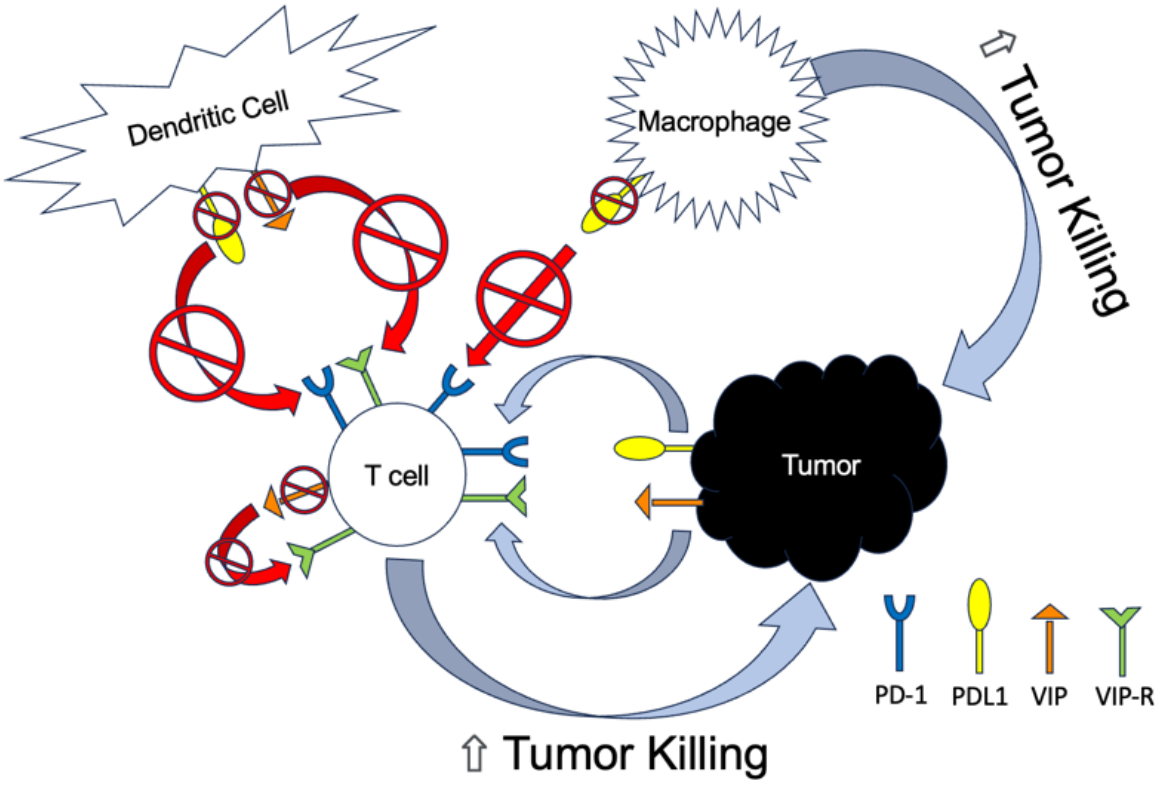


Figure 8. Depiction of hypothesized VIP PDL1 DKO anti-cancer mechanisms. Deletion of VIP and PDL1 paracrine signaling from dendritic cells, PDL1 paracrine signaling from macrophages, and VIP autocrine signaling, leads to a mediated T cell anti-cancer response. Furthermore, deletion of PDL1 expression on macrophages leads to a macrophage mediated anti-cancer response.

Our study on the generation of VIP PDL1 DKO has some limitations. In reference to the homologous recombination for KO generation, the neomycin mutant sequence used to replace the PDL1 sequence in PDL1 KO mice is the same as the one used in VIP KO mice. The primers targeting the neomycin sequence in PDL1 mice are not specific and bind to the neomycin mutant sequence in VIP KO mice. Thus, VIP +/- PDL1 +/- could not be differentiated from VIP +/- PDL1 +/+, and VIP -/- PDL1 +/- could not be differentiated from VIP -/- PDL1 +/+. A new primer design is being pursued to identify all genotypes to complete the Mendelian frequency table. Furthermore, more mice will be genotyped to generate statistically significant frequencies to compare with the expected Mendelian frequency.

Interestingly, mice in the same experimental groups for the tumor study have different outcomes. One explanation is the heterogeneity of the tumor. Factors include the initial cancer cell count of the injection and the depth of the tumor injection. To address the varying cancer cell counts, it may be useful to use a gradient of cancer cell injection doses to observe a wider range of initial tumor burden. For the depth variable, tumors in future studies that are not growing subcutaneously can be removed for consistency. Another explanation for the varying outcomes of the tumor study is the heterogeneity of the mice. Although the mice are inbred and theoretically genetically identical, environmental factors may still lead to differences in the makeup of their immune systems. Thus, it may be useful to characterize their immune phenotype further. For example, the response of isolated T cells to cancer cells in vitro may be characterized for activation and suppression markers.

The tumor study also had other limitations. First, the PDAC cell line used in this study expresses both VIP and PDL1. Indeed, KPC.Luc has previously been shown to express VIP and PDL1 as mechanisms of immune evasion[6,23]. It may be of value to repeat the tumor study with VIP PDL1 DKO cancer cell lines. This will allow for the investigation of anti-cancer responses solely dependent on the immune makeup generated from just VIP PDL1 DKO immune cells, absent the effects of VIP and PDL1 expression by the cancer. Additionally, studying mice that are knockout for the PD-1 and VIP-R can give insights into the VIP/VIP-R and PD-1/PDL1 pathways. Furthermore, immune checkpoints are characterized as important pathways across various cancers[24]. It may be useful to use cancers other than PDAC further to explore the biological effects of VIP, PDL1 DKO, and whether targeting these two immune checkpoints can be paired together for heightened anti-cancer responses. Lastly, a greater sample size is needed to determine the statistical significance of survival and tumor growth. The experiment will be repeated to accomplish this.

The immediate next steps of this study will be to continue to characterize the immune phenotype of the DKO mice. Flow cytometry of blood samples will be used to characterize the basal immune cell makeup and phenotype to expand on the data collected by the cell counter. Furthermore, blood samples after the tumor challenge will be collected, and flow cytometry will be used to characterize immune cell activity. Additionally, tumor samples will be collected for immunohistochemical staining to observe for T cell and macrophage infiltration into the tumor microenvironment.

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