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Vasoactive Intestinal Peptide Antagonist Enhances T cell Proliferation and Synergizes with PD-1 Antibodies to Promote Anti-Melanoma T cell Immune Responses

By

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B.S., Emory University, 2017

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An abstract of
A thesis submitted to the Faculty of the
James T. Laney School of Graduate Studies of Emory University
in partial fulfillment of the requirements for the degree of
Master of Science
in the Graduate Division of Biological and Biomedical Sciences
Cancer Biology and Translational Oncology
2018

Abstract

Vasoactive Intestinal Peptide Antagonist Enhances T cell Proliferation and Synergizes with PD-1 Antibodies to Promote Anti-Melanoma T cell Immune Responses

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Antagonism of vasoactive intestinal peptide (VIP) signaling is a novel approach to reverse immune check-point inhibitory pathways. Treatment with a peptide antagonist to VIP (VIPhyb) has been shown to enhance protective immune responses to mCMV and leukemia in murine models. While treatment with VIPhyb has reduced tumor burdens in human glioblastoma, lung cancer, and breast cancer engrafted in immune-deficient mice treated with concomitant chemotherapy, a stimulatory effect of VIPhyb on anti-cancer immunity to solid tumors has not been described. We hypothesized that VIPhyb treatment would enhance T cell activation and cytotoxicity in response to melanoma, which can be highly immunogenic. We describe herein a dose-dependent enhancement of *in vitro* T cell proliferation in response to stimulation with anti-CD3 antibodies following the addition of VIPhyb, and reversal of the suppressive effect of exogenous VIP peptide on T cell proliferation. VIPhyb synergistically enhanced T cell proliferation in vitro when combined with anti-PD1 antibodies.

To test the effect of blocking VIP-signaling on anti-cancer immune responses to solid tumors, mice bearing B16 melanoma tumors were treated with daily injections of VIPhyb. VIPhyb-treated mice had more effector CD8+ T cells compared with saline-treated controls, but single-agent VIPhyb treatment did not have a significant effect on the kinetics of tumor growth. Combining treatment with VIPhyb and PD-1 antibodies in mice with established melanoma, we found significantly enhanced suppression of tumor growth and improved survival compared with mice treated with either inhibitor alone or saline-treated controls. VIPhyb-and anti-PD1 antibody treatment synergistically enhanced T cell mediated immunity as assessed by increased numbers of effector CD8+ T cells.

Notably, VIPhyb treatment did not inhibit melanoma growth, invasiveness, or change expression of immunological surface ligands *in vitro*. We hypothesize that treatment of mice with VIPhyb induced adaptive immunity against melanoma by down-regulating co-inhibitory pathways and stimulating T cell survival through inhibition of NF-kB signaling leading to increased numbers of cytotoxic CD8+ T cells. Thus, blocking signaling through the VIP receptor represents a new strategy to induce anti-tumor immunity in solid tumor immune-oncology.

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Acknowledgements

The Waller Lab:

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Ernestine Mahar
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Introduction & Background

Melanoma and Clinical Management

Skin cancer is the most commonly diagnosed cancer in the US and melanoma accounts for the vast majority of all skin cancer deaths[1]. Melanoma is a malignancy of melanocytes, the pigment producing cells of the skin, which may originate as a benign nevus but when this occurs can eventually become malignant with a highly aggressive metastatic nature[1]. The development of a melanoma tumor is a complex process involving the escape of immune system detection, and "cancer-immunoediting" of emerging tumors[2]. The Cancer Genome Atlas Research network has divided melanoma into four subtypes based on the presence of mutations in the BRAF, RAS, and NF1 genes[3]. When diagnosed, melanoma lesions and surrounding normal tissue are removed, phenotyped, and, if appropriate, a sentinel lymph node biopsied to determine stage and treatment options [1]. Melanomas with deep invasion or that have spread to lymph nodes may be treated with surgery, chemotherapy, radiation, targeted therapy, or immunotherapy[1]. Targeted therapies are drugs have been developed to target driver mutations in melanoma including BRAF and MEK inhibitors[4].

Despite early detection and improved therapeutic interventions, like the aforementioned targeted therapies and immunotherapies, the incidence of melanoma is increasing and thus the number of individuals dying from metastatic disease continues to rise[5]. Melanoma is considered one of the most chemotherapy-resistant malignancies, and efficacy of existing therapies remains poor, with a five-year survival rate of 20% for patients with distant metastases[2,6]. Therefore, there is a paramount need to develop novel therapies that resolve the shortcomings of existing solutions. As a result, melanoma treatment in the clinic has experienced a paradigm shift, favoring the use of immunotherapy to treat disseminated disease.

Immunotherapy for the Treatment of Melanoma

Cancer immunotherapy aims to activate or enhance the immune system to kill tumor cells with antigen specificity[7]. In melanoma, a number of strategies have been tested, and modulating the immune system with immunotherapy to induce an antitumor immune response is becoming a standard approach to treat patients with advanced disease. Melanoma has a high rate of somatic mutations due to ultraviolet radiation, and thus a high level of neoantigens (antigens derived from mutated coding sequences)[6]. Successful responses to immunotherapy in melanoma, such as immune checkpoint blockade, is influenced by the neoantigen load resulting from high mutational burdens in melanoma[8]. As a result, though advanced melanomas are largely resistant to conventional chemotherapeutics, they have proven to be sensitive to immunotherapy. Melanomas often exhibit high numbers of infiltrating lymphocytes which can induce tumor regression, and the modulation of adaptive immunity has shown to induce remarkable long remissions in a minority of patients[9,10].

Current immunotherapeutic approaches for cancer include stimulation of an antitumor immune response with cytokines, active immunization, adoptive immunotherapy, and targeting immune checkpoints or immune regulatory molecules[11]. The antitumor effects of the T cell cytokine interleukin-2 (IL-2), in a subset of melanoma patients, provided early clinical evidence of the potential of immunotherapy for cancer[12]. In 2010, a novel antibody targeting a T cell checkpoint protein cytotoxic T-lymphocyte antigen 4 (CTLA-4), proved that immunotherapy can improve overall survival of patients with metastatic melanoma[13]. CTLA-4 is a receptor that downregulates a T cell response when it comes into contact with CD80 (B7-1) and CD86 (B7-2)[14].Later, an antibody against another checkpoint inhibitor, programmed death receptor-1 (PD-1), was developed and showed clinical activity in a range of different cancers[15]. PD-1 is also a

receptor that negatively regulates immune responses when it interacts with PD-L1 to exhaust T cells after long-term antigen expression[16]. Cytokines such as IL-2 and immune checkpoint inhibitors against CTLA-4 and PD-1, are currently the only FDA approved immunotherapeutic drugs for the treatment of advanced melanoma, with checkpoint inhibitors being the most effective immunotherapy. Cancers often hijack the immune system by expressing inhibitory immune checkpoint ligands that limit the effectiveness of an anticancer host immune response. While responses to IL-2 occur in only 10% of patients, an estimated 40% of patients initially respond to checkpoint inhibitors though 25-40% of those patients relapse within 3 years of treatment[10,17]. Therefore, although these drugs show significant improvement in overall survival, the investigation of more rigorous immunotherapies is of paramount importance.

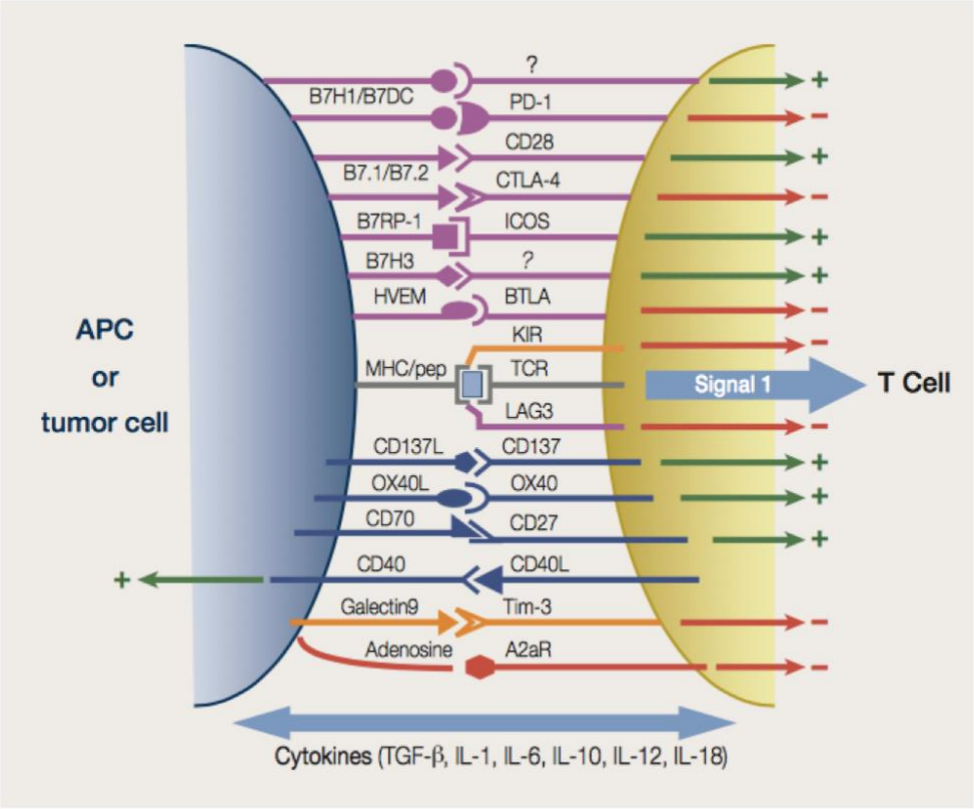


Figure 1. Costimulatory and Coinhibitory Interactions Between T cells and Tumors. Understanding the activating or inhibitory effects of immune checkpoint ligands aids in the development of novel immunotherapies to boost an immune response against cancer[18].

