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Inhibiting VIP Receptor Signaling Promotes T Cell Mediated Immune Response in Murine Melanoma Models

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Inhibiting VIP Receptor Signaling Promotes T Cell Mediated Immune Response in Murine Melanoma Models

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Bachelor of Science, Emory University, 2019

Advisor: Edmund K. Waller, MD, PhD

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Abstract

Inhibiting VIP Receptor Signaling Promotes T Cell Mediated Immune Response in Murine Melanoma Models

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Rohan Ketan Dhamsania

The most commonly diagnosed cancer in the US is skin cancer. Melanoma accounts for the vast majority of all skin cancer deaths. Current treatment for aggressive and metastatic melanomas includes targeted therapies including BRAF and MEK kinase inhibitors and immune checkpoint blockade therapies including anti-CTLA-4 and anti-PD-1 therapies. However, just 40% of the patients respond to these therapies and 25%-40% of these patients relapse within 3 years of treatment. As a result, it is imperative to explore more rigorous cancer therapies.

Vasoactive Intestinal Peptide (VIP) is a 28 amino acid immunosuppressive neuropeptide. Its potent immunosuppressive effects have been shown in leukemia and CMV mouse models, in which antagonism to VIP (ANT-08) has downregulated expression of PD-1 and PD-L1 in activated T cells and dendritic cells. In addition, ANT-08 has shown to significantly promote T cell proliferation *in vitro*. Interestingly, according to The Cancer Genome Atlas (TCGA), metastatic melanoma patients with low VIP mRNA expression has better survival then patients with high VIP mRNA expression in the tumor. Thus, we hypothesize that antagonizing VIP receptor signaling would activate the adaptive immune system. We describe herein that T cells from VIP-KO mice have dramatically increased levels of TNF-a and IL-6 and ANT-08 stimulates secretion of these proinflammatory cytokines in wildtype mice. In addition, ANT-08 promotes infiltration of proliferating CD4+ T cells in the murine melanoma tumor microenvironment.

Along with this, VIP causes phosphorylation of CREB, which is downstream to BRAF kinases – a target of several melanoma targeted therapies. It has been shown that VIP receptor antagonists are able to block phosphorylation of CREB. Thus, we hypothesize that antagonizing VIP receptor signaling would activate the adaptive immune system and block phosphorylation of CREB. We describe herein the direct cytostatic effects of ANT-08 on D4M, $BRAF^{v600E}$ mutation positive melanoma model.

Cumulatively, using ANT-08 to activate the adaptive immune system and block phosphorylation of CREB, we hypothesize that ANT-08 will elicit an anti-cancer effects against murine melanoma models. Thus, blocking VIP receptor signaling elicits a new strategy to activate the adaptive immune system and to develop novel immuno-oncology therapy.

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INTRONDUCTION AND BACKGROUND INFORMATION

What is Melanoma:

Skin cancer is the most commonly diagnosed cancer in the United States with 9,500 Americans diagnosed of the disease every day (American Cancer Society). While there are many types of skin cancer including Basal Cell Carcinoma (BCC), Squamous Cell Carcinoma (SCC), Merkel Cell Carcinoma, Melanoma is the most aggressive type of skin cancer as it can rapidly metastasize into distant organs and tissues (Turner, Ware and Bosenberg) Due to its aggressive nature, melanoma accounts for a vast majority of skin cancer deaths.

Melanoma is the cancer of melanocytes. Melanocytes are melanin-producing cells found on the upper layer of skin. Melanin gives the skin its color. Skin damage induced from Ultraviolet (UV) rays causes melanocytes to produce more melanin as a protective mechanism. Overexposure to UV radiation can cause DNA damage in melanocytes, thus rendering them to be cancerous.

Staging and Progression of melanoma:

Melanoma is classified using the TNM (Primary Tumor, Regional Lymph Nodes, Distant Metastasis) model. T category is assessed based on primary tumor thickness, mitotic rate and ulceration. N category is defined as the number of metastatic nodes, tumor burden and ulceration of the primary tumor. The criteria for M category is the site of distant metastasis and an elevated serum lactate dehydrogenase level (Balch et al.). Based on the melanoma classification, it is classified as different stages (Figure 1).

ANATOMIC STAGE/PROGNOSTIC GROUPS							
	Clinical Staging[3]				Pathologic Staging[4]		
Stage 0	Tis	N0	M0	0	Tis	N0	M0
Stage IA	T1a	N0	M0	IA	T1a	N0	M0
Stage IB	T1b	N0	M0	IB	T1b	N0	M0
	T2a	N0	M0		T2a	N0	M0
Stage IIA	T2b	N0	M0	IIA	T2b	N0	M0
	T3a	N0	M0		T3a	N0	M0
Stage IIB	T3b	N0	M0	IIB	T3b	N0	M0
	T4a	N0	M0		T4a	N0	M0
Stage IIC	T4b	N0	M0	IIC	T4b	N0	M0
Stage III	Any T	≥ N1	M0	IIIA	T1-4a	N1a	M0
					T1-4a	N2a	M0
				IIIB	T1-4b	N1a	M0
					T1-4b	N2a	M0
					T1-4a	N1b	M0
					T1-4a	N2b	M0
					T1-4a	N2c	M0
				IIIC	T1-4b	N1b	M0
					T1-4b	N2b	M0
					T1-4b	N2c	M0
					Any T	N3	M0
Stage IV	Any T	Any N	M1	IV	Any T	Any N	M1

Figure 1. Staging of Melanoma based on TMN model (American Joint Committee on Cancer). Clinical and pathological criteria to classify melanoma in patients. This classification is critical in the course of tumor treatment.

Early melanomas are classified as stage 0 and stage 1 where the cancerous cells are localized (Skin Cancer Foundation). Tumors in stage 0 and 1 are generally considered low risk. Stage 2 melanoma consist of localized but larger tumor volumes. Large tumor volumes have

certain characteristic traits like ulcerations that put stage 2 melanomas under high risk of spreading category. Stage 2 melanoma are considered intermediate to high risk tumors. Stage 3 melanoma is when the tumor starts to metastasize and spread to local lymph nodes. Stage 4 melanoma is when the cancer has advanced into distant lymph nodes, tissues and organs. Most common metastasis sites include lungs, liver, brain, bone and gastrointestinal tract. Due to the cancer having metastasized, stage 3 and stage 4 melanomas are considered advanced melanoma (Figure 1) (Skin Cancer Foundation). In the case of most cancers, it is the metastasis to distant sites that is fatal (Figure 2) (Gershenwald et al.). Course of treatment for melanoma is often dependent on tumor stage.

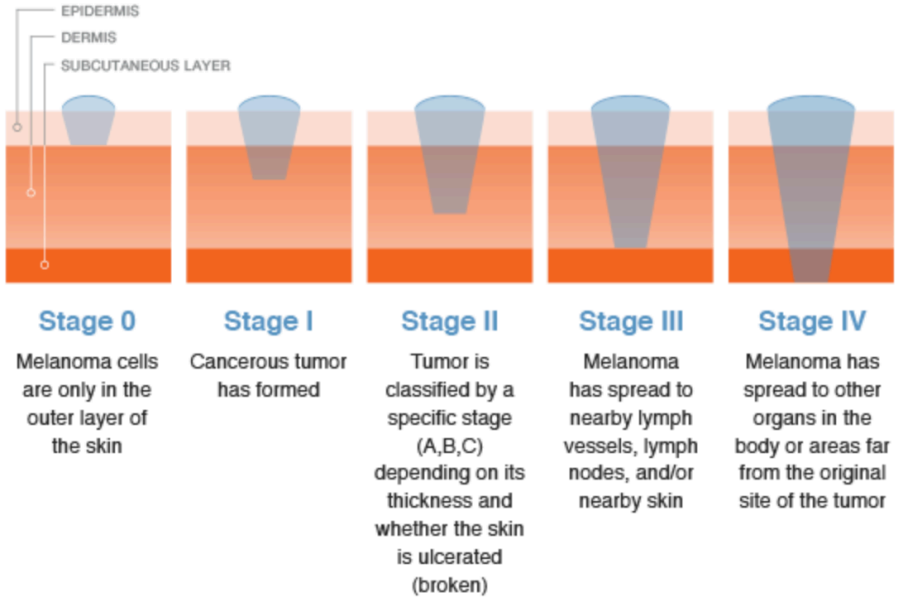


Figure 2. Schematic of melanoma tumor burden at corresponding tumor stage. Melanoma starts **(Takata, Murata and Saida).** Melanoma progresses in stages, starting in epidermis, spreading to regional lymph nodes before metastasizing to distant organs.

Current treatments for melanoma:

Melanoma is one of the most chemotherapy resistant tumors. Primary treatment options involve surgery, radiation and immunotherapy. The treatment course chosen by the physician is dependent on the stage of melanoma. Generally, stage 0 and 1 tumor are treated with excisional surgeries as the tumor is mainly topical. Intermediate to high-risk tumors are surgical removed and usually followed up radiation and immunotherapy. In the past, patients with stage 3 melanoma had a high risk for developing metastases (stage 4), and stage 4 melanoma had very poor outcomes, as metastatic tumors could not be cured with chemotherapy or radiation. More recently, the introduction of monoclonal antibodies that block signaling through immune checkpoints and treatment with ex vivo expanded tumor-infiltrating lymphocytes (Figure 3) have yielded durable disease-free remissions in some patients with advanced stage 3 and 4 melanoma (Pasquali et al.).

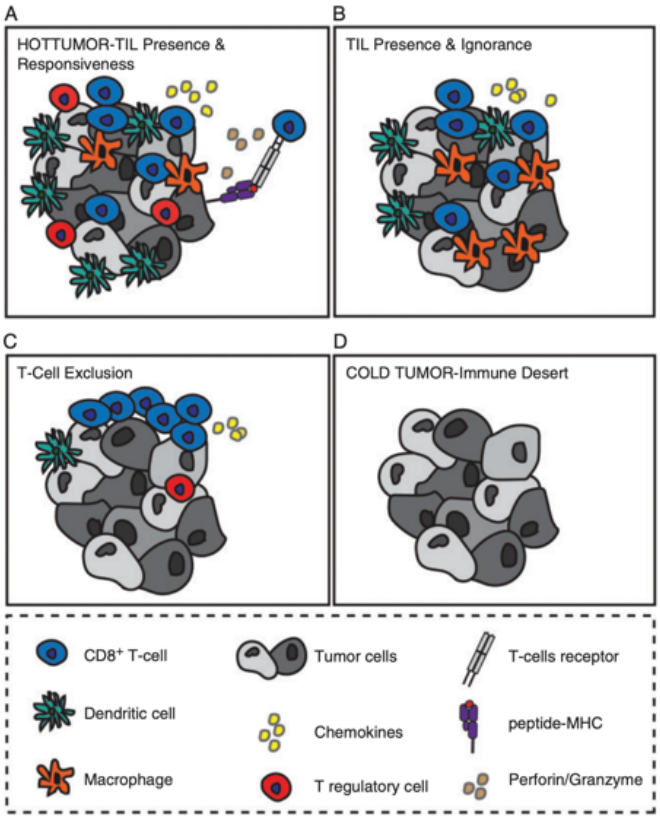


Figure 3. Tumor classification based on tumor-infiltration lymphocytes (TILs) (Lanitis et al.). Tumors infiltrated by the lymphocytes are referred to as hot tumors and some hot tumor respond to immunotherapies (A) while others don't (B). Lymphocytes can also be present in the periphery of the tumor (C) or nowhere close to the tumor as in the case of cold tumors (D).

The objective for using immunotherapies to treat melanoma is to activate the body's adaptive immune system. Since melanoma is already a hot tumor, there are relatively high proportion of Tumor-infiltrating Lymphocytes (TILs) in tumor microenvironment (TME) and thus immunotherapies have been relatively efficient in combating melanoma.

T cell Mediated Apoptosis of Melanoma Cells:

Melanoma cells are able to generate rapid innate immune response followed by adaptive immune response. Both these responses induce apoptosis of melanoma cells thereby central to counterbalance the growth of melanoma cells (Miller and Sadelain). In order to effectively mount an immune response against melanoma cells, four steps are essential: activation of T cells, homing of activated T cells into melanoma TME, recognition of melanoma cells and T cell mediated apoptosis of melanoma cells (F. Mahmoud et al.). Two types of T cells are essential to mount an anti-melanoma response, CD4 and CD8 T cells. CD4 T cells have two subsets, Th1 and Th2 positive cells. Upon activation, Th1 CD4+ T cells activate cytotoxic CD8+ T cells.

Dendritic cells (DCs), most potent antigen presenting cells (APCs), are recruited into the TME and they contribute towards phagocytosis of melanoma cells (Palucka and Banchereau). Following phagocytosis of tumor-associated proteins, proteins are digested into peptides which are loaded onto MHC molecules which then migrate to the plasma membrane where the tumor-associated peptide can be presented to T cells. Peptide-loaded MHC interact with T cell receptor (TCR) providing first signal for T cell activation. In addition to signal 1, when the CD28 receptors on the T cells bind the B7 molecule on DC, providing second signaling, cause the T cells to recognize the presented tumor antigen is not self (Palucka and Banchereau).

Following activation of T cells, homing of T cells into the melanoma tumor microenvironment (TME) is essential for an effective immune response. Adhesion molecules, integrins and chemokine ligands are essential for efficient homing.

Upon entering the TME, activated T cells bind to the same tumor associated antigens that had activated the T cells. On this binding, T cells initiate cytotoxic signaling against melanoma cells causing apoptosis of tumor cells (Garrido, Cabrera and Aptsiauri) (Figure 4).

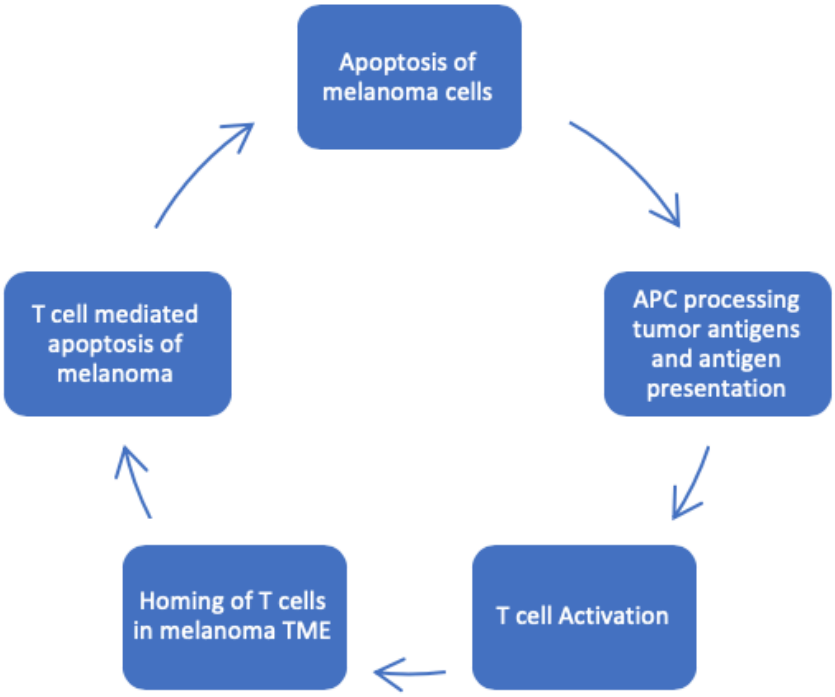


Figure 4. T cells mediated apoptosis of melanoma cells. APC present tumor antigens in order to activate T cells. Activated T cells home in the melanoma TME inducing T cell mediated apoptosis of melanoma cells.

Melanoma Immune Surveillance Escape:

However, melanoma cells are able to escape the immune system through immune editing. Immune editing is a dynamic process, that enable malignant melanoma cells to colonize distant sites (Passarelli et al.). Part of immune escape is immune editing to decrease presentation of tumor-associated antigens. Immune escape includes dysfunction in any of the four steps discussed above (activation of T cells, homing of activated T cells into melanoma TME, recognition of melanoma cells and apoptosis of melanoma cells) that limit the ability of the immune system to mount an effective anti-melanoma response. These processes include tumor-associated factors that lead to an immunosuppressed host, progressive exhaustion of T cells counterbalancing melanoma cells, defective antigen presentation and melanoma cells' increased resistance to apoptosis (Seliger et al.), (T. F. Gajewski, H. Schreiber and Y. X. Fu), (Crespo et al.).

T cells express on their surface checkpoint molecules that negatively regulate T cell activation when they bind a cognate ligand. Physiologic expression of immune checkpoint receptors on T cells help prevent autoimmune responses from developing in the face of damaged normal tissues. Exhausted T cells often start to these upregulate inhibitory receptors including CTLA-4, PD-1, LAG-3, TIM-3, VISTA and CD160 (Das, Zhu and Kuchroo), (Lines et al.), (Merelli et al.), (Lines et al.), and (Woo et al.). These inhibitory receptors interact with melanoma cells causing immunosuppressive downstream signaling (Passarelli et al.). As these receptors are targetable, blocking their interactions with ligands expressed in the TME and preventing inhibitory downstream signaling in T cells has provided the foundation for development of immunotherapies.