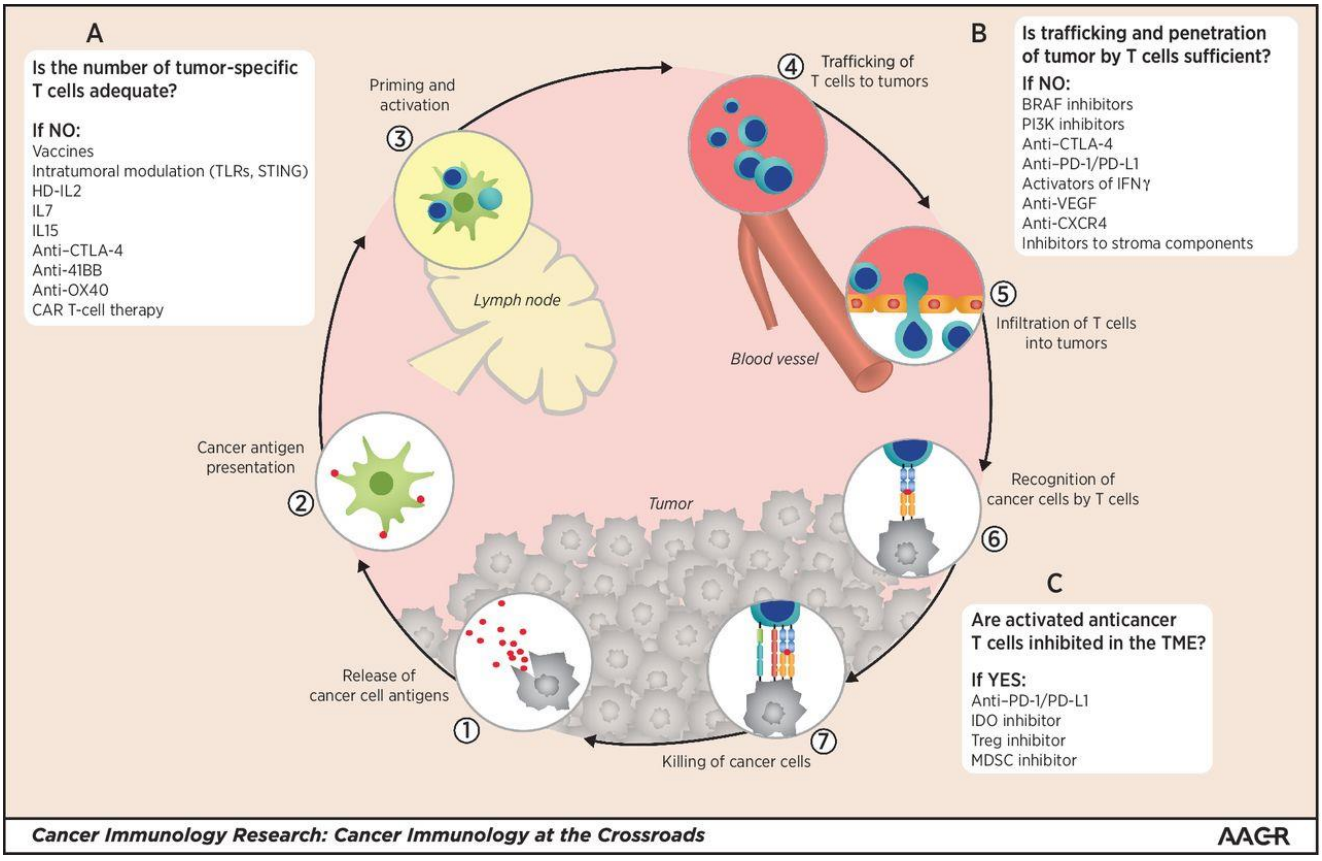


Figure 2. The Cancer Immunity Cycle. The relationship between cancer cells and the immune system involves complex interactions. As a result, numerous therapies have been developed to enhance anticancer immune responses at several points in this multistep process[19]

Vasoactive Intestinal Peptide

Vasoactive intestinal peptide (VIP) is a 28 amino acid immunosuppressive neuropeptide belonging to the gastrin/glucagon/secretin family of secretory peptides[20]. While VIP is predominantly secreted from nerve terminals and in the gastrointestinal tract, it is also secreted from various immune cells[21,22]. Activation of the VIP pathway mediates various physiological responses such as mast cell degranulation, plasma extravasation, vasodilation, and immunomodulation[23].VIP binds to VPAC1, VPAC2, and PAC1, the first two G-protein coupled receptors being expressed on T cells and dendritic cells (DC)[24]. VIP is synthesized by T cells, antigen presenting cells (APC) and is upregulated by injury, apoptosis, and proinflammatory cytokines such as IL-1, IL-6, and TNF[25,26,27]. VIP binding initiates a cAMP-protein kinase A (PKA) transduction pathway and activation of PI3K/PKC, and p38 MAPK[28,29].

Previous studies suggest that VIP signaling in response to inflammatory signals promotes an immunosuppressive environment by inducing the differentiation of regulatory T cells (Tregs), decreasing T cell proliferation and reducting the expression of pro-inflammatory cytokines such as TNF-a and IL-2[30]. Thus, VIP signaling has potent anti-inflammatory effects. In addition, VIP polarizes CD4+ T cells toward a Th2 response while suppressing the Th1 response and increasing Th2 associated cytokines[31,32]. Therefore, VIP antagonism may be efficacious as a therapy for immunogenic tumors.

Antagonist to Vasoactive Intestinal Peptide (VIPhyb)

Peptides with modified VIP sequences have been developed to antagonize the VIP receptor to regulate immune responses. Removing C-terminal amino acid residues in VIP leads to significantly less binding than the full-length peptide[33], and thus VIPhyb is a modified VIP, with the first 6 N -terminal amino acids of VIP (HSDAVF) being replaced with the sequence of neurotensin (KPRRPY). VIPhyb has activity against VIP, PHI, and PACAP binding to human and mouse VIP receptors: VPAC1, VPAC2, and PAC1, without adverse effects, and presents with fewer neurotoxicities than other VIP peptide antagonists[34].

This antagonism has been shown to further downregulate signaling through the cAMP/PKA, PLC/PKC, and p38 MAPK pathways[35]. In a mouse cytomegalovirus model, VIPhyb enhanced DC maturation, activated natural killer cells (NK), and increased serum levels of type-I interferons (IFNγ and IFNb)[26]. In allogeneic bone marrow transplant recipients, VIPhyb increased anti-leukemic activity of donor T cells, specifically CD8+ T cells, and longer survival without increasing graft-vs-host disease[36]. Further, VIPhyb treatment decreased expression of PD-1 and PD-L1 on donor T cells and DCs while increasing secretion of IFNγ, TNF, granzyme B and ICOS[27].

As a result, an absence of VIP signaling leads to an activation of adaptive cellular immunity and potential anti-cancer immunity. Further, VIPhyb directly inhibits proliferation of non-small cell lung cancer[37] and breast cancer[38]. The immunoadjuvant effect of VIPhyb can be further augmented by conjugation with a Pluronic-stabilized poly(propylene) sulfide (PPS)-core 30 nm nanoparticle (NP) which targets immune cells within the tumor draining lymph nodes (TDLN), a site that promotes tumor immune escape[39]. Given the identity in primary amino acid sequence between murine and human VIP[40], the clinical use of VIPhyb may be a novel approach

to enhance innate and adaptive cellular immunity against cancer, an effect that may be enhanced with a TDLN targeting NP.

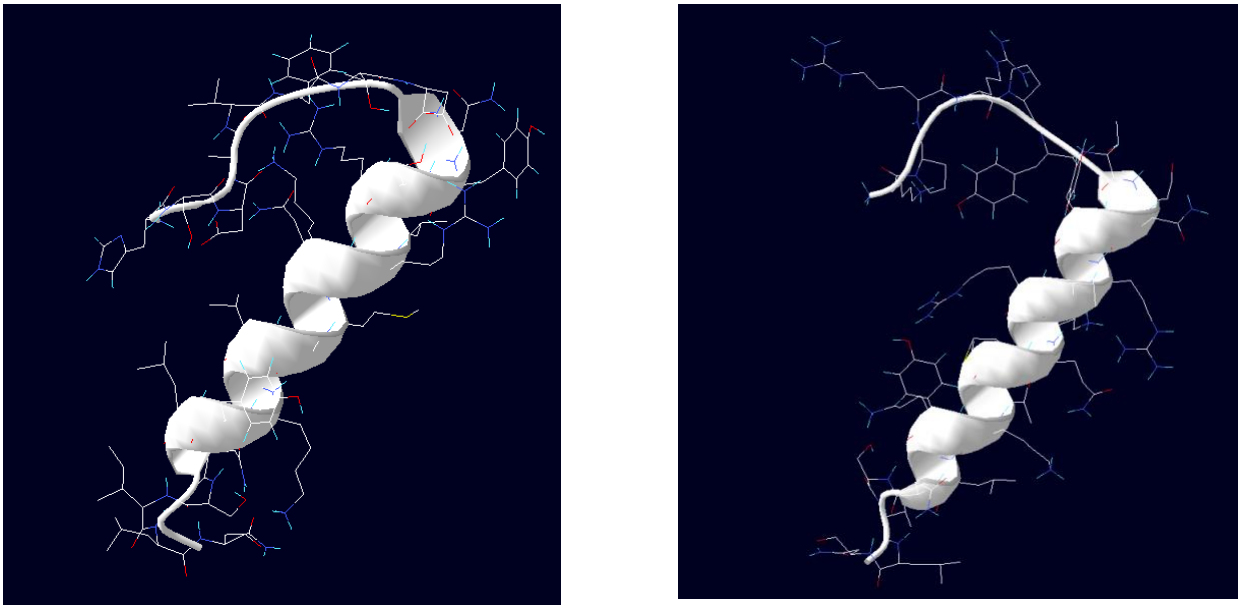


Figure 3. Protein Structures of VIP (left) and VIPhyb (right). VIP and VIPhyb have identical amino acid sequences, except for the first 6 N- terminal residues, making VIPhyb an antagonist of VIP receptors[41]

B16 Murine Melanoma Model System

Melanoma cells are surrounded by a microenvironment that includes blood vessels, extracellular matrix, and host immune cells, each of which play critical roles in the response of tumors to therapeutic agents[42]. Therefore, research models must not only recreate these features but also recapitulate natural tumor progression, from proliferation to invasion and metastasis. The most widely used preclinical model that employs the aforementioned characteristics is the murine model. Not only are mice easy to manipulate, they are widely available, and an immense knowledge base exists regarding their genetics[32]. The syngeneic model, in particular, has become the preferred method for studies on metastasis and immunotherapy[43].

Syngeneic transplantation models involve the induction and transplantation of melanoma cells into the same species and genetic background. This model is especially helpful in studying melanoma immunology, as it allows for the interaction of melanoma cells with competent T cells and B-cells found naturally in the human melanoma microenvironment[44]. A handful of cell lines are utilized in melanoma syngeneic transplantation models such as Harding-Passey, Cloudman S91, and B16. Though of all the murine melanoma models available, B16 is the most frequently used[45].

B16 was derived from a spontaneously arising melanoma of C57BL/6J origin, and has diverse subclones with different propensities for proliferation, invasion, and metastasis[1]. Two well-established subclones of the B16 melanoma cell line include B16F1 and B16F10. B16F1 demonstrates a low metastatic potential and is useful for studying primary tumor growth[46]. B16F10, however, has a high metastatic potential, especially to the lungs, and is ideal for in vivo studies because of its rapid growth pattern and high turnover[47]. Upon subcutaneous injection, B16 will form a palpable tumor in 5 to 10 days and grow to a 1cm × 1cm tumor in 14 to 21

days[48]. If grown larger, the tumors can become necrotic in the center, and begin to ulcerate or bleed[49].

Efficacy of immunotherapies against B16 is thought to be a reasonable predictor of the performance of immune therapies against human tumors[41]. B16 melanoma is poorly immunogenic, as it expresses low levels of MHC1, limiting their ability to be recognized by cytotoxic CD8+ T cells[50]. However, they express high levels of other targetable melanoma-associated antigens like tyrosinase related protein 2 (TRP-2) or gp100[51]. Although B16 tumors are poorly immunogenic, they have been used to test a multitude of immunotherapeautic interventions. Viral, DNA, dendritic cell, whole-tumor, and peptide vaccination regimens tested against established, palpable B16 tumors, reflects the response seen in the clinic[52,53]. As a result, it is possible to generate potent anti-tumor immune responses against these tumors when the therapy generates large T cell responses. With the help of several melanoma mouse models, there have been major advances in the treatment of melanoma.

Experimental Hypothesis

We hypothesize that treatment of B16 melanoma with VIPhyb will enhance an adaptive immune response by promoting T cell proliferation, an effect that could be augmented through combination therapy with an anti-PD-1 antibody.

Materials and Methods

Cell lines

The B16-F1 cell line was a generous gift from Dr. Jack Arbiser, who obtained it from American Type Culture Collection (ATCC). The B16-F10 cell line was obtained from the American Type Culture Collection (ATCC). B16-F1 and B16-F10 cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 4 mM L--glutamine, 1 mM sodium pyruvate, and 1500 mg/L sodium bicarbonate. Luciferase transfected cells were cultured in the same media, supplemented with 2 ug/mL of puromycin. All cell cultures were passaged at 80% confluency and tested negative for pathogens by Emory DAR. Only pathogen-free cells passaged under 30 times in the exponential growth phase were used for the experiments. All cell cultures were kept in a 37ºC 5% CO_2 humidified incubator.

B16 Cell Line Transfection

B16-F1 and B16-F10 cells were stably transfected with a luciferase vector (Promega- E6751) via nucleofection according to the manufacturer's instructions (Amaxa® Cell Line Nucleofector® Kit V). After nucleofection, both B16 cell lines were cultured in 6-well plates with DMEM containing puromycin concentrations ranging from 1 ug/mL-4 ug/mL and allowed to culture for 2 weeks to select for puromycin resistant clones. Individual colonies were harvested using cloning cylinders (Corning 3166-8) and expanded using the aforementioned media in a 37ºC 5% CO_2 humidified incubator.

B16 Phenotypic Analysis

250,000 B16-F1 and B16-F10 cells in duplicate were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 4 mM L-glutamine, 1

mM sodium pyruvate, 1500 mg/L sodium bicarbonate and supplemented with either 0 ng, 10 ng, or 20 ng of IFNγ in a 6-well plate. 3 µM of VIPhyb or 3 µM of VIP was added daily into each well and kept in a 37ºC 5% CO_2 humidified incubator. After 48 hours, the duplicate wells were washed and incubated with LIVE/DEAD™ Fixable Aqua Dead Cell Stain (ThermoFisher-L34957). After washing, samples were stained with MHCI, MHCII, CD80, CD86, PD-L1, PD-L2, and PD-1 (BioLegend 135215) (BD-553623, 558091, 557796). Samples were run on a FACS Aria (BD) and analyzed using FlowJo.

Cell Viability Assay

The effect of VIPhyb on the viability of B16-F1 and B16-F10 cells was determined with an MTT assay (Roche 11 465 007 001). Using a 96-well flat bottom plate, 100 µL of 100,000 B16-F1 or B16-F10 cells were seeded per well and incubated overnight with VIPhyb concentrations ranging from 1-3 µM. All measurements were conducted in triplicate. 1-3 µM of VIPhyb was then added daily for 3 days, and on day 3, 10 µL of 5 mg/mL MTT was added to each well and incubated for 4 hours. 100 µL MTT solubilization solution was added after 4 hours and incubated overnight at 37ºC in a 5% CO_2 humidified incubator. The absorbance was then read at 570 nm, with a higher absorbance corresponding to a higher amount of viable cells.

Transwell Migration Assay

Migration of B16-F10 cells treated with VIPhyb in response to complete media was measured in a transwell assay. 100,000 cells were serum starved overnight and suspended in serum-free media were placed in the upper compartment of an 8.5 µm pore transwells (Corning). Cells were

allowed to migrate for 24 h with complete media or serum free control media in the lower compartment, with the analysis of cell counts assessed using crystal violet staining.

Co-Culture Assay

100,000 splenocytes isolated from luciferase^{+} B6 mice were co cultured with 100,000 irradiated B16-F1 and B16-F10 cells in 96-well plates pre-coated with 1 µg/mL of functional anti-CD3 antibody and 30 U/mL of recombinant murine IL-2. VIPhyb (0.5-3 µM) was added daily and kept in a 37ºC 5% CO_2 humidified incubator. After 3 days, splenocyte proliferation was assessed by adding 150 µg/mL of luciferin into each well and analyzing bioluminescence using an IVIS Spectrum instrument and Living Image Software (PerkinElmer). Average radiance corresponds to cell number in each well. Cells were then stained for CD3, CD4, CD8, PD-1 (BioLegend 135215), CTLA-4, NK 1.1 for NK cells, CD11b, Gr-1, and I-A/I-E for MDSCs (BD- 551163, 553650, 561967, 565778, 562921, 564985, 562710, 743870). Samples were run on a FACS Aria (BD) and analyzed using FlowJo.