In addition, melanoma has a particularly high burden of mutations in genomic DNA termed "mutational burden" (Brown et al.). High mutational burden leads to formation of new tumor associated antigens (TAA) that can activate T cells as well as the subsequent loss of existing TAA

as a method of immune escape. Thus, T cells are no longer able to induce T cell mediated death of melanoma cells. As a result, acquiring new mutations enables melanoma cells to evade host immune system and progress (F. Mahmoud et al.).

Some of the most common mutations in melanoma include BRAF, NRAS, and MEK1(Carlino et al.). BRAF mutation, found in ~40% of human melanoma, constitutively activates BRAF kinase thereby causing increasing phosphorylation of CREB and consequently causing proliferation of melanoma cells (Zaretsky et al.) (Figure 5). As a result, these mutations provide targetable pathways for targeted therapies to counter melanoma cell proliferation.

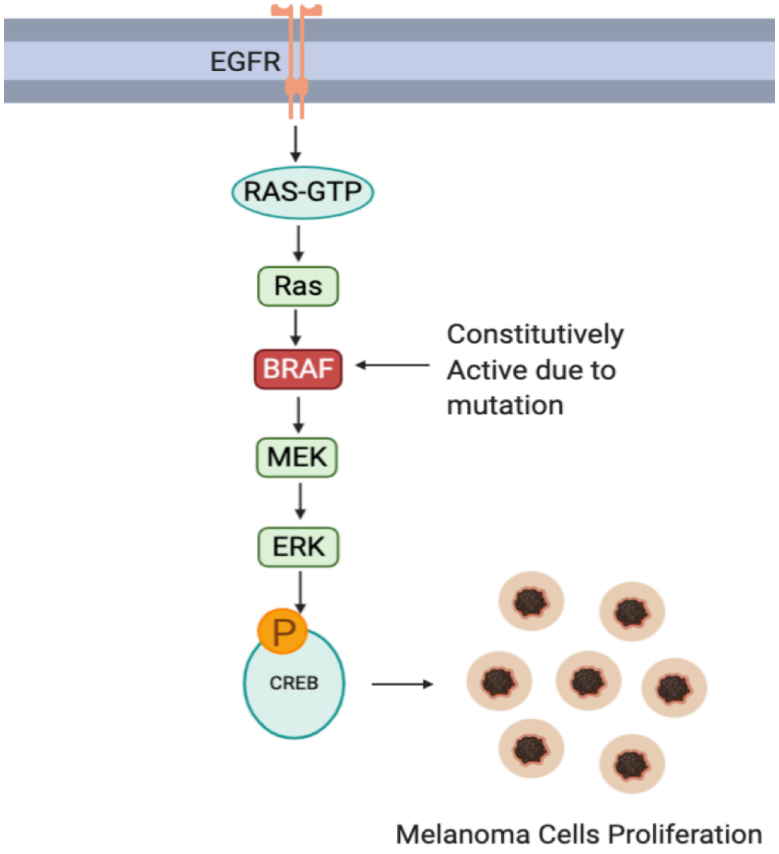


Figure 5. BRAF^{V600E} mutation causes proliferation of tumor cells. Constitutively activated BRAF causes activation of MEK kinase and ERK kinase leading to phosphorylation of CREB, which downstream results into melanoma cells proliferation.

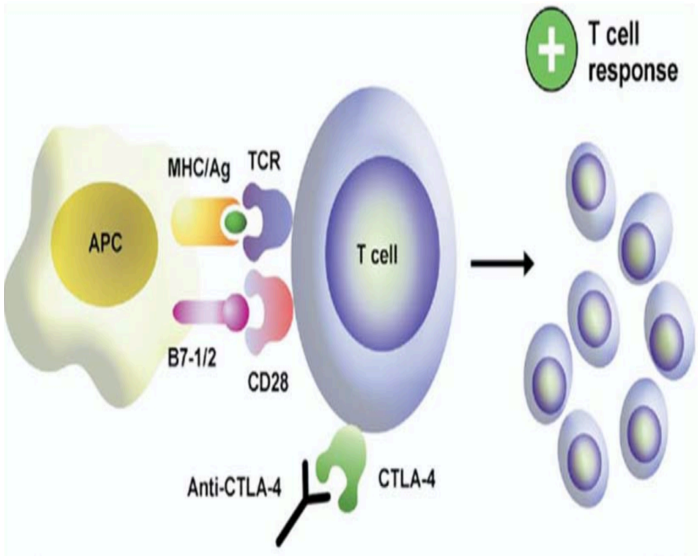
Melanoma current immunotherapies:

Since advanced melanomas are largely resistant to conventional treatments, checkpoint therapies have been the primary choice of treatment for advanced melanoma patients (Mellman, Coukos and Dranoff). Immunotherapy has had significant success, relative to conventional chemotherapy, in controlling the growth of advanced melanoma and extending life.

Patients with melanoma have had relatively high frequencies of response with checkpoint inhibition immunotherapies owing to the high mutational burden of melanoma induced by chronic exposure to mutagens like UV radiation (Rizvi et al.). Due to high mutational burden there are a plethora of neoantigens that enable lymphocytes to infiltrate the tumor microenvironment (TME) (Tsao, Atkins and Sober). As a result, melanoma is considered relatively immunogenic tumor owing to its ability to generate an immune response due to the mutation burden (Passarelli et al.). Previous studies have shown a significant increase in murine melanoma tumor burden on depletion of CD8+ cells (Farley et al.) and (S. M. Mahmoud et al.).

Two of the most widely used checkpoint inhibitors for melanoma include antibodies to Cytotoxic T-cell Lymphocyte Antigen-4 (CTLA-4) and Programmed Cell Death-1 (PD1) (Hodi et al.) and (Pentcheva-Hoang, Corse and Allison). Both these inhibitors were FDA-approved in 2011 and 2014, respectively, to treat advanced melanomas (Figure 6, 7).

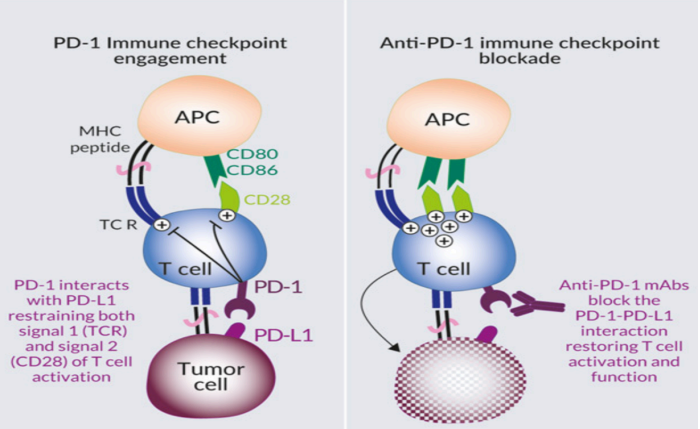
Mode of action for anti CTLA-4:



T cell activation requires two independent signals. First signal relays when an Antigen Presenting Cell (APC) presents an antigen to T Cell Receptor (TCR) via Major Histocompatibility Complex (MHC). The second signal is a costimulatory, antigen independent signal in which T cell receptor CD28 binds with B7-1/2 counter-receptor (Thompson, Allison and Kwon). T cells also have CTLA-4 receptor that inhibits T cell activation. CTLA-4 competes with CD28 for B7-1/2 interaction (van der Merwe et al.) and (Rudd, Taylor and Schneider). By making antibodies against CTLA-4 receptor, the objective is to block the CTLA-4/B7-1/2 interaction and thereby inhibit coinhibitory signaling. Therefore, anti-CTLA4 interferes with the activation phase of T cells and prevents an overall immunosuppressive environment. Ipilimumab is one such FDA-approved anti-CTLA4 antibody (Wolchok et al.).

Figure 6. Costimulatory and coinhibitory ligand-receptor interactions between APC/tumor cell and T cells. Anti-CTLA-4 antibody interferes with the interactions with B7-1/2 by binding with CTLA-4 receptor.

Mode of action for anti-PD1:



PD-1 receptor is present on the T cells and it interacts with the Programmed Cell-Death Ligand - 1 (PD-L1). This interaction leads to the generation of phosphatases that reverse T cell activation signals generated by tyrosine kinases, leading to T cell inhibition. As a result, PD-1 receptor upregulation is often a sign of an exhausted T cell (Beaver et al.). By developing antibodies against PD-1, it blocks the interaction PD-1 and PD-L1 thereby prevent T cell exhaustion. As a direct effect, this checkpoint blockade allows the T cell to be activated. Thus, unlike anti-CTLA4, anti-PD1 blockade targets the effector phase of T cells. Pembrolizumab and Nivolumab are two FDA-approved anti-PD-1 antibodies (Lepir et al.).

Figure 6. PD-1 blockade activates the T cells. Anti-PD1 antibody activates the T cell by preventing T cell exhaustion, as a result enabling T cells to combat tumor cells.

These immune checkpoint inhibition therapies have revolutionized treatments for metastatic melanoma patients. Around 40% of the patients can initially respond to these therapies (Zaretsky et al.).

Challenges for melanoma immunotherapies:

From the patients that respond to checkpoint blockade therapies, 25-40% relapse within 3 years (Zaretsky et al.) and (Said and Mutt). These relapsed patients often have extremely aggressive melanomas that are non-responsive to current treatments. There are several reasons for that including tumor immunogenicity and tumor microenvironment. Tumor immunogenicity is the ability of tumors to activate an immune response. Given melanoma's high mutational burden, melanoma is an immunogenic tumor. Owing to greater intratumor heterogeneity in melanoma, there is greater likelihood that a poorly immunogenic subclone can be selected. As a result, this causes resistant to immunotherapies (McGranahan et al.; Riaz et al.; Anagnostou et al.). In addition, greater infiltration of regulatory cells including regulatory T cells (Tregs) and myeloid derived suppressive cells (MDSCs) can cause resistance to immunotherapies (T. F. Gajewski, H. Schreiber and Y.-X. Fu; Sakaguchi et al.; Viehl et al.). Along with these, there are several other unstudied mechanisms through which the tumor cells are able to overcome immune checkpoint therapies.

Along with this, combination of CTLA-4 and PD-1 blockades often cause severe treatment-related adverse events (AEs). Collectively considering all these factors, it is imperative and necessary that directed efforts are continued to develop immunotherapies that can treat a broad-spectrum of patients and does not have severe AEs.

As only around 40% of the patients respond to these drugs, there is a clinical need to develop novel immunotherapies that can be applicable to broader patient population (Zaretsky et al.). Given the immunogenic nature of melanoma, it will be beneficial if the immunotherapies are able to activate the adaptive immune system resulting into greater tumor infiltrations as higher

proportion of TILs is associated with better clinical response in patients with melanoma (Goel et al.).

Current Targeted Therapies for Melanoma and its Challenges:

Melanoma has the highest mutational burden across all cancers (Alexandrov et al.). As a result of increased tendency to generate mutations, advanced melanoma can evolve to new mechanisms of growth-promotion and resistance to immune response. BRAF mutations is the most commonly mutated gene. Targeted therapies include therapeutic solutions to target these specific mutations including BRAF mutation.

BRAF mutation causes the kinase to be constitutively activated thereby causing phosphorylation of CREB, which in turn promotes tumor cell survival and proliferation (Dummer et al.) (Figure 3). Targeted therapies including vemurafenib and trametinib that inhibit functioning of mutated BRAF and MEK kinases respectively and thereby block constitutive activation of CREB (Zhang, Heinzmann and Grippo) (Figure 8).

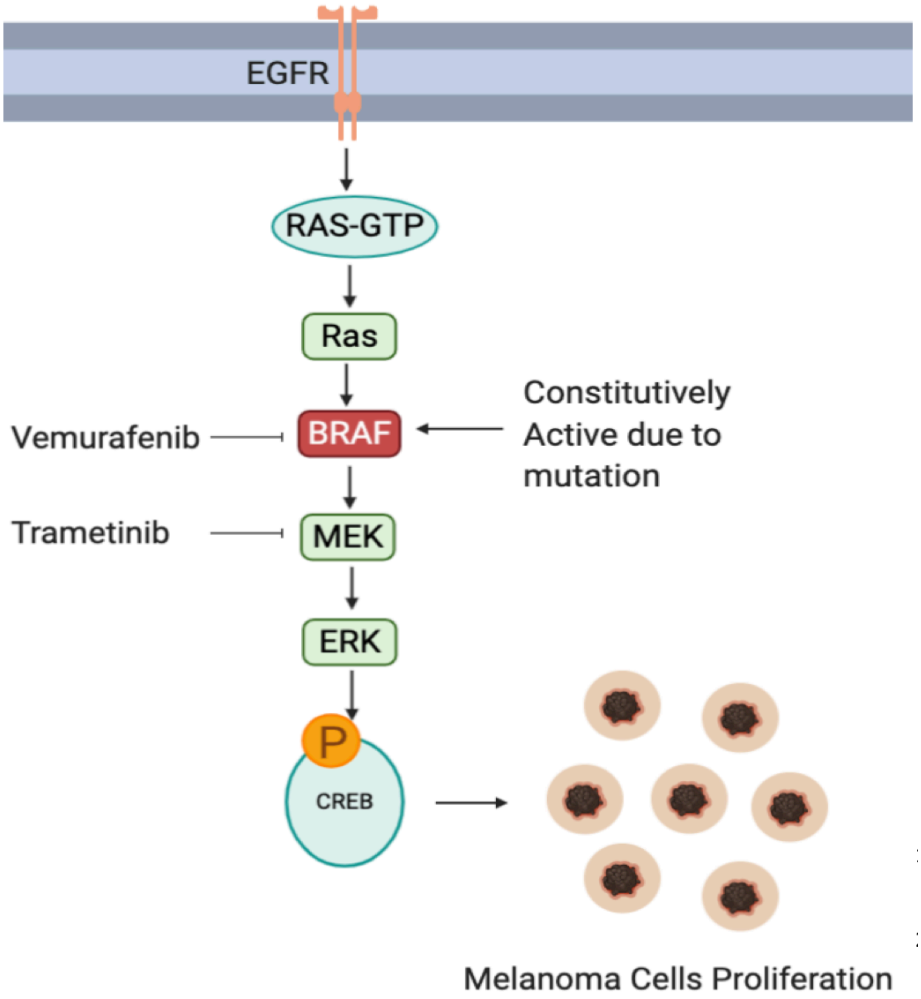


Figure 8. Vemurafenib and Trametinib inhibits activity of BRAF kinase and MEK kinase. Vemurafenib and trametinib inhibit respective kinases thereby preventing constitutive activation of CREB and its consequent downstream signaling pathways.

However, despite initial successes from the targeted therapies, patients often acquire resistance and relapse within a year or two in most cases (Das Thakur et al.). In addition, targeted therapies are also associated with treatment related adverse events that include skin toxicities (Sinha et al.).

As a result, in light of these shortcomings of the targeted therapies, it is with utmost urgency that we need to develop new therapies for melanoma.

Scope of Thesis:

Vasoactive Intestinal Peptide (VIP) is a 28 amino acid neuropeptide (Said and Mutt) and (Delgado, Pozo and Ganea). VIP's biological activities are mediated by three G-protein coupled receptors namely VPAC1, VPAC2, and PAC1. Off these receptors, VIP has 1000-fold greater affinity for VPAC1 and VPAC2 receptors then the PAC1 receptor (Jansen-Olesen and Hougaard Pedersen). All these receptors belong to gastrin/glucagon/secretin family (Delgado, Pozo and Ganea). After activation, peptide-receptor signaling stimulates diverse signaling cascades that are predominantly mediated by adenylate-cyclase, stimulation of protein kinase A (PKA), and stimulation of protein kinase C (PKC) via phospholipase C all resulting into phosphorylation of CREB and activation of p38 (Said and Mutt) and (Petersen, Li and Waller).

In addition to stimulate CREB phosphorylation, VIP modulates activity of the immune system (Figure 9). VIP-signaling has immunosuppressive effects against the innate and adaptive arms of the immune system. Macrophages treated with VIP secrete Th2-cytokines like IL-4 and IL-5 and reduces Th1-cytokines like IFN-gamma (Petersen, Li and Waller) and (Delgado, Leceta, et al.). VIP's effect on T cells includes a skewed response toward Th-2 effector function and increase in proportion of regulatory T cells (Petersen, Li and Waller) and (Delgado, Chorny, et al.). VIP has shown to block G1/S transition thereby causing cell cycle arrest in T cells (Anderson and Gonzalez-Rey). In addition, VIP-signaling leads to decreased T cell proliferation *in vitro* as a result of decrease in IL-2 production (Petersen, Li and Waller) and (Boudard and Bastide).

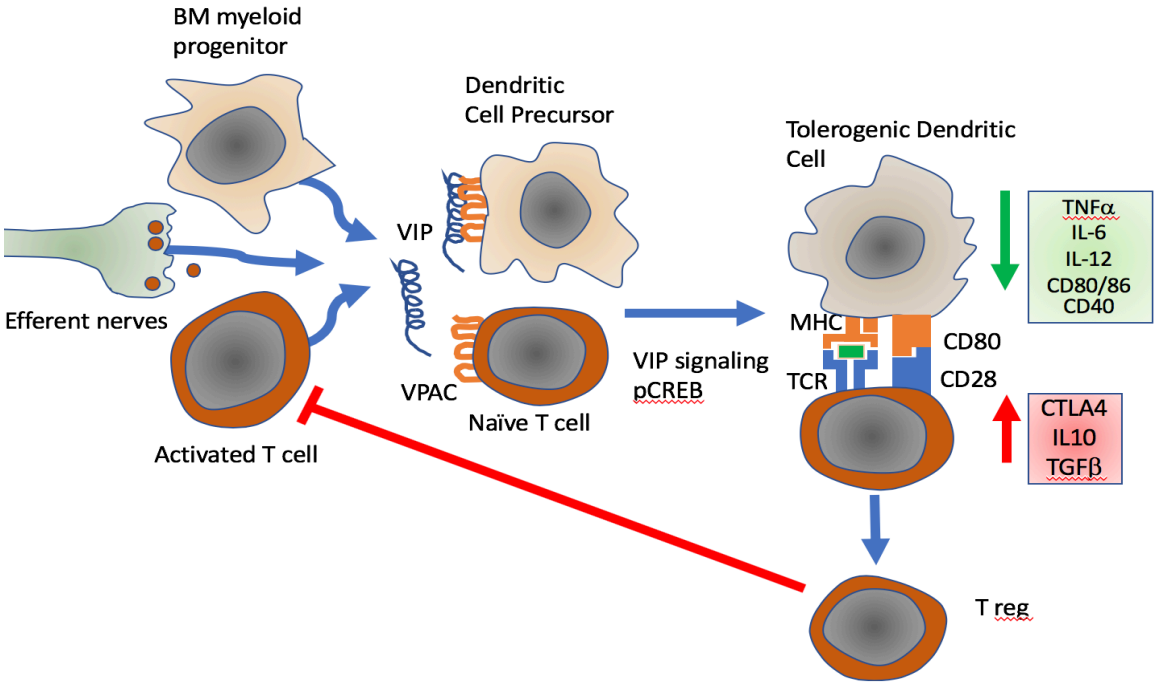


Figure 9. Schematic representation of the immunological effects of VIP on the adaptive immune system. VIP exerts immunosuppressive effects on the adaptive immune system by downregulating pro-inflammatory cytokines and upregulating anti-inflammatory cytokines (Delgado and Ganea).

Thus, I hypothesized that inhibiting VIP receptor signaling pathway would block tumor cell proliferation and survival by downregulating CREB phosphorylation (Figure 10, 11). In addition, I hypothesized that blocking the VIP receptor signaling pathway would activate the adaptive immune system leading to activation of T cells that challenge melanoma.

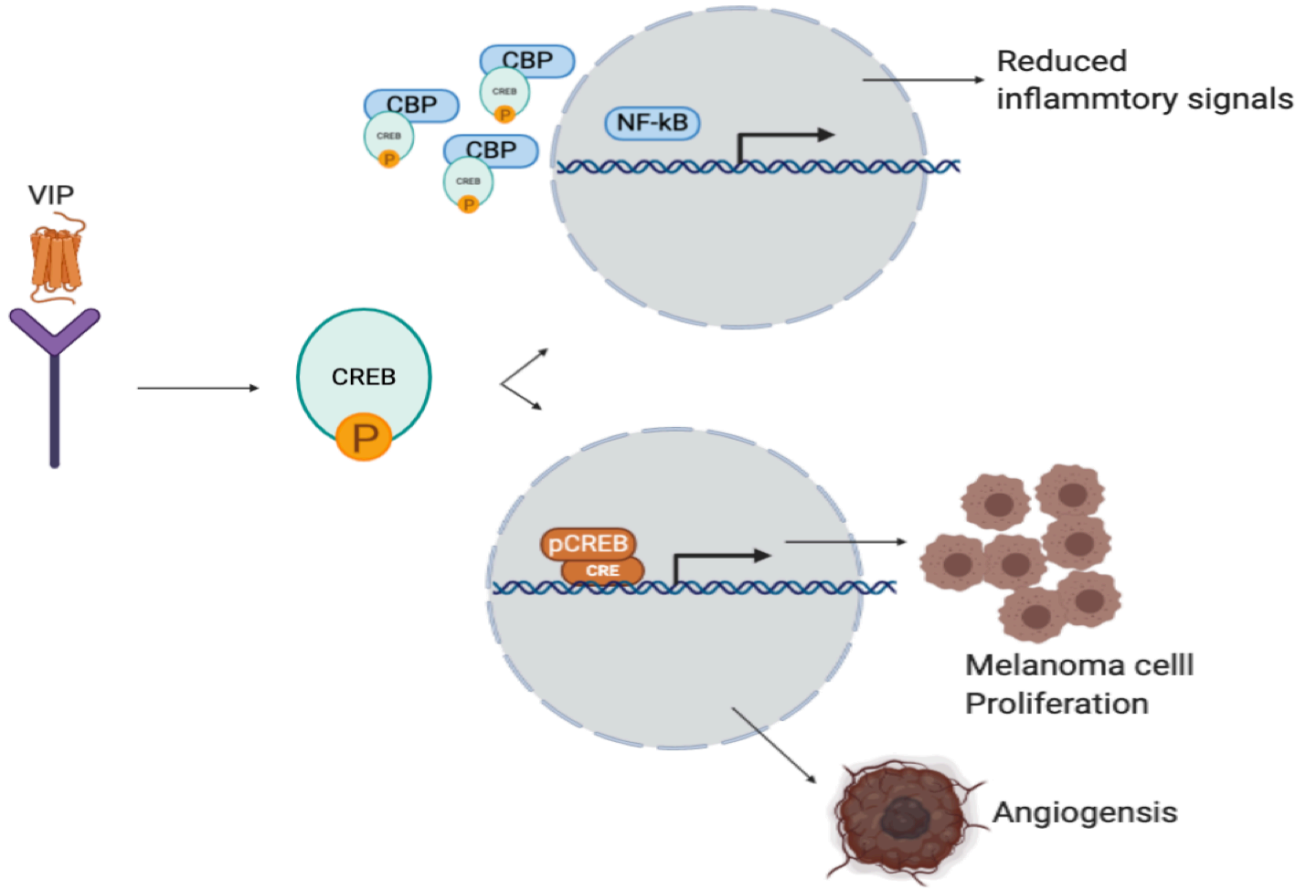


Figure 10. VIP Receptor Signaling promotes melanoma cell proliferation and blunts adaptive immune responses. VIP causes phosphorylation of CREB that downstream causes proliferation of melanoma cells and reduced inflammatory response via T cells.

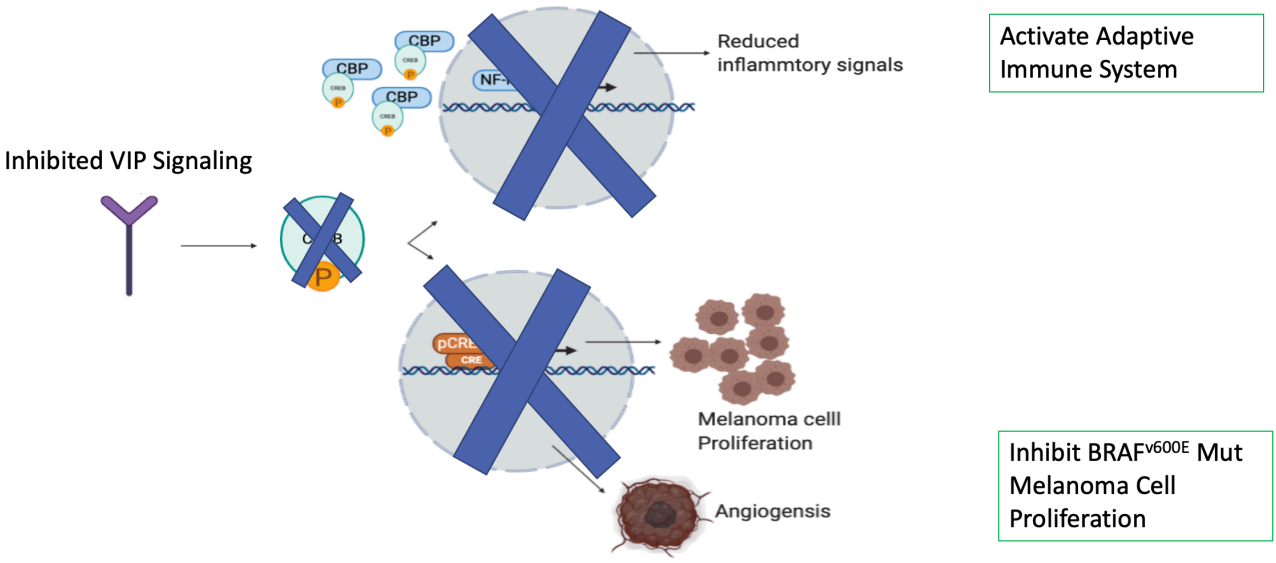


Figure 11. Inhibition of VIP Receptor signaling pathway causes reduced tumor cell proliferation and enhanced inflammatory signals in T cells. Blocking the VIP receptor signaling pathway leads to decreased CREB phosphorylation and decreased tumor cell proliferation. Lower levels of pCREB lead to higher levels of inflammatory signals due to increased T cell activation.

This work elucidates relationship between melanoma and VIP secretion, source of VIP secretion and how antagonizing VIP receptor signaling pathway can have activation effects on the adaptive immune system. In addition, it will strengthen our understanding of the possibility of using VIP receptor antagonist as therapeutic solution for melanoma.

Antagonizing Vasoactive Intestinal Peptide Signaling

To inhibit VIP receptor signaling pathway, pharmacological VIP receptor antagonists were developed. A peptide-based VIP receptor antagonist competitively binds the VIP receptor causing a pharmacological blockade of the VIP-signaling pathway.

Antagonizing VIP receptor signaling decreased CREB phosphorylation in T cells (Petersen, Li and Waller). Further, treatment of mice with a VIP receptor antagonist increased IFN-gamma and IFN-beta (type 1 interferons) levels in serum following infection with mouse cytomegalovirus (Symes et al.). In addition to the anti-viral effects of the VIP receptor antagonist, anti-tumor effects against leukemia, lymphoma, and solid tumors like pancreatic cancer have been noted. Early and late administration of VIP receptor antagonist significantly improved the survival rates in mice with leukemia (Petersen, Li and Waller). Treatment with a VIP receptor antagonist has transformed the "immunologically cold" TME of Pancreatic Ductal Adenocarcinoma (PDAC) to a "hot" TME with infiltration of CD4+ and CD8+ T cells and synergy with anti-PD-1 therapy in inhibiting tumor growth in mouse PDAC models (Sruthi Ravindranathan, Waller lab, unpublished data). Cumulatively, these results create a strong case to study the efficacy of a VIP receptor antagonist in the treatment of an immunogenic tumor like melanoma.

The Waller lab has developed its own novel VIP receptor antagonist called "ANT08" that has increased potency compared with the "VIPhyb" peptide antagonist which has been described in the literature (Petersen, Li and Waller). The VIP receptor antagonists studied in this thesis include VIPhyb and ANT-08.

Experimental model

B16 melanoma cell line was derived in 1954 when a tumor naturally occurred behind the ear of a C57BL/6 mouse (Teicher). Two well established subclones of this cell line include B16F10 and B16F1 (Knight GD) and (Herlyn and Fukunaga-Kalabis). B16F10 cells are less homotypically adhesive then B16F1 cells thereby making B16F10 cells highly metastatic (Fidler and Nicolson) and (Elvin and Evans) particularly to lungs and liver. Due to the high metastatic potential, transplantation of these tumors in mice cause death within 2-4 weeks (Elvin and Evans). In addition, B16F10 cell line has been responsive to translational immunotherapies (Yamano et al.) and (Ingram et al.). Because of its metastatic potential, strong pigmentation, and translational response to immunotherapy, B16F10 is a popular choice to study immunotherapy in a preclinical model of melanoma in mice (Herlyn and Fukunaga-Kalabis) and (Elvin and Evans).

$BRAF^{V600E}$ mutation is one of the most common mutations in melanoma (Bisschop et al.). Due to the mutation, BRAF oncogene is constitutively active, thereby resulting into high levels of phosphorylated ERK (Ziogas et al.). D4M cell line is a murine melanoma that recapitulates $BRAF^{V600E}$ mutation (Jenkins et al.). This cell line is transplantable in C57BL/6 mice, thus allowing to be used in immune-oncology research. D4M cell line is an unpigmented, aggressive murine cell line that results into tumor associated death in 2-4weeks (Jenkins et al.). To understand interaction of immunotherapies with targeted therapies in melanoma, D4M is a popular research model.

Syngeneic mice models are commonly used for immune-oncology studies (Zhong et al.). Syngeneic models allow for transplantation of melanoma cells into mice with the same genetic background. This allow for interaction of the immune cells from the host species with the melanoma cells.

To understand VIP receptor antagonist's response to tumor cells and immune cells, it is crucial to recapitulate the tumor microenvironment that includes blood vessels, extracellular matrix and a variety of cells (Kuzu et al.). As a result, mice models are used to recapitulate tumor progression and immune response to these tumor cells. Along with this, mice models are relatively easy to manipulate given our vast knowledge base on mice genomics (Wilmanns et al.). Owing to the immense data on mouse genome, it allows for genomic manipulations like gene knockouts (KO) and knock-ins (Joung et al.). We are thankful for the generous gift of VIP-KO mouse model from Dr. James A. Waschek from the University of California, Los Angeles, CA.

METHODS AND MATERIALS

Methods and Materials

Cell lines

Four melanoma cell lines were used to study the response of melanoma to VIP antagonist treatment. The four melanoma cell lines include B16F10, B16F1, D4M and SM1. B16F1 cell line was a generous gift from Dr. Jack Arbiser, who had obtained it from American Type Culture Collection (ATCC) (Manassas, VA, USA). All other cell lines were obtained from ATCC. B16F10 and B16F1 cell lines were cultured in Dulbecco's Modified Eagle Medium (DMEM) with L-glutamine and sodium pyruvate, supplemented with 10% fetal bovine serum (FBS), 100U/mL penicillin, 100ug/mL streptomycin, and 1500mg/L sodium bicarbonate. D4M cells were cultures in DMEM F-12 advanced medium supplemented with 5% fetal bovine serum (FBS), 100U/mL penicillin, 100ug/mL streptomycin, and 4nM/L L-glutamine. SM1cells were cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS), 100U/mL penicillin, 100ug/mL streptomycin, and 4nM/L L-glutamine.

All the cell lines were passaged when 80% confluent and were tested negative for pathogens from Emory Division of Animal Resources. All the cell cultures were maintained in 37℃ 5% CO_2 humidified incubator. Tumor cell lines used for experimentation were passaged less then 50 times to maintain consistency of the cell line.

Cytostatic Assay

To determine any cytostatic effects of ANT-08 on B16F10 and D4M cells, colorimetric MTT assay from Roche (Basel, Switzerland) was obtained. Cell suspension of B16F10 and D4M cells were made such that there are 100,000 cells per 100uL. In a black 96 well,

flat-bottom plate, 4000 or 2400 B16F10 or D4M cells were seeded for 24 hours or 48 hours with treatment with concentrations of ANT-08, ranging from 1-5uM, every 24 hours. All treatments were done in replicates of three. After the last round of treatments, 10uL of 5mg/mL MTT was added to each well and incubated for 4 hours in 37℃ 5% CO_2 humidified incubator. Color of the solution in the wells will change to purple indicating reduction of MTT dye by NADPH-dependent cellular oxidoreductase enzymes. Following 4 hours incubation, 100uL of solubilization buffer was added to each well and incubated overnight in 37℃ 5% CO_2 humidified incubator. This will result into change in color to yellow. Using a colorimeter, obtained from Bio rad, absorbance was measured at 570nm. Higher absorbance corresponds to higher proportion of viable cells.

Splenocyte Harvestation

Naïve or tumor-inoculated VIP-WT or VIP-KO mice were euthanized, and their spleens were harvested. Spleen was gently crushed with the back of the syringe plunger to obtain single cell suspension. The cell suspension was passed through 70um cell strainer to remove fat particles. Following, the cells were incubated in RBC lysis buffer for 10 minutes at room temperature to lysis RBCs from the cell suspension. The single cell suspension – now splenocytes – were counted using a hematocytometer. Splenocytes were either used for consequent experiments or up to 30 million splenocytes were stored down in 1mL cryostore.

T cell Isolation Assay

Naïve VIP-WT or VIP-KO mice were euthanized, and their spleens were harvested A single cell suspension of splenocytes was obtained by gently crushing the spleen against a wire mesh with the back of the syringe plunger. The cell suspension was passed through 70um cell

strainer to remove aggregates and larger particles. RBCs were lysed by incubating the cell suspension with RBC lysis buffer for 10 minutes at room temperature. The single cell suspensions were counted using a hematocytometer.

Pan T cell isolation kit II from MACS Miltenyi Biotec (Bergisch Gladbach, Germany) was used to isolate T cells from the splenocyte cell suspension. All dilutions were based on 10,000,000 or fewer splenocytes. The splenocyte cell pellet was resuspended in 40uL of auto MACS rinsing solution and then incubated in 10uL of Biotin-Antibody cocktail for 5 minutes at 4℃. Following which, 5000uL of the auto MACS rinsing solution was added and incubated in 20uL (per 10,000,000 cells) of Anti-Biotin MicroBeads for 10 minutes at 4℃. The LS column from MACS Miltenyi Biotec (Bergisch Gladbach, Germany) was placed in the magnetic field of MACS Separator. The column was rinsed with 3mL of auto MACS rinsing solution. Then the cell suspension was applied on to the column and the flow through was captured. The column was washed again with auto MACS rinsing solution and flow through was captured. The flow through – containing enriched T cells – was resuspended in Peripheral Blood Mononuclear Cell (PBMC) medium (Petersen et al.) for T cell culture experiments.