Western Blot

VPAC1 and VPAC2 protein levels were determined by western blot. B16F1 and B16F10 protein lysates were loaded onto a 4-20% gradient gel (BioRad- 4561096). The samples were then transferred to a nitrocellulose membrane and probed with 1:1,000 dilutions of anti-VPAC1 and anti-VPAC2 polyclonal antibodies overnight (Santa Cruz Biotechnologies- sc-30019, 30020). The membrane was imaged using an Azure Biosystems c600.

T cell Proliferation Assay

100,000 purified splenic T cells from luciferase$^+$ B6 mice were cultured in RPMI-1640 supplemented with 10% fetal bovine serum, 50 µM 2-mercaptoethanol and 30 U/mL of recombinant murine IL-2 in 96-well plates pre-coated with 1 µg/mL of functional anti-CD3 antibody (eBioscience- 16-0032081). PBS, VIP (1 pM-1µM) and/or VIPhyb (0.5-3 µM) were added daily and kept in a 37^{o}C 5% CO_2 humidified incubator . After 3 days, T cell proliferation was assessed by adding 150 µg/mL of luciferin into each well and analyzing bioluminescence using an IVIS Spectrum instrument and Living Image Software (PerkinElmer). Average radiance corresponds to cell number in each well. Cells were then stained for CD3, CD4, CD8, PD-1 (BioLegend 135215), and CTLA-4 (BD- 551163, 553650, 561967, 565778). Samples were run on a FACS Aria (BD) and analyzed using FlowJo.

Mice

Six- to eight-week-old female albino B6 mice (B6(Cg)-*Tyrc-2J*/J) mice were used for the B16-F1(luc) and B16-F10(luc) models and purchased from Charles River Laboratories. Cell viability was assessed before injection via trypan blue exclusion and was always greater than 95%. Cells were injected in PBS subcutaneously on the right flank. Tumors were measured twice weekly with digital calipers and imaged once a week starting at day 7, once the tumors were palpable. Tumor volume was calculated as the product of twice the width and length, divided by 2. Tumor burden was also assessed using bioluminescent imaging once a week. Inoculated mice were monitored every day until the tumor size reached the IACUC limit of 1 cm in any direction and then euthanized by CO_2 asphyxiation. All procedures were approved by the Emory University

Institutional Animal Care and Use Committee and conformed to the *Guide for the Care and Use of Laboratory Animals.*

VIPhyb Treatment

VIPhyb peptide (H2N-KPRRPYTDNYTRLRKQMAVKKYLNSILN-amide), was purchased from New England Peptide. The desiccated peptide was kept in a -20^{o}C freezer, reconstituted in sterile deionized water and diluted in sterile PBS for *in vitro* use. A 10 µg dose in a 100 µL injection volume was administered to each mouse subcutaneously. Treatment began the day before tumor inoculation and continued daily until the tumors measured 1 cm in any direction. An equal volume of PBS was injected in control mice.

VIPhyb Nanoparticle

VIPhyb with a C-Terminal six amino acid linker (KPRRPYTDNYTRLRKQMAVKKYLNSILNGGGGSC) was conjugated to a 30 nm Pluronic-stabilized polypropylene sulfide (PPS) nanoparticle by inverse emulsion polymerization. VIPhyb-NP was generously provided by Dr. Susan Thomas.

Bioluminescent Imaging

Tumor burden was assessed using bioluminescent imaging. Mice were first anesthetized by isofluorane inhalation. Prior to imaging, the flanks of the mice were shaved with an electric razor to decrease interference. Luciferin was injected intraperitoneally at a dose of 150 mg/kg (GoldBio- 115144-35-9). Images were acquired 3 minutes after luciferin injection using an IVIS Spectrum (Perkin Elmer) and radiance quantified with Living Image software (Perkin Elmer) by

tracing a region of interested over the location of the tumor on the mouse. Measurements are reported as p/sec/sr/cm^2.

Flow Cytometry

Spleen samples were collected from mice at necropsy, and prepared for flow cytometric analysis. Spleens were dissociated in RPMI incomplete media using the back of a syringe plunger, and the contents passed through a 70 µm strainer. Red blood cells were lysed in ammonium chloride buffer. Samples were then incubated with LIVE/DEAD™ Fixable Aqua Dead Cell Stain (ThermoFisher- L34957). After washing, samples were incubated in the presence of an anti-CD16/32 Fc receptor-blocking antibody followed by staining with three separate panels: PerCP-Cy5.5 CD3, FITC CD4, APC-Cy7 CD8, BV421 NK1.1 PE CD366 (Tim-3), APC CD223 (Lag-3), PE-Cy7 CD279 (PD-1) (BioLegend 135215), APC-R700 CD152 (CTLA-4), and BV421 CD278 (ICOS) (BD- 551163, 53650, 561967, 562921, 566346, 562346, 565778, 564070). PE-Cf594 Gr-1, APC-R700 CD11c, FITC I-A/I-E, APC B220 (BioLegend- 103212), PE PD-L1, BV421 PDCA-1 and PE-Cy7 CD19 (BD- 562710, 565872, 553623, 558091, 566431, 553786). Intracellular cytokine staining was performed on splenocytes using Leukocyte Activation Cocktail with Golgiplug, BD Cytofix/Cytoperm kit, and antibodies toward IFNγ, TNF, IL-2, IL-4, IL-17, and IL-22 (BioLegend- 25-7021-82) (BD- 562921, 554413, 558000). All samples were run on a FACS Aria flow cytometer (BD) and analyzed using FlowJo software.

Immunohistochemistry

Melanoma tumors were excised from mice at the endpoint for immunohistochemical analysis. Tumor tissue was fixed in 10% neutral buffered formalin, stained with H&E, CD4, CD8, PD-L1

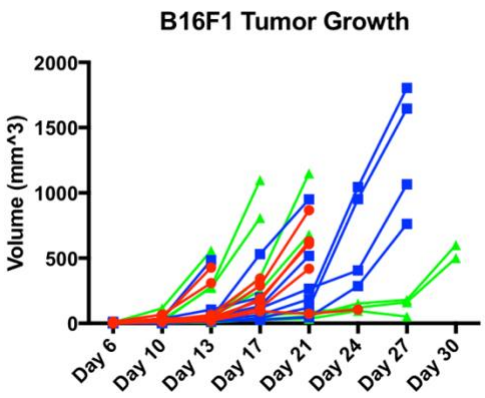
(Cell Signaling- 64988), and FoxP3 (Abcam- ab183685, ab203035, ab54501), and embedded in paraffin. Immunohistochemical staining was performed by the Pathology Core Laboratory at Winship Cancer Institute of Emory University.

Results

VIPhyb Has No Effect on B16F1 Tumor Growth and Survival Despite Different Routes of Administration

To investigate VIPhyb as an immunotherapy for melanoma, and to determine the best route of administration, we injected luciferase-expression B16F1 cells into B6 Albino syngeneic recipients and gave 10 µg of VIPhyb peritumorally or subcutaneously on the contralateral flank beginning 1 day before tumor inoculation and continuing daily until the experimental endpoint. Mice were monitored for tumor growth and survival, and no difference in tumor burden was found between the two different routes of administration. Fig. 3A shows no significant change in tumor volume between the treated and untreated mice as measured twice weekly with digital calipers. In addition, there was no significant difference in survival (Fig. 3B). This nonsignificant effect on tumor volume was supported with once weekly bioluminescent imaging to quantify the overall tumor burden, showing no tumor reduction in the VIPhyb treated mice. (Fig. 3C)

A



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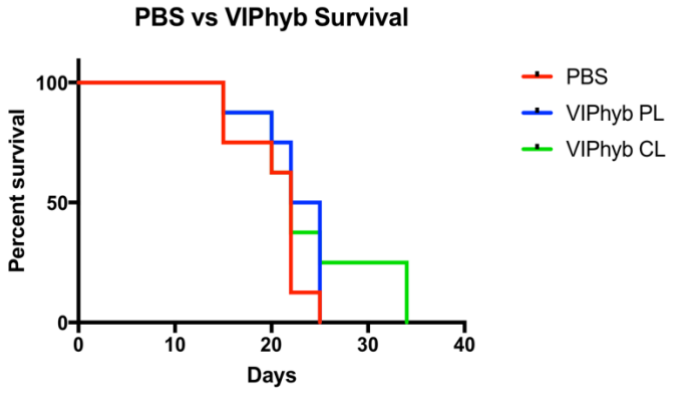