T cell proliferation assay

100,000 purified splenic T cells were cultured in PBMC medium in 96 wells plate pre-coated with 1ug/mL of functional anti-CD 3 antibody. Anti-CD3 antibody was obtained from eBioscience (San Diego, California, USA)). T cells growth was stimulated by adding 3.75U/mL of recombinant murine IL-2. Recombinant murine IL-2 was obtained from Peprotech (Rocky Hill, New Jersey, USA). T cells were treated daily by the daily addition of 0.3uM or 3.0uM of VIP, scrambled peptide, or ANT-08. After 72 hours culture in 37℃ 5% CO_2 humidified incubator, cells

were counted manually using hematocytometer or bio-luminescence imaging of luciferase positive splenic T cells after adding 150 ug/mL of luciferin into each well and analyzing using IVIS Spectrum instrument and Living Image software to identify and quantify T cell activation/proliferation. T cells were later stained with a cocktail of flow antibodies and the samples were run on FACS Aria BD and analyzed using FlowJo 2 software.

Bioluminescent Imaging of mice

Bioluminescent imaging was used to quantify tumor burden in mice inoculated with luciferase+ melanoma cell lines. Mice were anesthetized by isoflurane inhalation. The site of tumor inoculation was shaved to have the least possible interference. 150mg/kg luciferin, obtained from GoldBio (St. Louis, Missouri, USA), was intraperitoneally injected into mice. Images were acquired at 2 minutes and 4 minutes time points using IVIS Spectrum instrument and analyzed using Living Image software.

Animal care

Six to eight weeks old female C57BL/6 mice were obtained from Charles River Labs (Wilmington, Massachusetts, USA). Mice were housed in Emory University Division of Animal Resources (DAR) facilities. All experimentation on mice was done 7 days after their date of arrival to give them adequate time to acclimatize. Endemic infections were monitored by Emory DAR using cages of sentinel mice. Mice were euthanized when the tumor volume reached the IACUC endpoint of $500mm^3$. Mice with minor ulcerating tumors were treated with topical Neosporin on recommendation by the veterinarian. In the case of major tumor ulceration, mice were euthanized.

Tumor Transplantation Models

250,000 B16F10 or D4M cells were injected subcutaneously on to the shaved right flank of mice. Following inoculation, mice were monitored daily and treatments were started after the tumors were palpable. Tumor measurements were taken every 48 hours using Vernier calipers and tumor volume was calculated with an approximation of $4/3*\pi*r^3$ using the formula: (length*breadth*height)/2.

VIP Receptor Antagonist Treatment

VIPhyb, ANT-00, ANT-08 and scrambled peptide were purchased from RS synthesis (Louisville, Kentucky, USA). All peptides were dissolved in di-ionized water and diluted with PBS to make stock solutions. Stock solutions were stored at -20℃. Stock solutions were further diluted in PBS to make working solutions of 10ug/100uL. Treatments were started after tumors were palpable. 10ug of the VIP receptor antagonist or scrambled peptide were injected subcutaneously peritumorally or between the shoulder blades, into mice for 10 days or until the time of euthanasia.

Anti-mouse PD1 Treatment

InVivoMAb anti-mouse PD-1 (CD279) was obtained from Bio X Cell (West Lebanon, New Hampshire, USA). The anti-mouse PD1 treatment was started after the tumors were palpable. The control mice received isotype IgG2a, obtained from Bio X Cell (West Lebanon, New Hampshire, USA). 200ug of the respective treatment was delivered through intra-peritoneal injection once every three days.

Murine Tissue Harvestation

At the time of mice euthanasia, mice were terminally bled and blood collected into a micro-centrifuge tube purchased from Thermo Fisher Scientific (Waltham, MA, USA). Blood samples were centrifuged at 2700rpm for 10 minutes at 4℃. The cell pellet was discarded and serum as collected. Tumors from the mice were resected and half of the tumor was formalin-fixed for Immunohistochemistry (IHC) staining. The other half of the tissue was utilized for flow cytometric analysis. T cells from splenocytes of euthanized mice were isolated and stored at -80 in media containing 10% v/v DMSO for future experiments.

Immunohistochemistry (IHC)

Formalin-fixed tumor samples were stained with H&E, CD4, CD8 and VIP mouse monoclonal antibody and embedded in paraffin. CD4 and CD8 monoclonal antibodies were obtained from Santa Cruz Biotechnology (Dallas, Texas, USA) and the VIP monoclonal antibody was obtained from OriGene (Rockville, Maryland, USA). IHC staining was performed by Pathology Core Laboratory at Winship Cancer Institute of Emory University.

Co-culture Assay

Luciferase positive mice (luc+) splenocytes (effectors) were isolated as per the steps listed above. Luc+ splenocytes were co-cultured with irradiated B16F10 cells (targets) at E:T ratios of 1:0.1, 1:0.3, 1:1, 1:3, and 1:10 in 96 wells plate pre-coated with 1ug/mL of functional anti-CD 3 antibody (eBioscience, San Diego, Califronia, USA). T cell proliferation was augmented by the addition of 3.75U/mL of recombinant murine IL-2 in cultures maintained at 37℃ in a 5% CO_2

humidified incubator. After 72 hours, the supernatants from all the samples were collected and the cells were stored down at -80℃ for flow cytometric staining and analysis.

Western Blotting

B16F10, B16F1 and D4M lysates were prepared using the standard lysate preparation protocol. VPAC1 and VPAC2 protein expressions were measured from the cell lysates. The lysates were loaded on to the 4%-20% gradient gel obtained from BioRad (Hercules, California, USA), electrophoresed, and then proteins from the gel were transferred on to the nitrocellulose membrane. The membranes were probed with 1:500 dilutions for the anti-VPAC1 and anti-VPAC2 antibodies overnight at 4℃. After washes, the membrane was incubated in the secondary antibody at 1:2000 dilutions for 60 minutes at 4℃ and then washed 3 times with 1X TBS. The primary and secondary antibodies were obtained from Santa Cruz Biotechnology (Dallas, Texas, USA). The membranes were imaged using Azure Biosystems c600 and protein expression was quantified using Azure Biosystems c600.

VIP-Enzyme Immunoassay (EIA)

Mouse VIP-EIA kit was obtained from RayBiotech (Peachtree Corners, Georgia, USA). Human serum, mouse serum, and melanoma cell lines supernatants were probed for the VIP levels using the kit. The 96 wells plate was coated overnight with VIP antibody. The diluted or undiluted samples and standards were adding to wells followed by biotinylated VIP and the non-specific binding sites were blocked with HRP. Following washes and incubations the plate was read for bioluminescence using BioRad instrument. Higher bioluminescence reading is proportional to

greater VIP concentration. Only the test with r-squared value of standard curve greater than 0.95 were considered reliable.

V-PLEX

Supernatants from T cells treated with VIP, ANT-08, or scrambled peptide were stored down at -80℃ to measure levels of the proinflammatory cytokines including IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, KC/GRO, and TNF-α. All the antibodies were precoated in a 96 well plate obtained from Meso Scale Diagnostics (Rockville, Maryland, USA). V-PLEX cytokine data collection was done in collaboration with Emory Genomics Core.

Flow Cytometry

Spleen, tumor and blood samples were collected from mice upon euthanasia. In order to get single cell suspensions, spleen and tumor samples were dissociated in RPMI complete media using the back of a syringe plunger, and the contents were passed through a 70um strainer. Following, red blood cells from all spleen, tumor and blood samples were lysed using lysis buffer. Samples were counted to determine White Blood Cell count using Beckman Coulter cell counter (Brea, CA, USA). 500,000 cells of respective samples were then incubated with LIVE/DEAD™ Fixable Aqua Dead Cell Stain from Thermo Fisher Scientific (Waltham, MA, USA), for 10 minutes in 4℃. After washing, samples were incubated in the presence of an anti-CD16/32 Fc receptor-blocking antibody for 5 minutes at room temperature in dark. After blocking, samples were stained with following antibodies: APC-Cy7 CD3, FITC CD4, PerCP Cy 5.5 CD8, PerCP CD25, BV421 NK1.1, PE Ki67, APC CD223 (Lag- 3), PE-Cy7 CD279 (PD-1), and PE Texas red

FoxP3. All flow antibodies were purchased from Biolegend (San Diego, CA, USA). All samples were run on a FACS Aria flow cytometer (BD) and analyzed using FlowJo software.

RESULTS

Murine Melanoma cell lines and tumor tissue does not endogenously secrete VIP.

In order to determine the source of the VIP in tumor-bearing animals, we first measured VIP levels in cell culture supernatants of various melanoma cell lines, namely, B16F10, B16F1, SM1 and D4M. 300,000 cells of B16F10, B16F1, SM1 and D4M cells were incubated for 24 hours in 37℃ 5% CO_2 humidified incubator. Collected supernatants were centrifuged to remove any floating dead cells. VIP levels were determined using mouse VIP-EIA. No appreciable VIP levels were detected from the supernatants of all four murine melanoma cells, as shown in figure 12 (a). Additionally, 100,000 cultured B16F10 cells were plated on a slide in a monolayer using cytospin. The slides were stained using immune-fluorescent VIP monoclonal primary antibody and the slides were imaged using confocal microscopy. As shown in figure 12 (b), melanoma cells didn't secret appreciable VIP levels. In addition, formalin-fixed melanoma tumor tissue samples were sectioned and stained with monoclonal VIP antibody to probe for VIP secretions in the tumor microenvironment. Consistent with our previous findings, appreciable VIP signal was not detected from tumor tissues (Figure 12c).

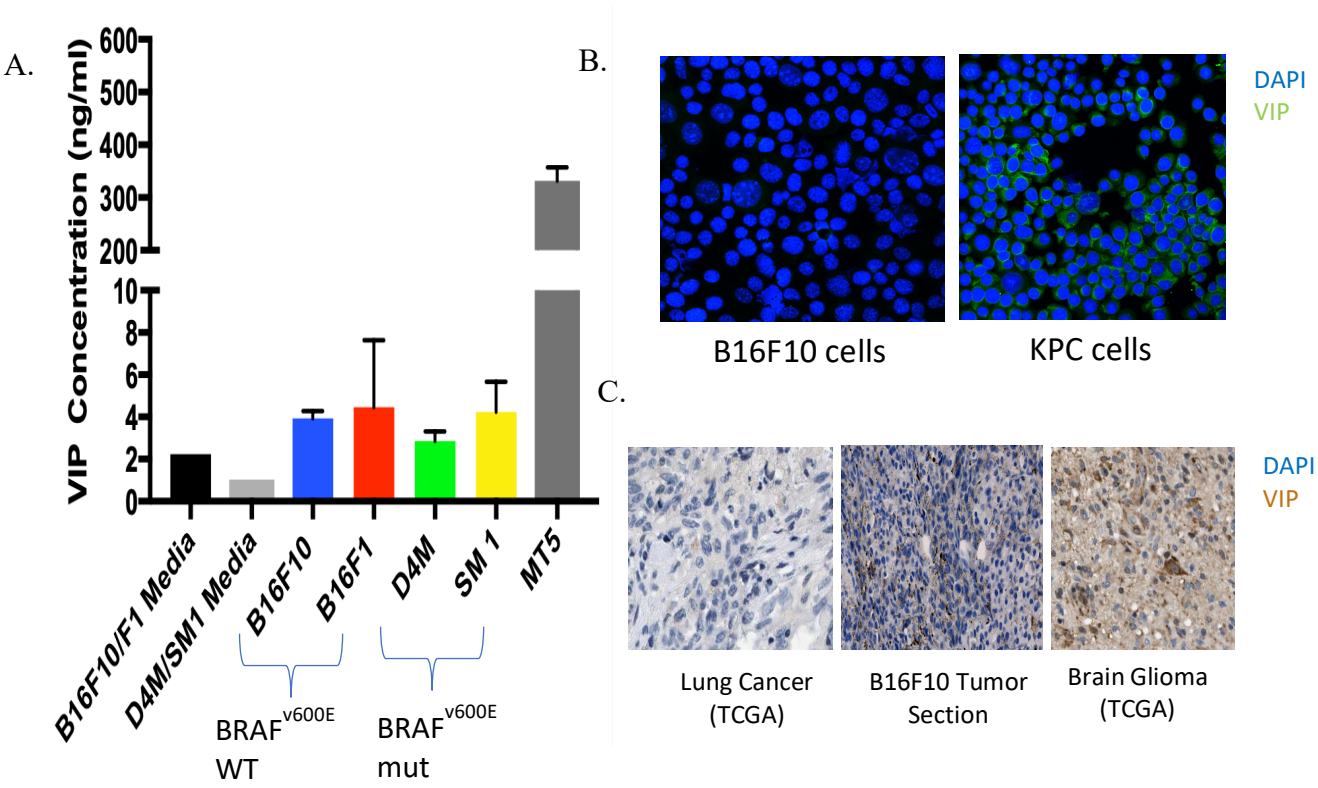


Figure 12. Murine melanoma cell lines and tumor tissue do not endogenously secrete VIP. (A) 300,000 B16F10, B16F1, D4M and SM1 cells were cultured for 24 hours in 3mL of media. Following, supernatants were collected, and VIP levels were measured using VIP-EIA kit. (B) To visualize secretion of VIP, 100,000 B16F10 melanoma cells or KPC pancreatic cells (positive control) were spun down using cytospin. Staining with anti-VIP antibody and immunofluorescence was visualized using confocal fluorescent microscopy. Blue represents DAPI and Green represents VIP. (C) Resected murine melanoma tumors were formalin-fixed and were stained with anti-VIP antibodies using immunohistochemistry. Lung cancer and brain glioma were negative and positive control for The Cancer Genome Atlas (TCGA).

Elevated levels of VIP in serum of B16F10 tumor bearing mice

To determine if VIP levels change with B16F10 tumor inoculation, we injected 250,000 B16F10 tumor cells subcutaneously. Mice were regularly monitored for tumor growth using Vernier calipers and were terminally bled and euthanized when the tumors reached the predetermined volumes of 200, 400, 600, 800 and 1000mm^3 .

As shown in figure 13 (a), there was no significant correlation between tumor volume and VIP levels in mice serum. However, overall, tumor-bearing mice had significantly elevated levels of VIP when compared to naïve tumor-free mice (Figure 13B).

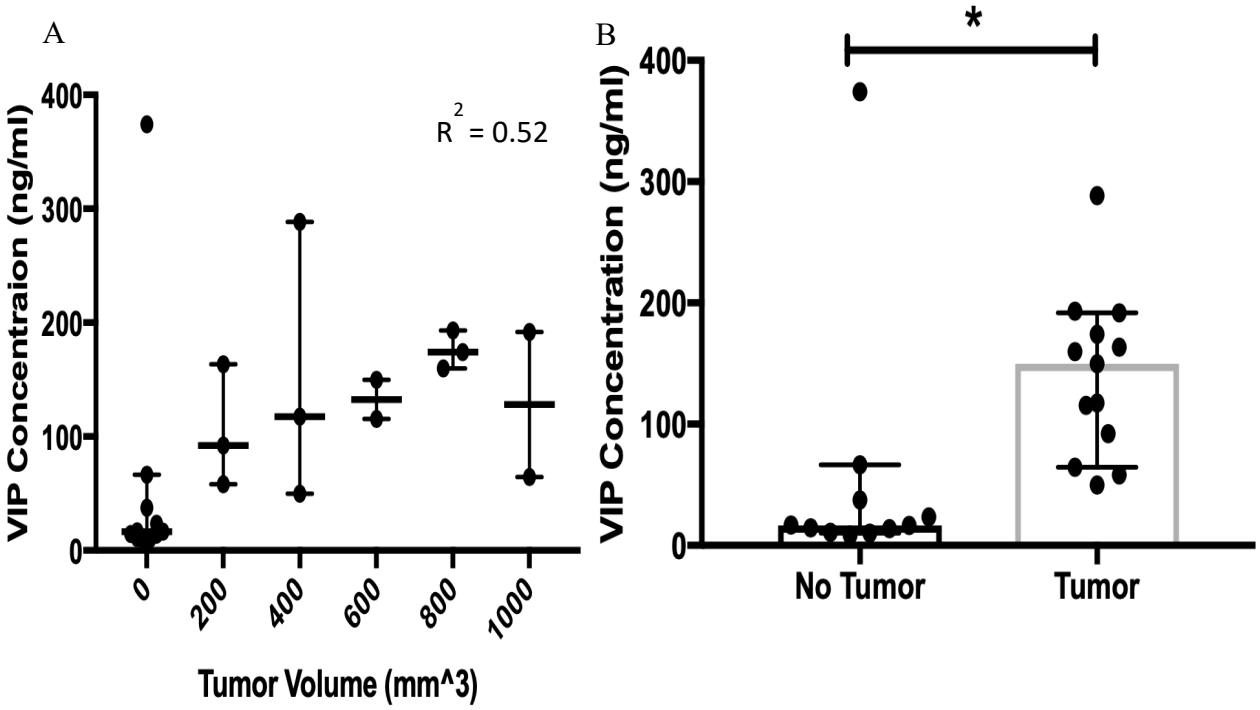


Figure 13. Mice with B16F10 tumors have elevated VIP levels than tumor-free mice. (a) 250,000 B16F10 cells were injected subcutaneously into mice. No treatment was given to the mice and the mice were euthanized at predetermined tumor volumes of 0, 200, 400, 600, 800 and 1000 mm^3. Serum was collected at the time of euthanasia and VIP levels were measured using VIP-EIA.

(B) Serum VIP levels were pooled from all the mice inoculated with the tumors and compared with the serum VIP levels from mice without tumors. *$p<0.05$

ANT-08 promotes in-vitro proliferation of murine T cells

To determine if T cell proliferation was regulated by VIP, mouse splenic T cells were stimulated in culture with anti-CD3 in media containing 30U/ml of IL-2, with/without 3uM peptide containing scrambled VIP sequence (VS-2), native VIP or the VIP antagonist ANT-08. As anticipated based on the immunosuppressive properties of VIP, the addition of VIP to cell cultures resulted in decreased levels of T cell expansion when compared to the cells treated with the scrambled peptide. On the other hand, the addition of ANT-08 to the culture media resulted in significantly enhanced expansion of T cells when compared to scrambled or VIP treated T cells. (Figure 14A and B). The augmentation of T cell proliferation by a VIP antagonist that blocks signaling through the VIP receptor indicates autocrine synthesis of VIP by activated T cells.

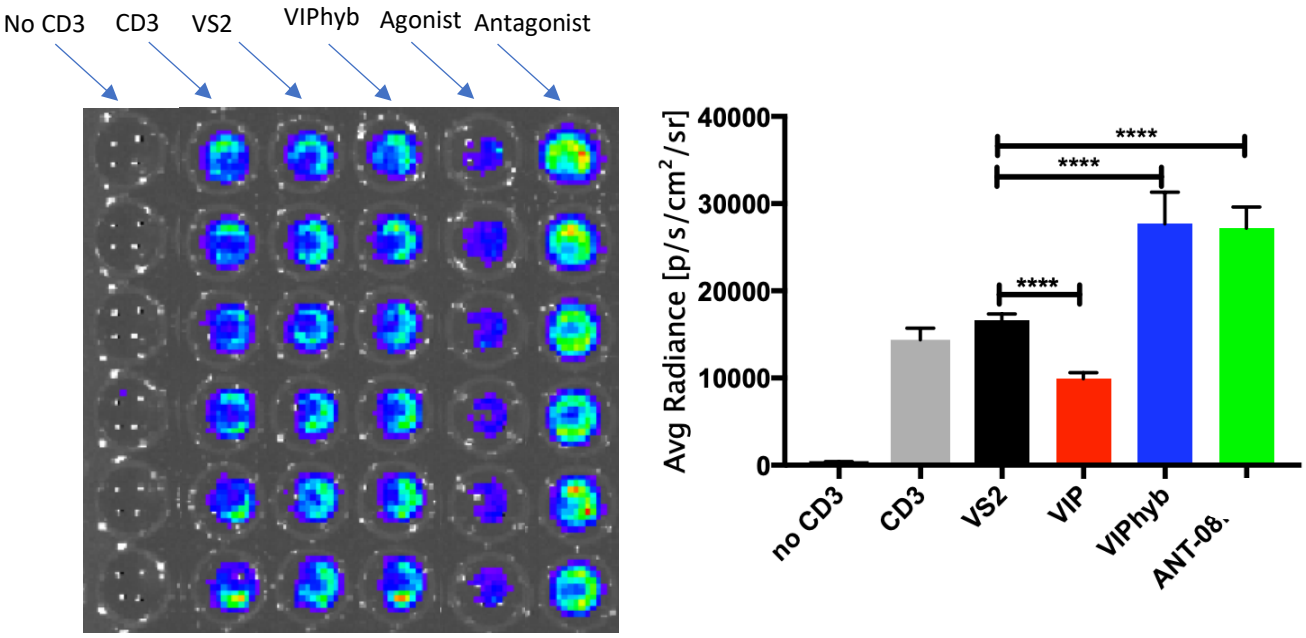


Figure 14. Antagonizing VIP receptor signaling promotes proliferation of T cells. (A) Splenic luciferase positive T cells were treated with VIPhyb and VIP at varying concentrations. BLI representative image of T cells' anti-proliferatory or proliferatory response to respective treatments. (B) Quantification of the BLI image showing. represents the VIP. ****P<0.0001. (Yiwen Li, unpublished data, Waller lab).

VIP is secreted by activated T cells

To document T cell synthesis of VIP, the cytospin technique was used to plate 100,000 T cells in a monolayer on a slide and stained with monoclonal VIP antibody and imaged with confocal microscopy. T cells were isolated from human PBMCs. They were cultured for 72 hours in media or media supplemented with 10ng/ml PMA and 0.5ug/ml ionomycin (iono). As shown in figure 15A, unstimulated enriched T cells did not secrete VIP. However, when the T cells are stimulated in culture with PMA/iono. VIP secretion is observed (Figure 15B).

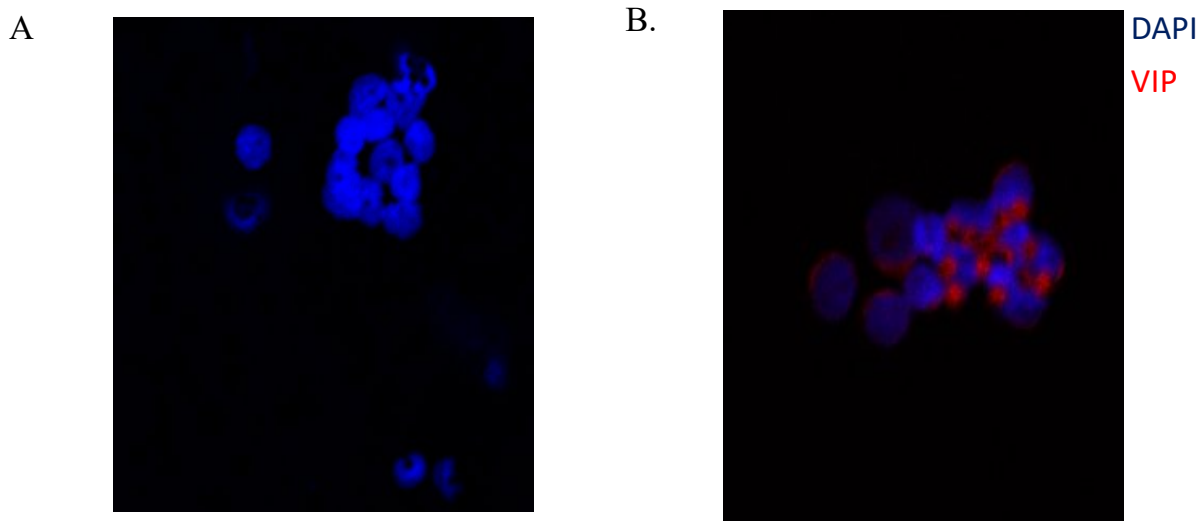


Figure 15. Stimulated human T cells secrete VIP. T cells were isolated from human PBMCs. (A) T cells were cultured for 72 hours without any stimulation. (B) T cells were cultured for 72 hours and stimulated with PMA/ionomycin in culture. 100,000 of these purified and cultured T cells were plated on a slide in a monolayer using cytospin. Following, the slides were stained for VIP. Confocal microscopy was used to image the slides.

ANT-08 inhibits T cells autocrine VIP receptor signaling

To test if ANT-08 blocks autocrine VIP receptor signaling in T cells, splenic T cells from 3 VIP-WT and 3 VIP-KO mice were isolated and cultured with VIP, scrambled peptide and ANT-08 at varying concentrations. A 72 hours proliferation assay was used to measure T cell activation. The splenic T cells were counted before and after the treatment using hematocytometer. As expected, VIP inhibited proliferation of splenic T cells from VIP-WT mice, and ANT-08 promoted proliferation of splenic T cells from VIP-WT mice 08 (figure 16A). There was no significant difference in the T cell proliferation when treated with VIP scrambled peptide. Interestingly, ANT-08 did not promote proliferation of splenic T cells from VIP-KO as shown in Figure 16B.

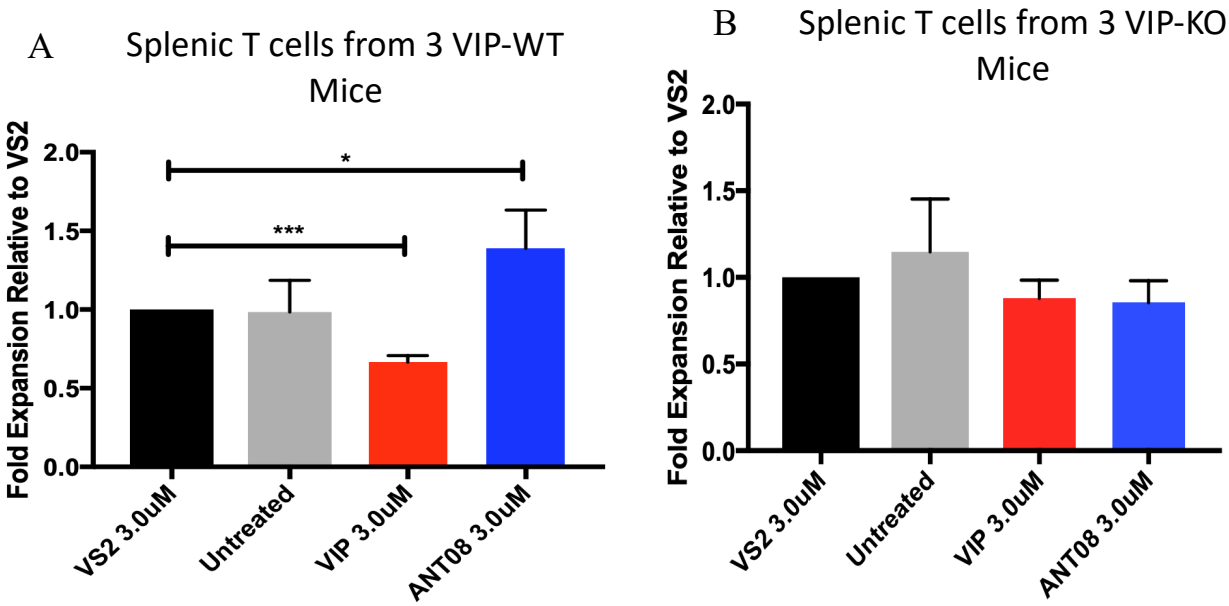


Figure 16. ANT-08 blocks the autocrine VIP receptor signaling in T cells. (A) 1,000,000 purified splenic T cells from VIP-WT mice are expanded for 72 hours and were treated with VIP or VIP scrambled peptide or ANT-08 every 24 hours. The T cells were counted before and after expansion to quantify suppressive or stimulatory effects of the treatment. (B) 1,000,000 purified

splenic T cells from VIP-KO mice are expanded for 72 hours and were treated with VIP or VIP scrambled peptide or ANT-08 every 24 hours. The T cells were counted before and after expansion to quantify suppressive or stimulatory effects of the treatment.

ANT-08 promotes secretion of TNF-a in enriched T cells from VIP-WT mice

T cells were isolated from 3 VIP-WT and 3 VIP-KO naïve mice. Following isolation with magnetic beads and columns, T cells were treated with 3uM of VIP or VIP scrambled peptide (VS2) or ANT-08 every 24 hours for 72 hours. Supernatants were collected following 72 hours expansion. The supernatants were tested for levels of TNF-a using VPLEX plate. VIP-KO T cells, regardless of the treatment group, VIP-KO had significantly higher levels of TNF-a secretion (Figure 17A). VIP-WT T cells treated with ANT-08 showed significantly increased secretion of TNF-a and when treated with VIP showed significant downregulation (Figure 17B). At the same time, enriched VIP-KO T cells did not have any effects upon treatment with ANT-08, while VIP treatment caused significant downregulation of TNF-a secretion (Figure 17C).

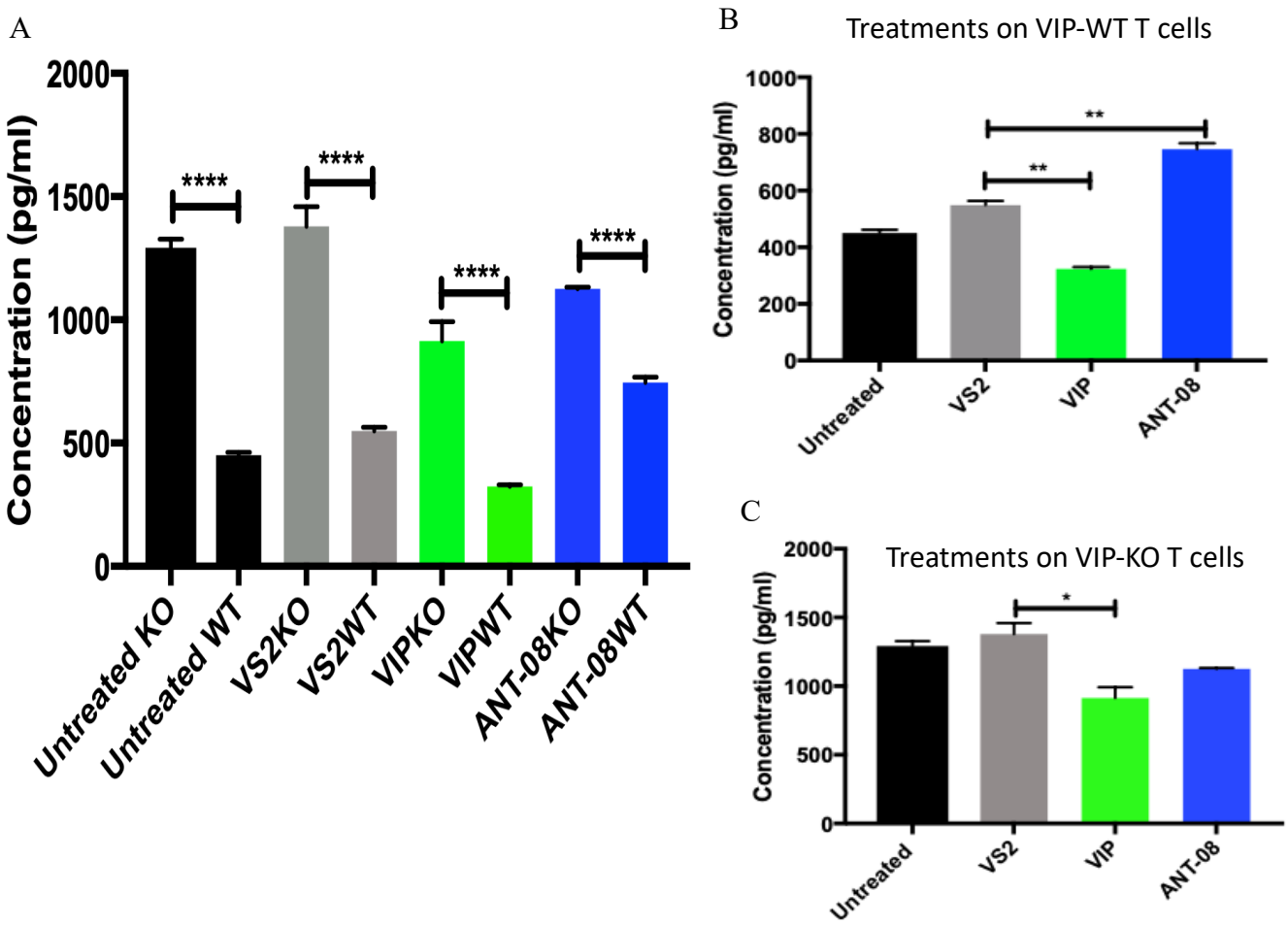


Figure 17. ANT-08 promotes secretions of TNF-a from enriched T cells. T cells were isolated from spleens of 3 VIP-WT and 3VIP-KO mice. Following isolation, T cells were cultured and treated with VIP or scrambled peptide or ANT-08 for 72 hours and tested for TNF-a using VPLEX. (A) T cells from VIP-KO mice has significantly higher levels of TNF-a. (B) ANT-08 treated VIP-WT T cells had significantly higher levels of TNF-a. (C) VIP-KO T cells treated with VIP had significantly lower levels of TNF-a levels (Figure 15C). *P<0.05; ****P<0.001

ANT-08 did not promote secretion of TNF-a in B16F10 tumor bearing mice serum

250,000 B16F10 tumor cells were inoculated in VIP-WT and VIP-KO mice. Once the tumors were palpable, mice were treated with 10ug scrambled peptide (VS-2) or ANT-08 for 10 days. At the time of euthanasia, serum was collected to measure TNF-a levels. There were no significant differences in TNF-a levels in the sera of VS-2 treated mice or ANT-08 treated mice (Figure 18A-B).