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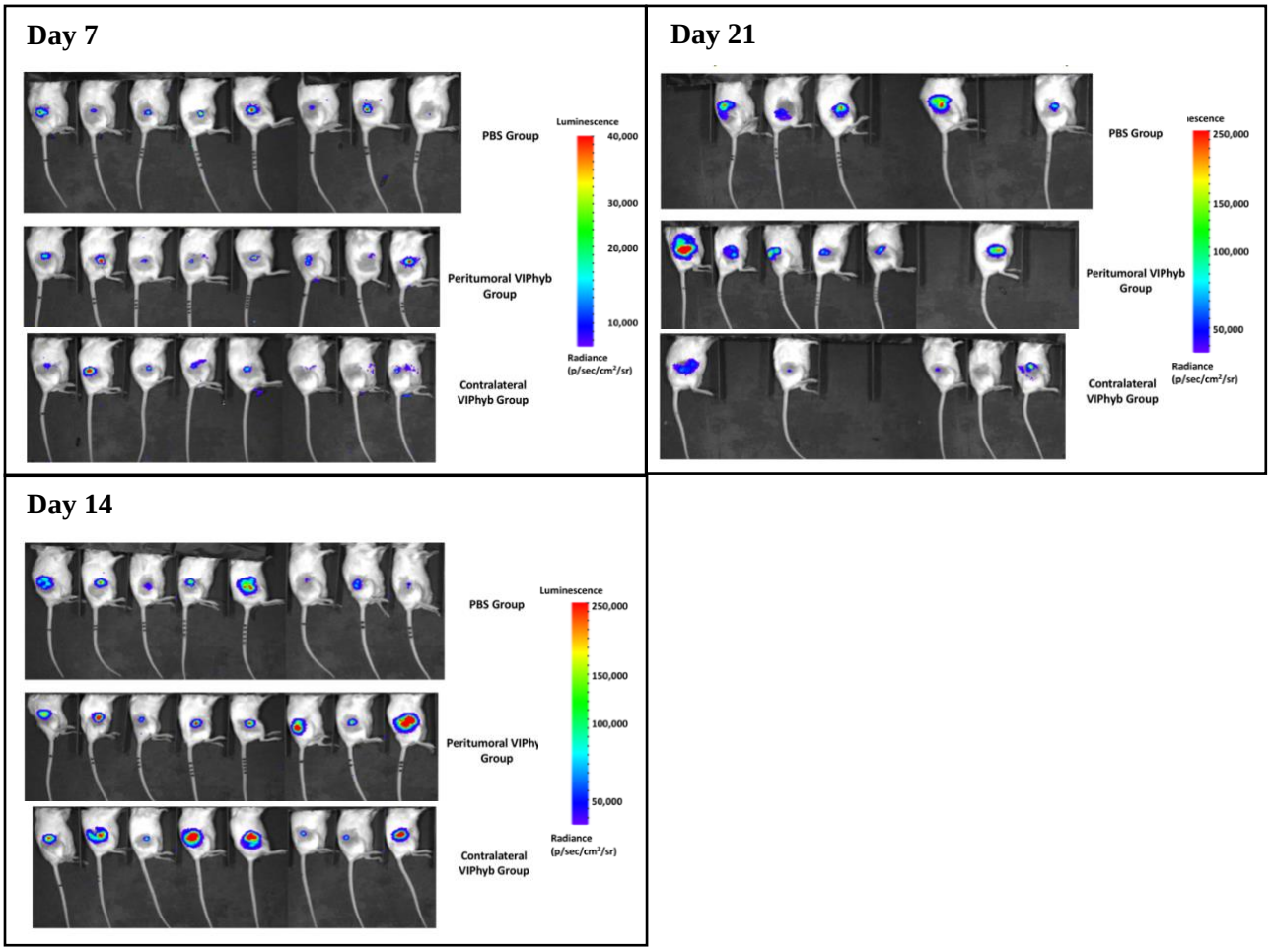
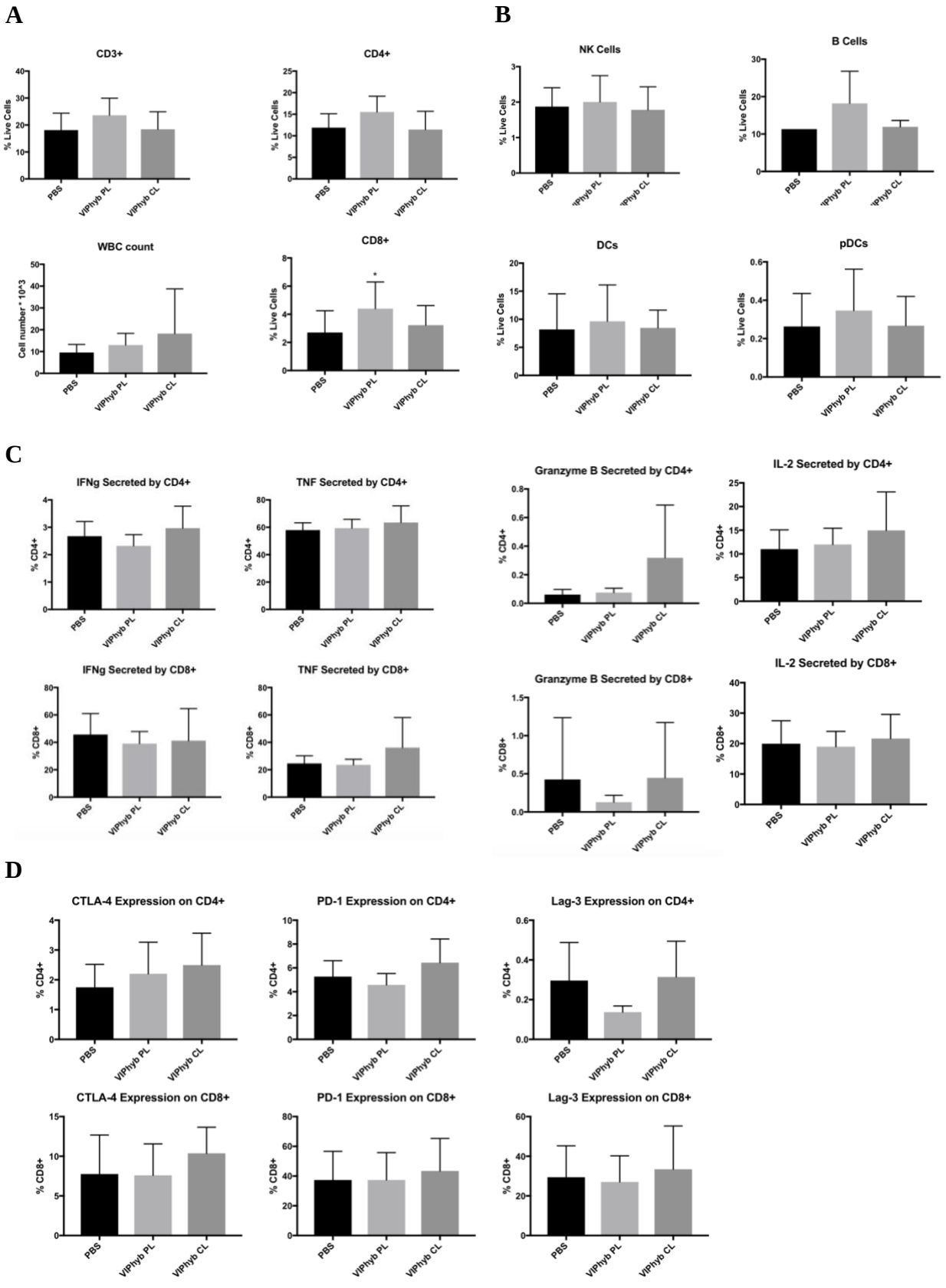


Figure 3. VIPhyb Does Not Affect Overall Tumor Burden and Survival in Mice with B16F1 Tumors. (A) Individual tumor volumes from mice treated with PBS (n=8), VIPhyb peritumorally (n=8), and VIPhyb contralaterally (n=8). Volumes were calculated as (length*width)/2. (B) Survival of B16F1 bearing, VIPhyb-treated B6 Albino mice injected peritumorally or contralaterally, compared with B16F1 bearing, PBS treated mice. (C) Representative BLI images of B16F1 bearing B6 Albino mice treated with either PBS or VIPhyb throughout treatment. The scale indicates the intensity of the signal emitted from B16F1 cells.

VIPhyb Enhances CD8+ T cell Proliferation and Non-Significantly Activates an Adaptive Immune Response in a B16F1 Model

Although VIPhyb does not reduce tumor burden, we examined whether VIPhyb may still have an effect on adaptive immunity. Mice with tumors that exceeded 500 mm^3 in volume were euthanized and their splenocytes phenotyped using flow cytometry. Blood was also collected for a complete blood count (CBC) to determine amount of peripheral white blood cells (WBCs). Of particular interest was the expansion of CD8+ T cells to corroborate the *in vitro* data, and expression of the inhibitory molecule PD-1. Though there was a nonsignificant modest increase in CD4+ T cells and peripheral WBCs, there were significantly more CD8+ T cells from VIPhyb-treated mice injected peritumorally (Fig. 4A). Further, there was a nonsignificant increase in NK cells, B cells, and different dendritic cell subtypes (Fig 4B). We next analyzed the functional capacity of T cells from the spleens of VIPhyb and PBS-treated B16F1-bearing mice by intracellular cytokine staining. There was no significant difference in the frequency of IFNγ, TNF, Granzyme B, and IL-2-producing cells in both the CD4+ and CD8+ compartments comparing VIPhyb treated mice with PBS treated controls (Fig. 4C). Further, there was no significant difference in the frequency of CD4+ and CD8+ T cells expressing the PD-1, CTLA-4, and Lag-3 co-inhibitory ligands (Fig. 4D).