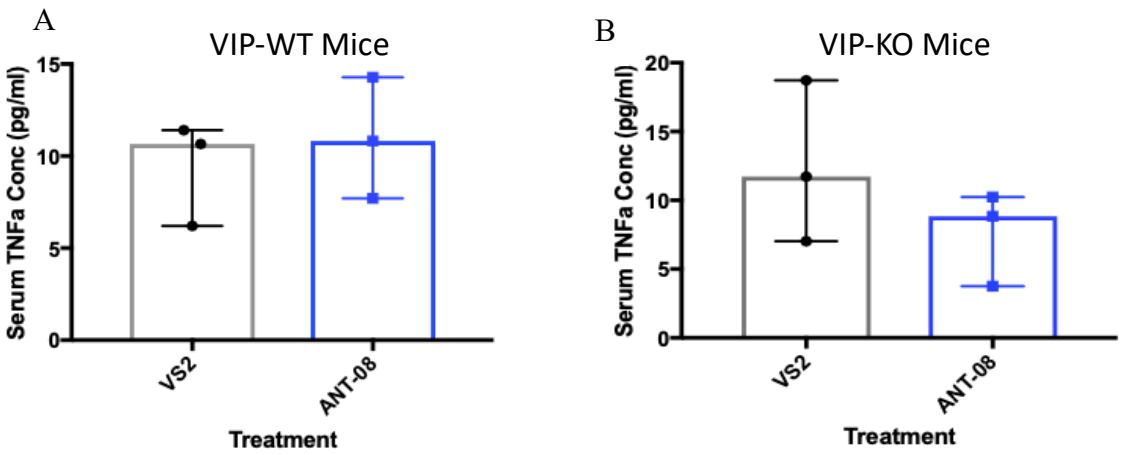


Figure 18. ANT-08 treated mice do not have significantly different TNF-a levels in the serum. B16F10 melanoma tumor bearing mice were treated with VS-2 or ANT-08. Upon euthanasia, serum was collected to measure TNF-a levels. (A) Tumor-bearing VIP-WT mice treated with ANT-08 had no difference significant difference in the serum TNF-a levels. (B) Tumor-bearing VIP-KO mice treated with ANT-08 had no significant difference.

ANT-08 promotes secretion of IL-6 in enriched T cells from VIP-WT mice

T cells were isolated from 3 VIP-WT and 3 VIP-KO. All were naïve mice. Following T cell isolation, they were treated with 3uM of VIP or VIP scrambled peptide (VS2) or ANT-08 every 24 hours for 72 hours. Supernatants were collected following 72 hours expansion. The supernatants were tested for levels of IL-6 using VPLEX plate. VIP-KO T cells, regardless of the treatment group, had significantly higher levels of IL-6 secretion than WT T cells (Figure 19A). VIP-WT T cells treated with ANT-08 showed significantly increased secretion of IL-6 (Figure 19B). At the same time, ANT-08 treatment did not have any effects on IL-6 secretion in VIP-KO T cells, while VIP treatment caused downregulation of IL-6 secretion (Figure 19C).

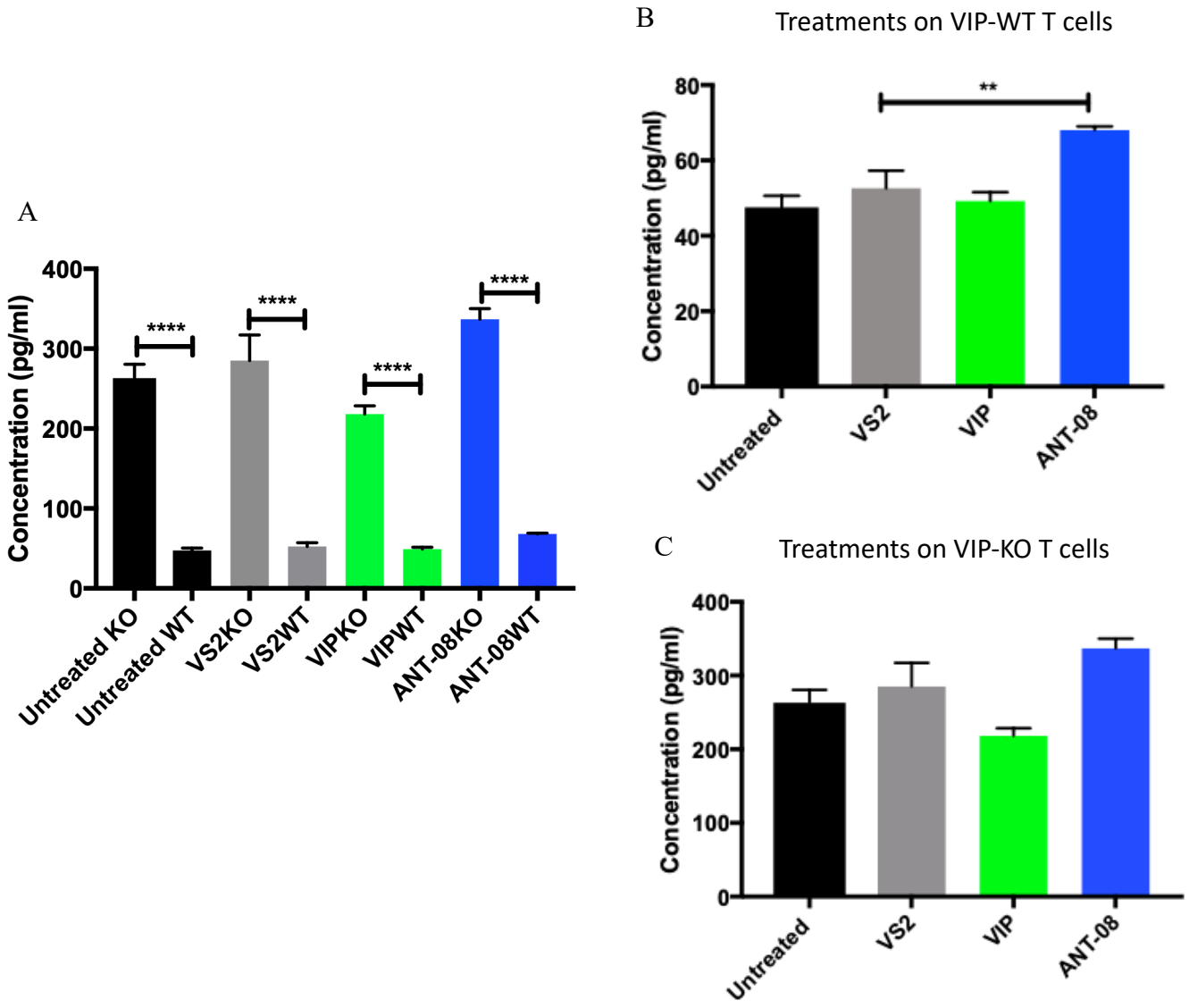


Figure 19. ANT-08 promotes secretions of IL-6 from enriched T cells. T cells were isolated from spleens of 3 VIP-WT and 3VIP-KO mice. Following isolation, T cells were cultured and treated with VIP or scrambled peptide or ANT-08 for 72 hours and tested for IL-6 using VPLEX. (A) T cells from VIP-KO mice has significantly higher levels of TNF-a. (B) ANT-08 treated VIP-WT T cells had significantly higher levels of IL-6. (C) VIP-KO T cells did not have any significant differences in IL-6 levels (Figure 17C). ****P<0.001

ANT-08 promotes secretion of IL-6 in VIP-WT mice serum

250,000 B16F10 tumor cells were inoculated in VIP-WT and VIP-KO mice. Once the tumors were palpable, mice were treated with daily subcutaneous injections of 10ug VIP scrambled peptide (VS-2) or ANT-08 for 10 days. At the time of euthanasia, serum was collected to measure IL-6 levels. IL-6 levels were significantly higher in serum of WT VIP-treated mice, (Figure 20A). On the contrary, there was no difference in the IL-6 levels in the VIP-KO mice sera (Figure 20B)

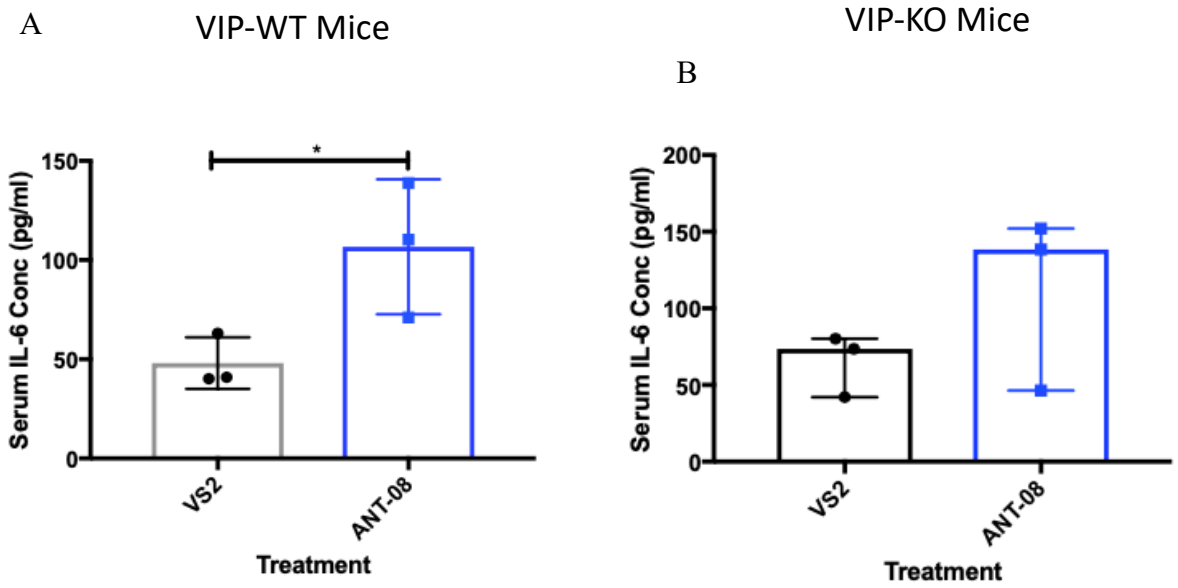


Figure 20. ANT-08 treated VIP-WT mice had elevated IL-6 levels in serum. B16F10 melanoma tumor bearing mice were treated with VS-2 or ANT-08. Upon euthanasia, IL-6 levels were measured in serum. (A) Tumor-bearing VIP-WT mice treated with ANT-08 had significantly increased serum IL-6 levels. (B) Tumor-bearing VIP-KO mice treated with ANT-08 had no significant difference in the IL-6 levels in serum. *$p<0.05$.

ANT-08 caused significantly higher proportion of proliferating CD4+ cells in TILs from B16F10 tumors

250,000 B16F10 tumor cells were inoculated in mice. Once the tumors were palpable, mice were treated with control treatment or VIP antagonists (VIPhyb or ANT-08). At the time of euthanasia, tumor, spleen, and blood were harvested for flow cytometry. Regardless of the treatment group, tumors had significantly higher frequency of proliferating CD+ T cells then spleen or blood (Figure 21 A-C). In addition, tumors from the treatment group had significantly higher proportion of CD4+/Ki67+ T cells (Figure 21A).

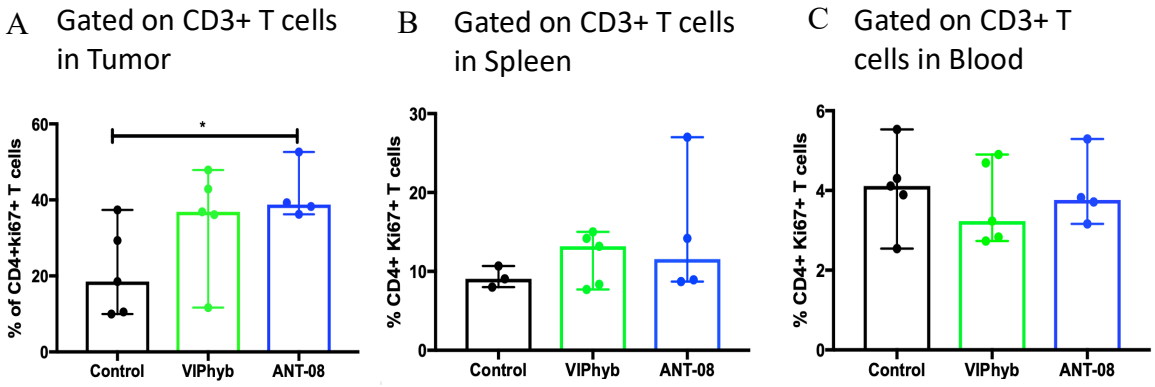


Figure 21. VIP antagonists treated mice had significantly higher proportion of CD4+/Ki67+ TILs. Tumors were resected at the time of euthanasia and processed in single cell suspensions for flow cytometry. N = 3-5 per group. (A) TILs from the treated groups had significantly higher proportion of proliferating CD4+ cells. (B) No significant differences in the phenotype of T cells were observed in the spleen samples. (C) No significant differences in the phenotype of T cells were observed in the blood samples.

ANT-08 as a monotherapy agent did not yield significant survival differences in B16F10 melanoma model

To test single-agent efficacy of ANT-08 against murine melanoma, 20 VIP-WT mice or 20 VIP-KO mice were subcutaneously injected with B16F10 melanoma cells on the right flank. Mice were monitored daily to test for tumor growth. Once the tumors were palpable, mice were treated with subcutaneous daily injections of 10ug scrambled peptide or ANT-08 for 10 continuous days palpable (Figure 22A). Tumor volumes were measured every 48 hours using Vernier calipers. There were no significant differences between survival rates or tumor growth rates in VIP-WT groups (Figure 22B, C) or VIP-KO groups (Figure 22B, D). There were no significant differences in survival between VIP-WT and VIP-KO mice (Figure 22B, E).

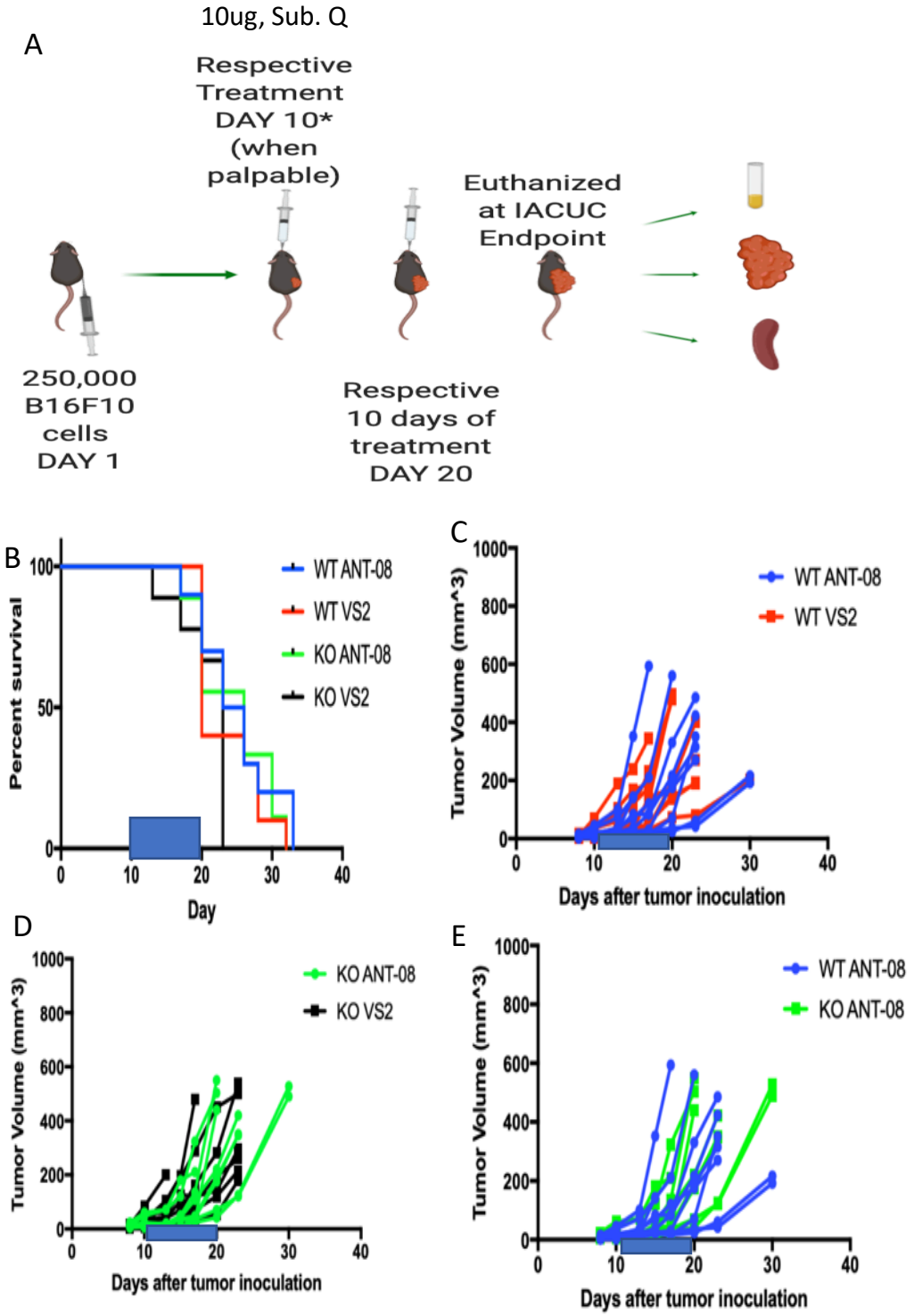


Figure 22. ANT-08 did not have single-agent anti-tumor activity against B16F10 melanoma in tumor-bearing mice. (A) VIPWT and VIP-KO mice were inoculated with 250,000 B16F10 cells. 10ug of ANT-08 or VS-2 was administered subcutaneously for 10 days after tumors became palpable. (B) There was no survival difference between the treated and untreated groups. (C) Tumor volumes compared between VIP-WT mice treated with ANT-08 and VS-2. No significant differences were observed. (D) Tumor volumes compared in VIP-KO mice treated with ANT-08

and VS-2. No significant differences were observed. (E) Tumor volumes compared between VIP-WT and VIP-KO treatment groups.

Anti-PD1 did not improve survival rates in VIP-KO mice bearing B16F10 tumors

With the eventual goal of synergizing ANT-08 and anti-PD1, we wanted to test if the anti-PD1 therapy would improve survival rates in VIP-KO mice, an optimal case of VIP antagonism. 20 VIP-WT mice or 20 VIP-KO mice were subcutaneously injected with B16F10 melanoma cells on the right flank. Mice were monitored daily to test for tumor growth. Once the tumors were palpable, mice received intraperitoneal treatment injections every 3 days of 200ug of anti-PD1 antibody or an isotype-matched control antibody over a 10-day period (4 doses in all) after tumors became palpable (Figure 23A). Tumor volumes were measured every 48 hours using Vernier calipers. There were no significant differences between survival rates or tumor growth rates in VIP-KO groups (Figure 23B, C) or VIP-WT groups (Figure 23B, D). There was no significant difference between VIP-WT and VIP-KO (Figure 23B, E).

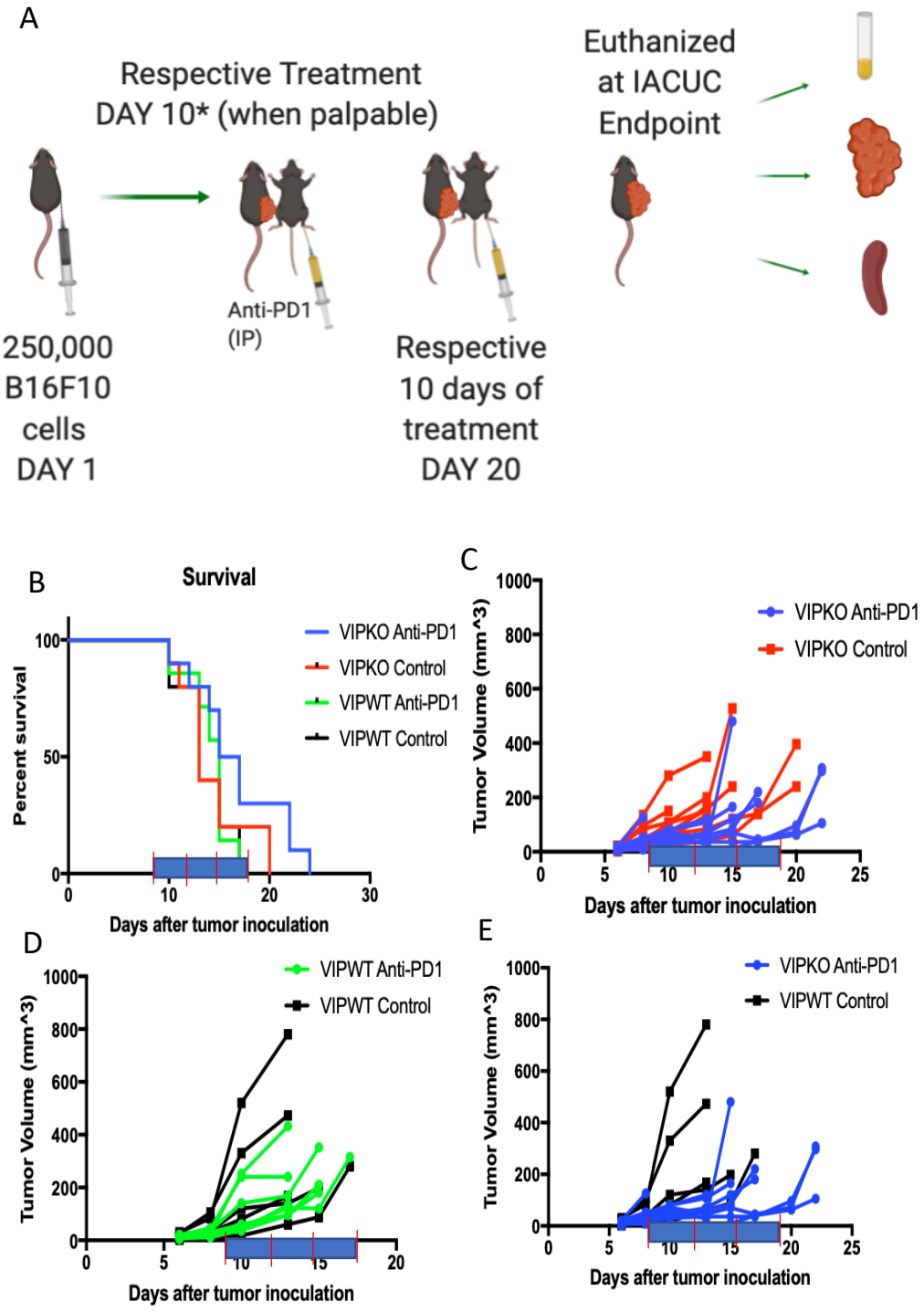


Figure 23. Anti-PD1 treatment did not have improve survival in tumor-bearing VIP-KO mice. (A) VIPWT and VIP-KO mice were inoculated with 250,000 B16F10 cells. 200ug of anti-PD1 or igG2a control isotype was intraperitoneally administered every 3 days over 10 days after tumors became palpable. (B) There was no survival difference between the treated and untreated groups. (C) Compares tumor volumes in VIP-KO mice treated with anti-PD1 or igG2a control. No

significant differences were observed. (D) Compares tumor volumes in VIP-WT mice treated with anti-PD1 or igG2a control. No significant differences were observed. (E) Compares tumor volumes between VIP-WT and VIP-KO from the treatment group. No significant differences were observed.

D4M and B16F10 cells express VIP receptors

D4M and B16F10 cell lysates were prepared by culturing 1,000,000 respective cells for 24 hours and levels of VPAC1 and VPAC2 expression were measured by western blotting. Both the VIP receptors were expressed on the tumor cells at similar levels (Figure 24).

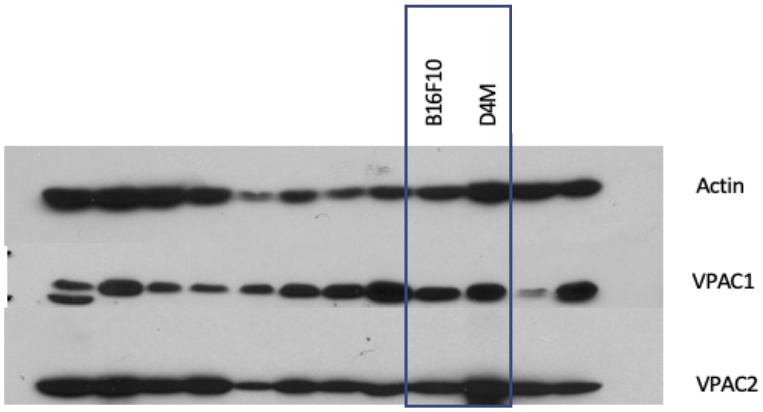


Figure 24. D4M and B16F10 melanoma cells express VPAC1 and VPAC2 receptors. 1,000,000 respective tumor cells were cultured in 3ml media for 24 hours. Following incubation, the cell lysates were made. The cell lysates were probed for VPAC1 and VPAC2 expression. D4M and B16F10 cells express VIP receptors.

ANT-08 has direct cytostatic effects on the growth of D4M cells *in vitro*

To test for any direct cytostatic effects of VIPhyb on D4M and B16F10 cells, the MTT assay was performed on cells cultured with VIP antagonist. 2400 D4M or B16F10 cells were plated on a 96 wells plate and treated daily by the addition of 3uM VIP antagonist peptide of (VIPhyb) for 48 hours. Addition of MTT reagent prompted a change in color that was proportional to the cells that were alive. Changes in color were measured using colorimeter and absorbance was measured at 570nm. The addition of 1uM to 5uM VIPhyb had significant cytostatic effect on D4M cells following 48 hours incubation (Figure 25A, B). In contrast, VIPhyb did not have direct cytostatic effect on B16F10 cells (Figure 25C, D). Vemurafenib (Vem) and Trametinib (Tram) were used as the positive controls for cytostatic effects of BRAF mutation positive murine melanoma model.

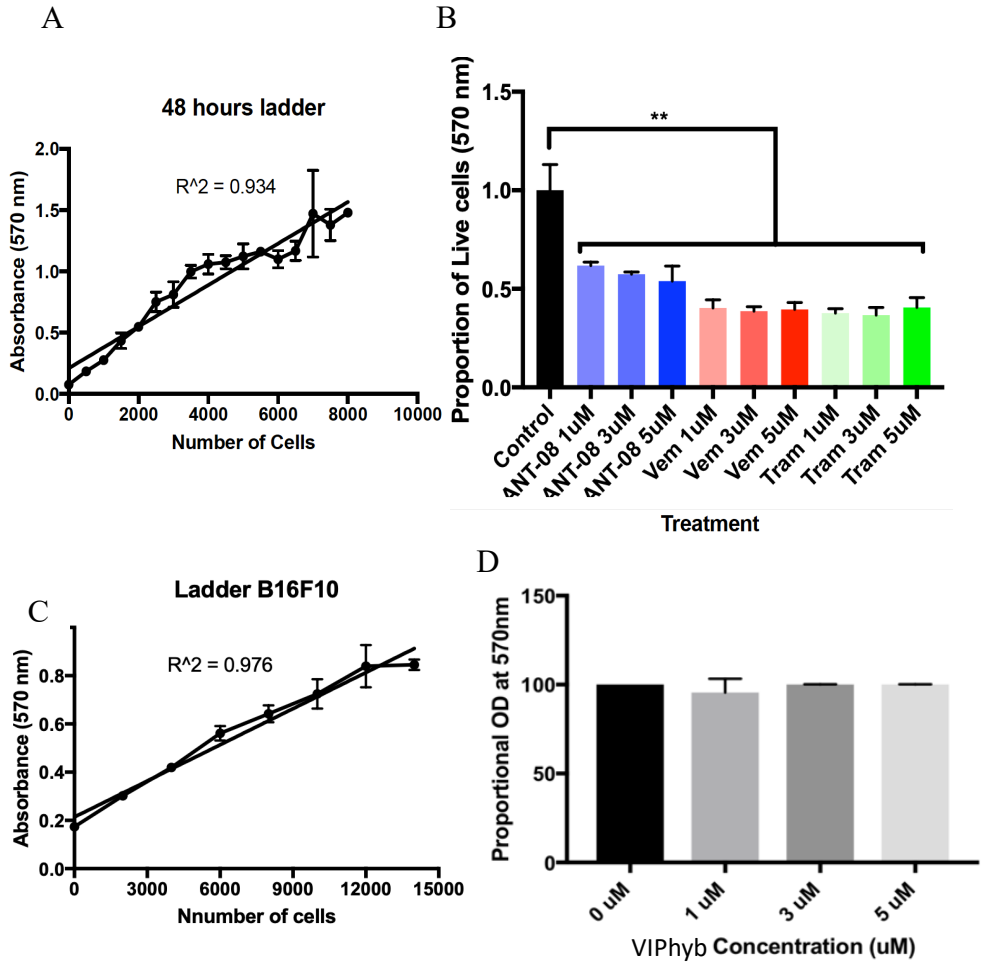


Figure 25. ANT-08 has direct cytostatic effects on D4M cells *in vitro.* (A) number of D4M cells are proportional to absorbance OD for D4M cells. Cells used to make dose-response in panels A and C were not treated with VIPhyb. (B) VIPhyb had significant direct cytostatic effects on D4M after 48 hours of treatment. (C) number of B16F10 cells are proportional to absorbance OD for D4M cells. (D) B16F10 cells did not show cytostatic effects following VIPhyb treatment for 48 hours. Vemurafenib (Vem) and trametinib (tram) were used as positive controls for treatment-induced cell death as they are the standard of care of BRAF mutation positive melanomas.

ANT-08 does not have mono-therapeutic or synergistic effect with anti-PD1 on tumor growth and survival rates against the D4M inoculated tumors in mice

250,000 D4M cells were subcutaneously injected into mice. Treatments were started only after the tumors were palpable and treatments included ANT-08 or anti-PD1 or combination. 10ug of ANT-08 was subcutaneously injected every day for 10 days while 200ug of anti-PD1 was intraperitoneally injected once every three days or a total of 4 doses (Figure 26A). Tumors were monitored daily and tumor volumes were measured every 48 hours using Vernier calipers. The combination-treated group and anti-PD1 treated group had significantly better survival rates. The anti-PD1 treated group have 20% tumor free mice (Figure 26B, C) and combination treated group have 30% tumor free mice. (Figure 26B, E). However, group receiving ANT-08 monotherapy did not show any differences in tumor volumes (Figure 2D).

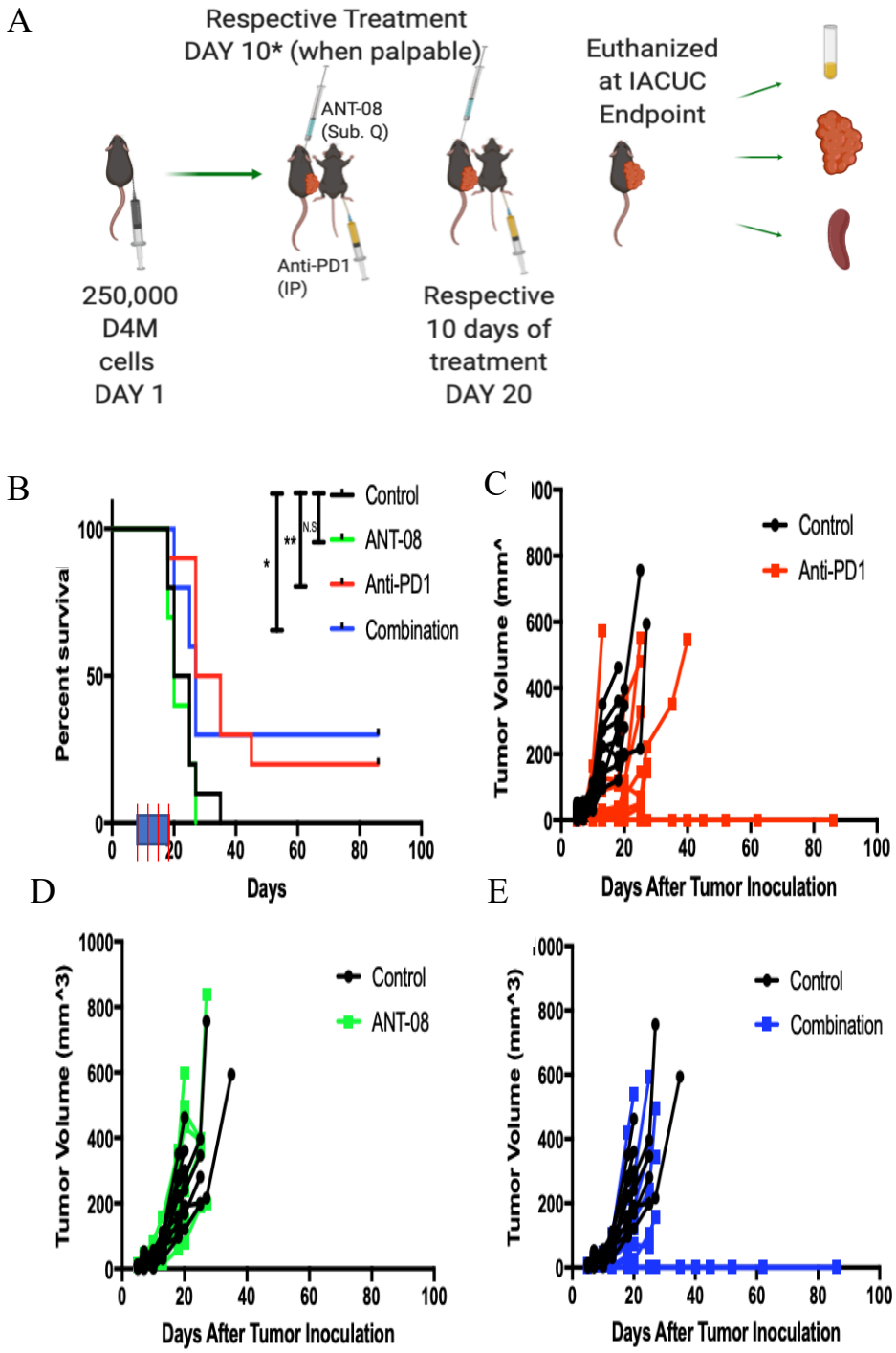


Figure 26. ANT-08 did not have single-agent *in vivo* activity in mice with D4M melanoma. (A) 250,000 D4M cells were subcutaneously inoculated in B6 mice and treatment was started once tumors were palpable. The treatment continued for 10 days, with daily subcutaneous injections of 10ug of ANT-08 or control and 200ug of anti-PD1 or isotype control antibody injected i.p. every

third day. (B) 30% of mice were tumor free in the combination group an 20% of mice were tumor free in anti-PD1 group. (C) Tumor growth rates were significantly slower in the anti-PD1 treated group. (D) ANT-08 did not have reduced tumor growth rate. (E) Combination treated group had significantly reduced tumor growth rate. $^{**}p<0.01$, $^{*}p<0.05$.