A
CD3+
% Live Cells
PBS
VIPhyb PL
VIPhyb CL
CD4+
% Live Cells
WBC count
Cell number * 10^3
CD8+
% Live Cells
*
B
NK Cells
% Live Cells
B Cells
% Live Cells
DCs
% Live Cells
pDCs
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C
IFNg Secreted by CD4+
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TNF Secreted by CD4+
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IFNg Secreted by CD8+
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PD-1 Expression on CD8+
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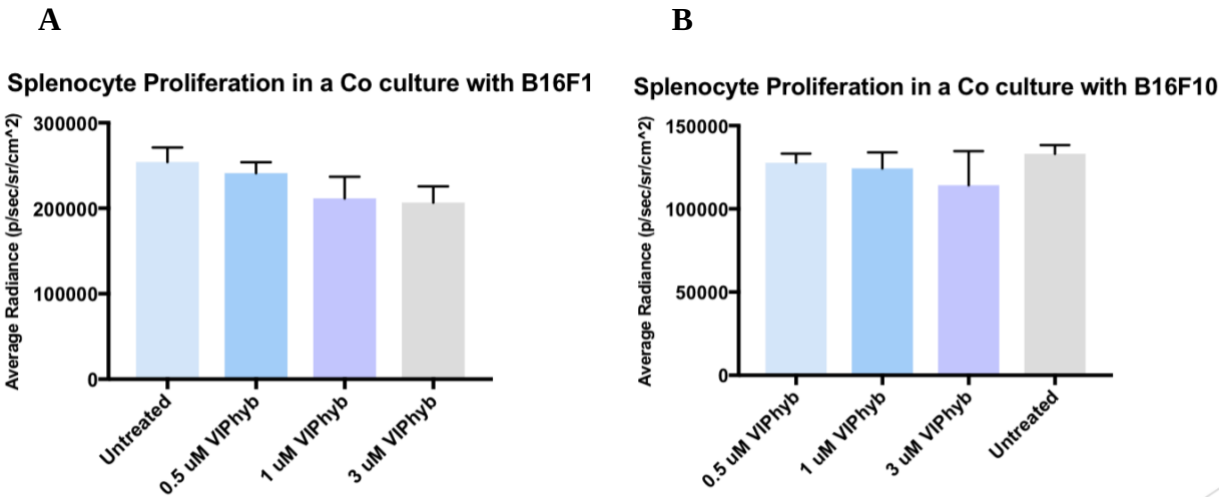
Figure 4. VIPhyb Increased CD8+ T cell Proliferation but Did Not Significantly Promote an Anti-Tumor Immune Response (A) Frequency of CD3+, CD4+ and CD8+ T cells in the spleen and WBC count in peripheral blood upon euthanasia. (B) Frequency of NK cells, B cells, dendritic cells, and plasmacytoid dendritic cells in the spleen upon euthanasia. (C) Expression of IFNγ, TNF, Granzyme B, and IL-2 in splenic T cells from B16F1-bearing mice treated with PBS or daily consecutive doses of VIPhyb. (D) Expression of PD-1, CTLA-4, and Lag-3 on subsets of splenic T cells treated with PBS or daily consecutive doses of VIPhyb. *p <.05

VIPhyb Alone Does Not Enhance Splenocyte Proliferation and T cell Expansion in a B16F1 and B16F10 Co Culture Unless Combined with an Anti-PD-1 Antibody

Due to the lack of tumor regression in the B16F1 model, we sought to evaluate if in combination with a PD-1 inhibitor, VIP could enhance an adaptive immune response to the more aggressive B16F10 model *in vitro*. Therefore, we co-cultured irradiated B16F1 and B16F10 cells with luciferase expressing splenocytes from B6 mice. Cells were placed in 96-well plates pre-coated with anti-CD3 antibody and low-dose recombinant murine IL-2 for 3 days and assessed proliferation by BLI. Administration of VIPhyb was not enough to promote expansion of splenocytes (Fig. 5A,B). However, when added with 1 dose of 10 µg of anti-PD-1 antibody, splenocyte proliferation increased dose-dependently (Fig. 5C,D), particularly with the cytotoxic CD8+ T cell subset when co-cultured with B16F10, with no significant effect on expansion of CD4+ T cells (Fig. 5E,F).



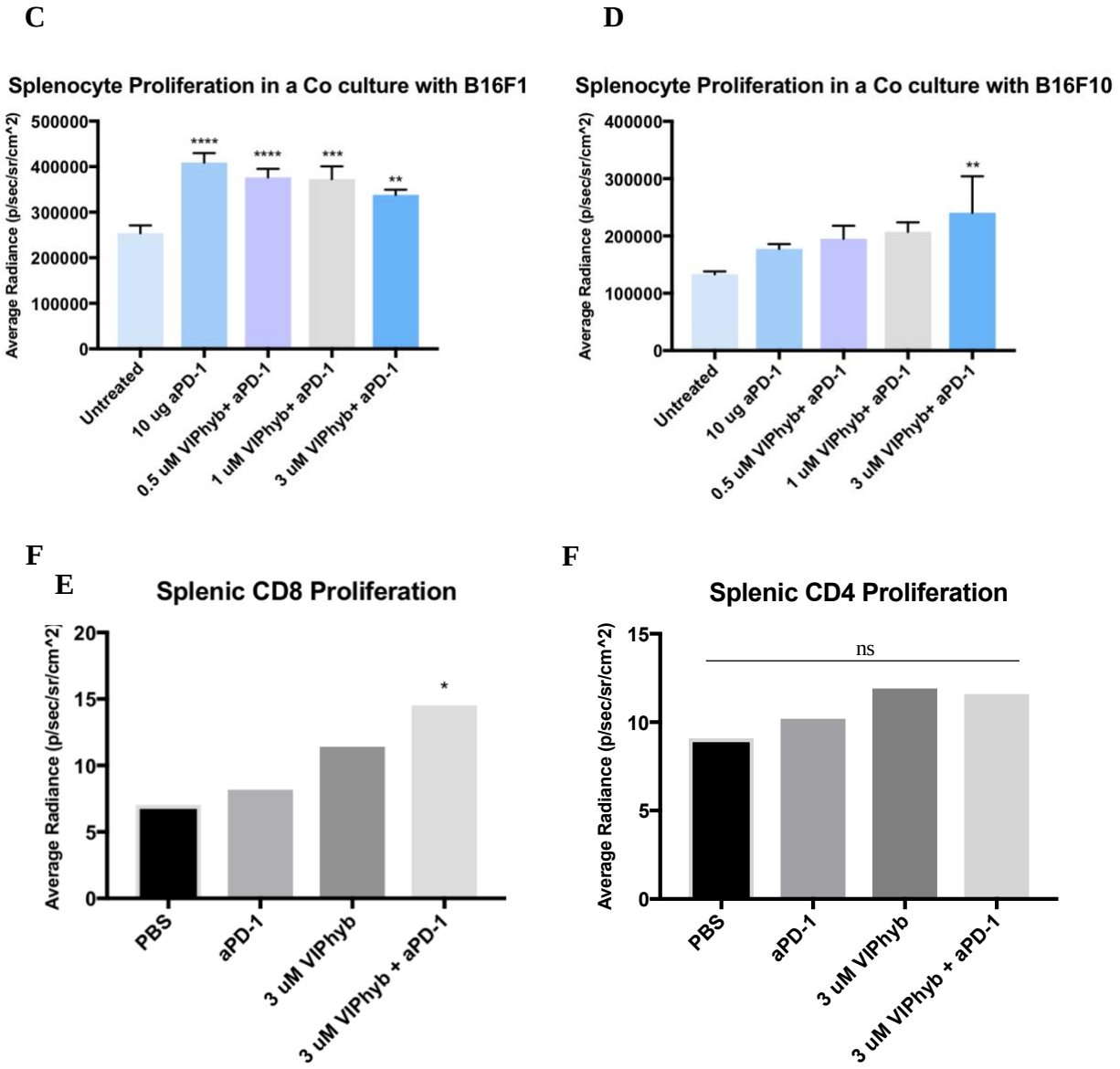
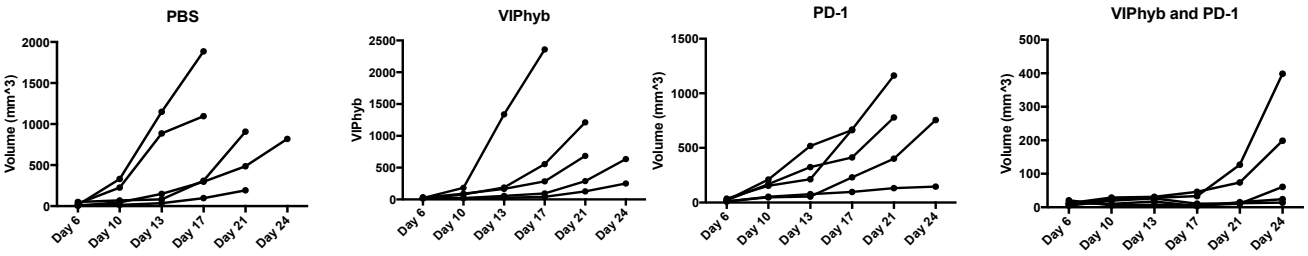