DISCUSSION

Discussion:

Human melanoma has two striking characteristics; firstly, melanoma is an extremely immunogenic tumor (Tucci et al.). Its high immunogenicity is one of the key reasons for success of immunotherapies against melanoma (Yunger et al.). Secondly, melanoma has a high mutational burden. In particular, around 40% of human melanomas are BRAF mutation positive (Dankort et al.). BRAF mutation causes constitutive phosphorylation of CREB. Phosphorylated or activated CREB promotes tumor cell proliferation and survival. Thus, blocking phosphorylation of CREB can counter melanoma tumor growth.

Combining these characteristics of melanoma, antagonizing VIP receptor signaling is an exciting approach as it can activate the adaptive immune system and reduce phosphorylation of CREB. Previously published work from Waller lab has shown a CD8 T cell-dependent anti-tumor activity of the antagonist against myeloid and lymphoblastic leukemia cell lines in mice (Petersen, Li and Waller). Previously published research has also shown how VIP receptor antagonist can stimulate the adaptive immune response in the mouse cytomegalovirus (mCMV) model (Li et al.). My work focused on understanding the source of VIP in tumor-bearing mice and the ability of VIP antagonists to activate the adaptive immune in murine melanoma models. In addition, this work also explores ANT-08 as a potential inhibitor of CREB phosphorylation in BRAF ^{V600E} mutation positive murine melanoma.

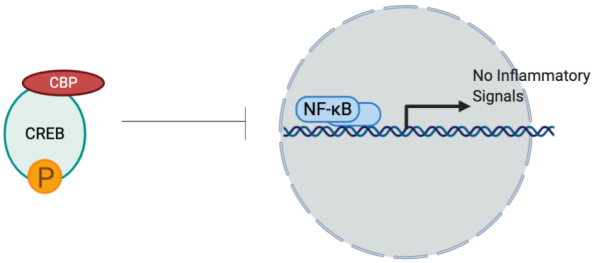
Melanoma cell lines or tumor tissue do not secrete VIP. Interestingly, inoculation of mice with B16F10 melanoma cells caused serum VIP levels to rise more than 10 times the baseline levels recorded in tumor-free mice. This significant increase in the VIP levels raised an important question as to where VIP is coming from. Multiple lymphocytic populations can secrete VIP (Verma et al.). Given the high proportion of tumor infiltration lymphocytes (TILs) in melanoma

(Fischer et al.) and expression of VIP receptors on T cells (Mosley et al.), we hypothesized that T cells secrete VIP.

VIP expression was induced in human T cells stimulated in vitro with PMA/ionomycin (Chatila et al.), suggesting that autocrine secretion of VIP by activated T cells may be a negative regulator of T cell proliferation. To test this hypothesis, we cultured T cells activated with plate-bound anti-CD3 antibody with VIP and the VIP antagonist ANT-08, and measured T cell proliferation. We reasoned that blocking VIP receptor signaling using ANT-08 would block autocrine signaling by VIP and thereby enhance activation and proliferation of T cells. Furthermore, we hypothesized that ANT-08 would be ineffective in enhancing T cell activation in VIP KO mice, in which autocrine secretion of VIP does not occur. In line with our hypothesis, VIP inhibited T cell proliferation while ANT-08 promoted proliferation of T cells from VIP-WT mice, and the stimulatory effects of ANT-08 were absent in T cells from VIP-KO mice. This suggests that ANT-08 is able to block the VIP receptor signaling pathway and thereby increase proliferation of T cells. In addition, because ANT-08 did not have any effect on VIP-KO T cells, it suggests that ANT-08 by itself does not directly elicit enhanced immune responses.

In addition to increase proliferation of T cells, we were also interested to study functional changes in T cells following ANT-08 treatment. Since cytokines are the immunomodulatory signaling molecules used by T cells to interact with a variety of cells, we measured pro-inflammatory cytokines levels secreted by enriched T cells treated with VIP, ANT-08 or VIP scrambled peptide. Surprisingly, T cells from VIP-KO mice had dramatically higher levels of the proinflammatory cytokines, particularly TNF-a and IL-6 than WT mice. In addition, ANT-08 treatment promoted secretion of TNF-alpha and IL-6 in T cells from VIP-WT mice. As TNF-a and IL-6 are proinflammatory cytokines (Gonzalez-Rey, Ganea and Delgado), (Chen et

al.), and (Kadowaki et al.), this finding supports our hypothesis that ANT-08 promotes activation of the adaptive immune response. Notably, ANT-08 blocks phosphorylation of CREB downstream of the VIP receptor. The absence of phosphorylation on CREB limits CREB association with the CREB binding protein (CBP) (Fernandez et al.). As a result of the lack of the heterologous association between CREB and CBP, CBP binds NF-kB and stimulates the downstream signaling pathway of NF-kB (Figure 27). Downstream targets of NF-kB that were promoted by ANT-08 include TNF-a (Hayden and Ghosh) and IL-6 (Brasier).


CBP
CREB
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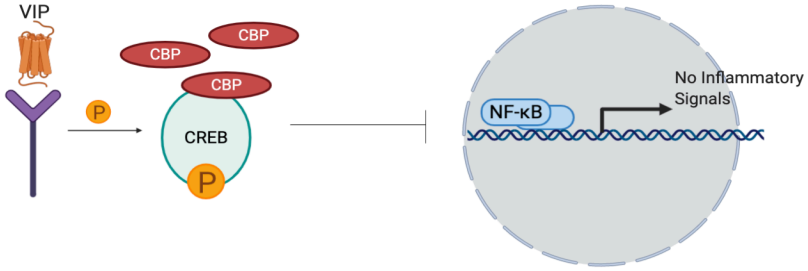
Signals


VIP
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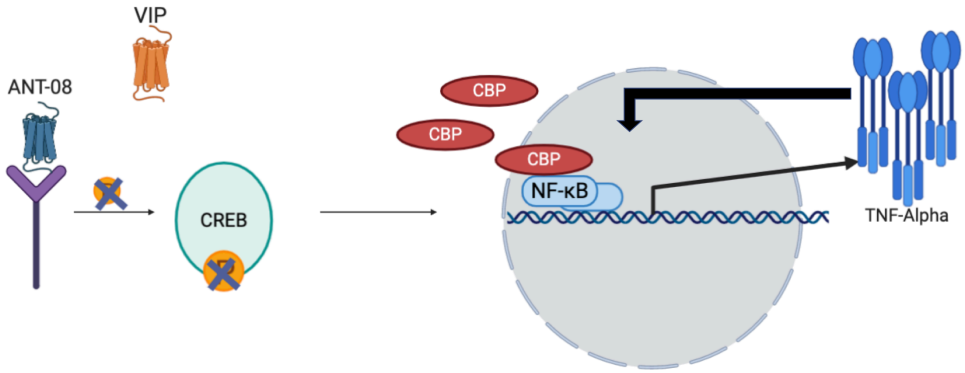
No Inflammatory
Signals


VIP
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TNF-Alpha

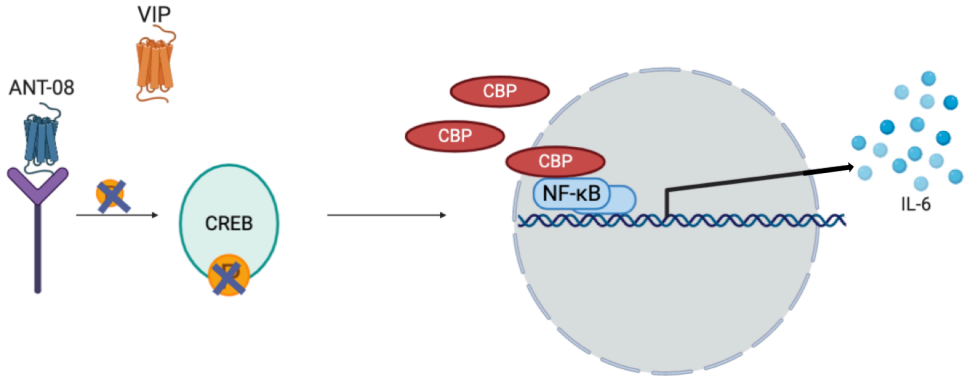
VIP
ANT-08
CREB
CBP
CBP
CBP
NF-κB
IL-6

Figure 27. ANT-08 promotes upregulation of TNF-alpha and IL-6 via NF-kB. (A) Phosphorylated CREB inhibits NF-kB activity. (B) VIP binding to the VIP-R leads to phosphorylation of CREB thereby asserting brakes on NF-kB-mediated transcription of inflammatory cytokines. ANT-08 blocks CREB phosphorylation thereby releasing brakes off NF-kB transcription activity, upregulating TNF-alpha secretion (C) and IL-6 secretion (D).

To further our understanding of T cell activation, we were curious to see if the upregulation of TNF-a and IL-6 was maintained in B16F10 tumor bearing mice serum. Excitingly, mice treated with ANT-08 had significantly higher levels of IL-6 in the serum than control mice treated with scrambled peptide. There was no difference in TNF-a serum levels, potentially due to its short half-life of 30 minutes, in contrast to half-life of IL-6 of 30 hours (Zahn and Greischel) and (Kuribayashi).

High levels of IL-6 in ANT-08 treated tumor bearing mice serum was an extremely important finding as it has been shown that IL-6 can cause proliferation of CD4 T cells by two mechanism: retaining function of BcL-2 expression in CD4 T cells thereby preventing CD4 T cells apoptosis and acting as a co-stimulatory molecule even in the absence of IL-2 (Rincon), (Sabio and Davis), Hara et al.), (Brasier), and (Dutzan et al) (Figure 28). Therefore, we analyzed proliferating CD4+ T cells levels in tumor, spleen and blood of B16F10 tumor bearing mice from the treated and contrl groups. Tumors had dramatically high levels of proliferating CD4+ T cells compared to blood and spleen. In addition, tumors from ANT-08 treated mice had signifcanly higher levels of proliferating CD4+ T cells than tumors from control mice.

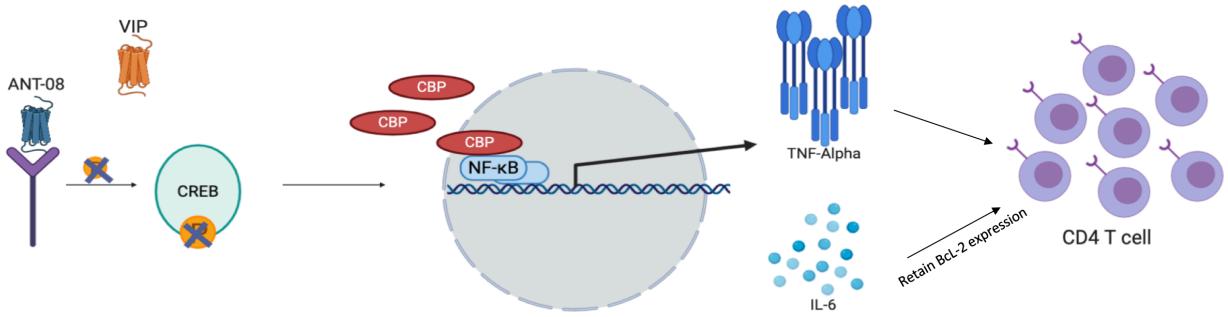

Figure 28. ANT-08 promotes Ki-67+/CD4+ T cells in B16F10 tumor bearing mice via the proposed pathway in T cells. ANT-08, by blocking CREB phosphorylation causes upregulation in IL-6, which in turn retains expression of BcL-2 and thereby preventing apoptosis of CD4 T cells.

Collectively, these results gave us new information, that antagonizing VIP receptor signaling pathway by treatmnet with the VIP antgaonist ANT-08 activates the adaptive immune system *in vitro* and *in vivo.* Surprisingly, the ANT-08 activated adaptive immune system did not result in a measurable and significant anti-cancer effect in mice with melanoma. ANT-08 treatment did not have monotherepeutic or synergistic effects with anti-PD1 against B16F10 melanoma. The low expression of MHC-II in the B16F10 cell line may be one reason that increased tumor infiltration of proliferating CD4+ T cells did not translate into survival differences (Merritt et al.). Thus, activated CD4+ T cells may not able to recognize tumor cells due to reduced antigen presentation. Thus, we propose to further investigate this hypothesis by transfecting B16F10 cells with IFN-y to increase MHC-II expression and then inoculating tumors in mice and treating them with ANT-08 (Marijt et al.).

Since we know that VIP causes phosphorylation of CREB, in which downstream signaling leads to proliferation of melanoma cells, we were curious to see if ANT-08 treatment had direct cytostatic effects against BRAF mutation positive D4M murine melanoma model (Kelley) and (Ruiz-Garcia et al.). We showed expression of the VIP receptors VPAC1 and VPAC1

on D4M cells thereby suggesting that ANT-08 might block VIP receptor signaling in tumor cells. Interestingly, ANT-08 treatment had a significant direct cytostatic effect on D4M cells. Unfortunately, these cytostatic effects did not translate into survival benefits in mice. This might be because there is a competition between ANT-08 and BRAF kinase; ANT-08 blocks phosphorylation of CREB while BRAF kinase constitutively phosphorylates CREB. Based on the data, this competition is being "won" by constitutively active BRAF kinase (Figure 29). As a result, due to constitutively active BRAF kinase, there is downstream signaling of phosphorylated CREB, which provides a pathway for tumor cell survival and proliferation.

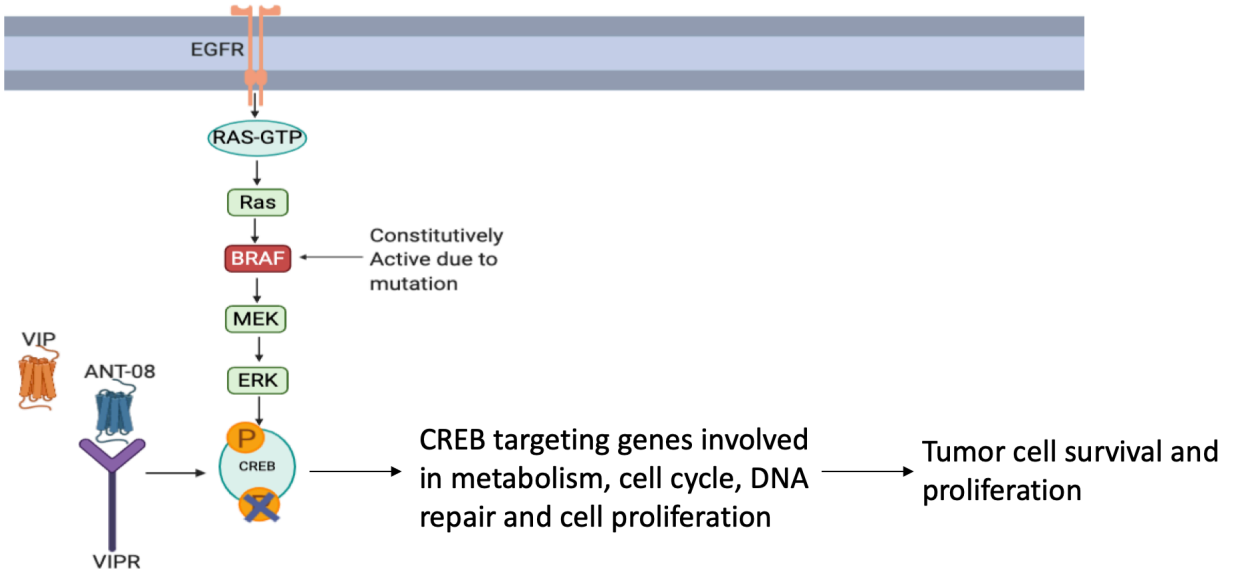

Figure 29. Race to phosphorylate and unphosphorylated CREB. ANT-08, by inhibiting VIP receptor signaling, blocks phosphorylation while at the same time, constitutively activate BRAF kinase downstream phosphorylates CREB.

Thus, we hypothesize that combining ANT-08 treatment with BRAF and MEK kinases inhibitors like vemurafenib and trametinib may elicit a strong anti-melanoma response, and these experiments are being planned.

In conclusion, we have shown ANT-08 activates the adaptive immune system *in vitro* and *in vivo.* We have hypothesized reasons for lack of ANT-08 efficacy on B16F10 and D4M *in vivo* models and have highlighted approaches to test these hypotheses for future study to bolster anti-melanoma immune response using ANT-08. In addition, owing to short half-life of VIP, we are curious to study effects of PEGylating ANT-08 to increase its stability and improve subcutaneous absorption (Zbyszynski et al.) and (Sethi et al.). Lastly, it is clear that ANT-08 is able to activate the adaptive immune system by causing proliferation of T cells and by functionally changing the T cells to secrete pro-inflammatory cytokines. Thus, VIP is a novel target for immunotherapy in a variety of solid and hematologic malignancies.

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