Figure 5. VIPhyb Synergistically Enhances Splenocyte Proliferation and T cell Expansion in a B16F1 and B16F10 Co Culture in Combination with an Anti-PD-1 Antibody. (A) Splenocytes isolated from 3 naïve luciferase^{+} B6 mouse were cultured with irradiated B16 cells and with or without VIPhyb in triplicate, and proliferation measured with BLI. Splenocyte culture was performed in triplicate and the results were expressed as mean ± SD. (B) The co-culture in Fig.A was repeated with or without added anti-PD-1 antibody and the results were expressed as mean ± SD. (C) Quantification of CD8 subset in splenocytes co-cultured with irradiated B16 cells and VIPhyb and/or an anti-PD-1 antibody. *p <.05 **p <.01 ***p <.001 ****p <.0001 indicate significant differences between the control and treated groups.

VIPhyb Synergizes with an Anti-PD-1 Antibody to Reduce Tumor Burden

Because VIPhyb alone did not activate a robust anti-tumor immune response in a B16F1 tumor model, we tested if combination with an anti-PD-1 antibody in the B16F10 model would stimulate a synergistic effect. We injected B16F10 cells in B6 Albino mice and administered 10 µg of VIPhyb subcutaneously everyday for 10 days, or 3 doses of 200 µg of an aPD-1 antibody intraperitoneally every 3 days, or a combination of administrating both VIPhyb and the PD-1 antibody. Fig. 9 shows the tumor growth through day 17 of the *in vivo* experiment. B16F10-bearing mice treated with VIPhyb or an aPD-1 antibody have a lower average tumor volume than untreated B16F10-bearing mice. However, mice treated with a combination of VIPhyb and an aPD-1 antibody had a significantly lower tumor volume on average (Fig. 6A) and increased survival (Fig 6B).

A



B

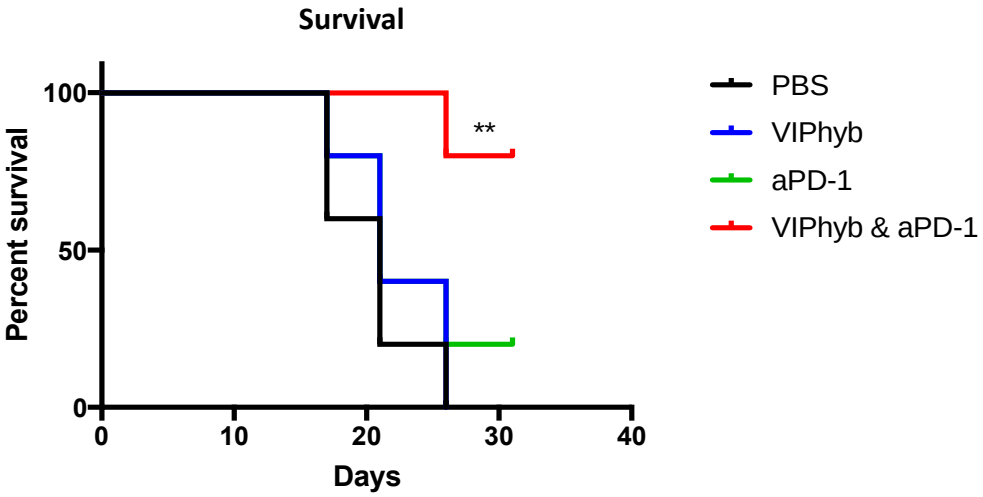


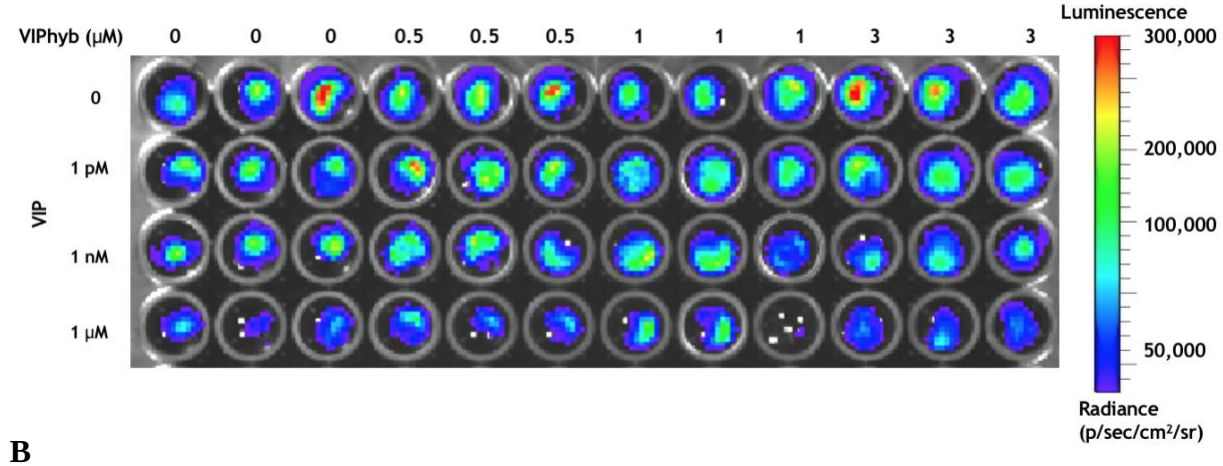
Figure 6. VIPhyb Synergizes with an aPD-1 Antibody to Significantly Reduce B16F10 Tumor Growth. (A) Tumor volumes were calculated as (length*width)/2. (B) Survival of B16F10 bearing, VIPhyb-treated, PD-1 treated, and both VIPhyb and PD-1 treated B6 Albino mice, compared with B16F10 bearing, PBS treated mice through Day 25 $**p < .01$

VIPhyb and VIPhyb-NP Increases T cell Proliferation In Vitro

To determine if VIPhyb's mechanism works by increasing T cell proliferation, we cultured luciferase expressing B6 T cells in 96-well plates pre-coated with anti-CD3 antibody and low-dose recombinant murine IL-2 for 3 days and assessed proliferation by BLI (Fig. 7A). Addition of VIPhyb dose-dependently promoted proliferation in response to CD3 stimulation, while VIP resulted in a dose-dependent reduction of proliferation. Further, addition of VIPhyb to cultures containing exogenous VIP partially reversed VIP-mediated suppression of T cell proliferation up to 1 μM (Fig. 7C).

VIPhyb can only partially reverse the effects of low-dose exogenous VIP, and has questionable stability due to the presence of proteases in cell media. Therefore, we next tested whether VIPhyb coupled to a 30 nm pluronic-stabilized polypropylene sulfide nanoparticle (VIPhyb-NP) could enhance the stimulatory effect of VIPhyb on T cells. Administration of VIPhyb-NP dose-dependently and more consistently promoted proliferation in response to CD3 stimulation, as measured by BLI (Fig. 7B). In addition, administration of VIPhyb-NP to cultures with exogenous VIP partially reversed VIP-mediated suppression of T cell proliferation up to 10 μM (Fig. 7D).

A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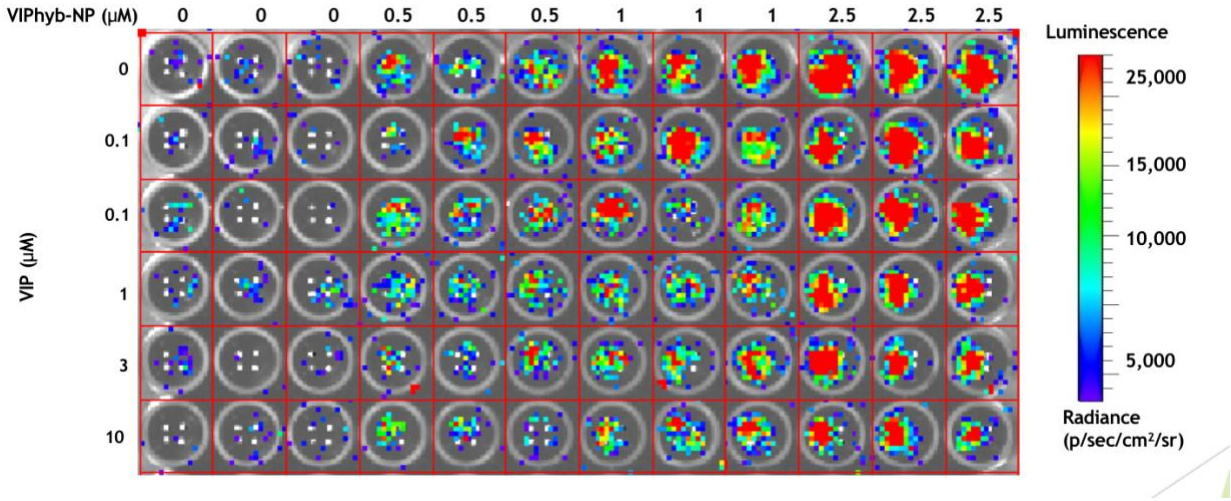
B


VIPhyb-NP (μM)
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VIP (μM)
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Luminescence
25,000
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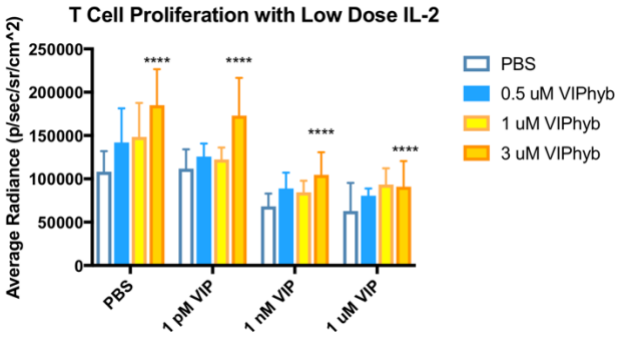
5,000
Radiance
(p/sec/cm²/sr)

C


T Cell Proliferation with Low Dose IL-2
Average Radiance (p/sec/sr/cm^2)
250000
200000
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PBS
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1 uM VIP
PBS
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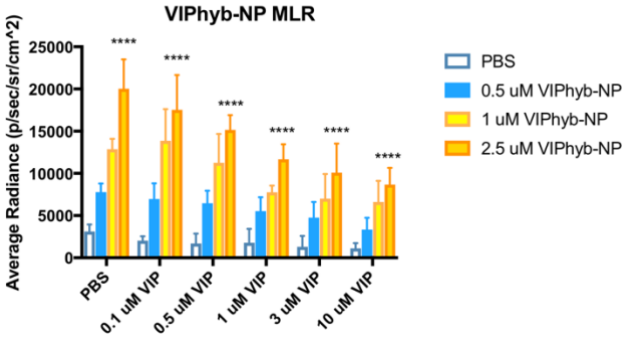
1 uM VIPhyb
3 uM VIPhyb

D


VIPhyb-NP MLR
Average Radiance (p/sec/sr/cm^2)
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PBS
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0.5 uM VIP
1 uM VIP
3 uM VIP
10 uM VIP
PBS
0.5 uM VIPhyb-NP
1 uM VIPhyb-NP
2.5 uM VIPhyb-NP

Figure 7. VIPhyb and VIPhyb-NP Enhanced T cell Proliferation *In Vitro.* (A) Representative BLI image of luciferase expressing T cells treated with either PBS, VIPhyb, or VIP. The scale indicates the intensity of the signal radiance emitted from T cells. (B) Representative BLI image of luciferase expressing T cells treated with either PBS, VIPhyb-NP, or VIP. The scale indicates the intensity of the signal emitted from T cells. (C,D) Quantification of proliferation reported as average radiance emitted from the T cells. ****p <.001 indicate significant differences between the control and treated groups.

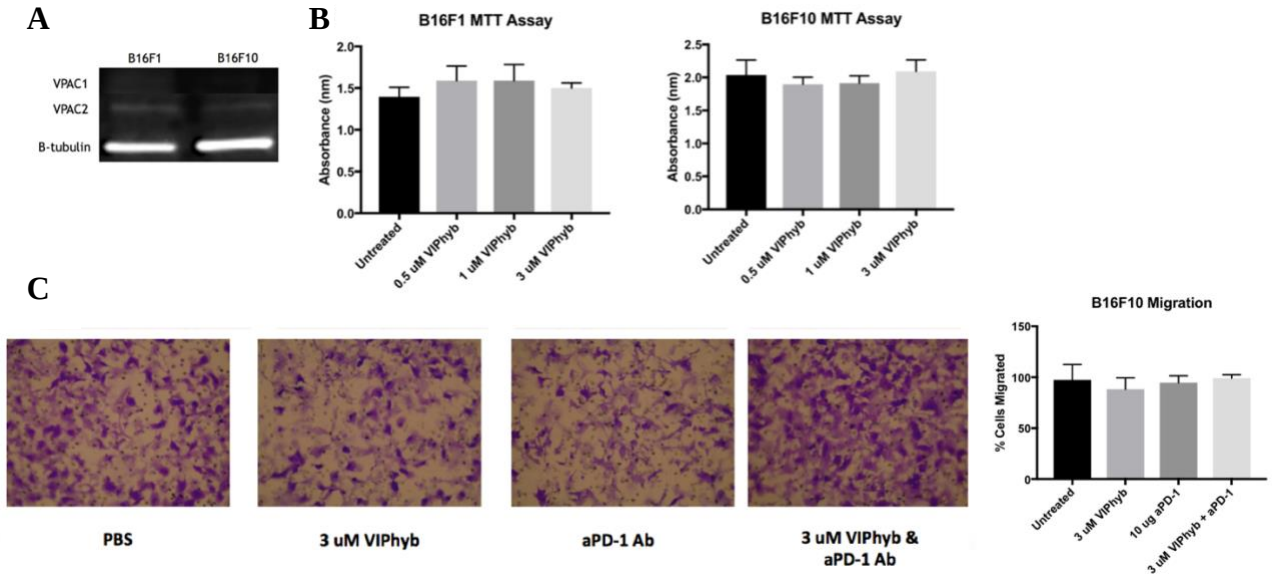
B16F1 and B16F10 Express PD-L1, PD-L2, MHCI, and VPAC2, but do not Respond to VIPhyb

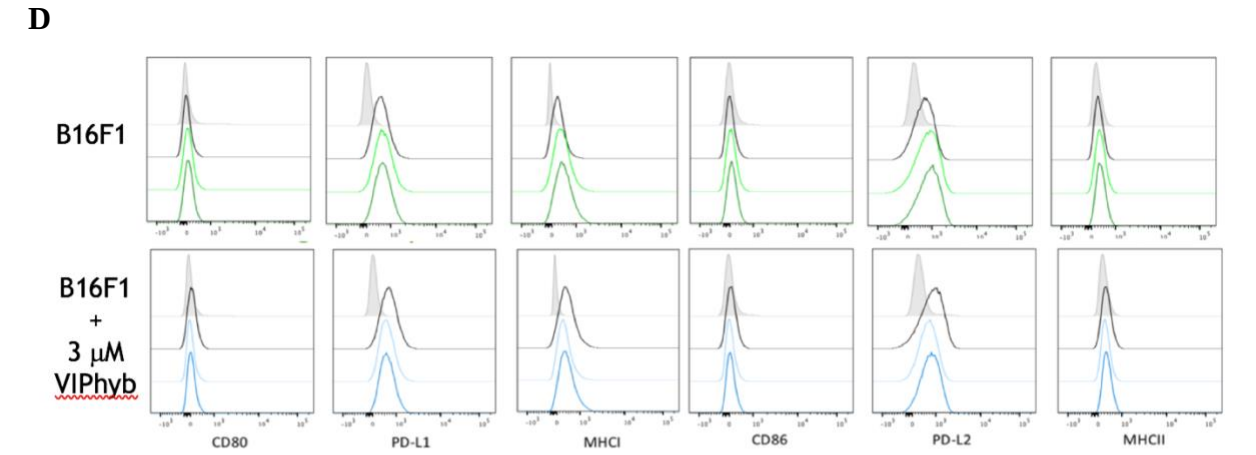
To identify if VIPhyb has a direct anti-cancer effect on murine melanoma, we tested whether the peptide impacted the immunological or growth properties of B16F1 and B16F10. We performed western blots using polyclonal antibodies to VPAC1 and VPAC2 to determine protein expression of the VIP receptors (Fig. 8A). No bands were detected when membranes were probed for VPAC1, but probing for VPAC2 yielded a single band. These results show that both B16F1 and B16F10 cells only express VPAC2.

Since inhibition of this receptor with VIPhyb could lead to decreased growth and invasiveness, we next examined the viability and invasive potential of B16F1 and B16F10 cells *in vitro* in the presence or absence of VIPhyb. Cell viability did not significantly differ with increasing concentrations of VIPhyb compared to the negative control as measured by MTT assay (Fig. 8B).

The B16 cell lineage is a known PD-L1-expressing cell line[54]. Because melanoma often upregulates PD-L1 to escape immune recognition, we investigated if culture with VIPhyb could reduce expression of co-inhibitory ligands following IFNγ stimulation. As shown in Fig. 8C and 8D, the expression of co-inhibitory ligands was not prevented or reduced by treatment with 3 μM

of VIPhyb. As a result, a B16 melanoma tumor has the potential to signal through the PD-1 pathway to induce anergy in T cells even when administered VIPhyb.





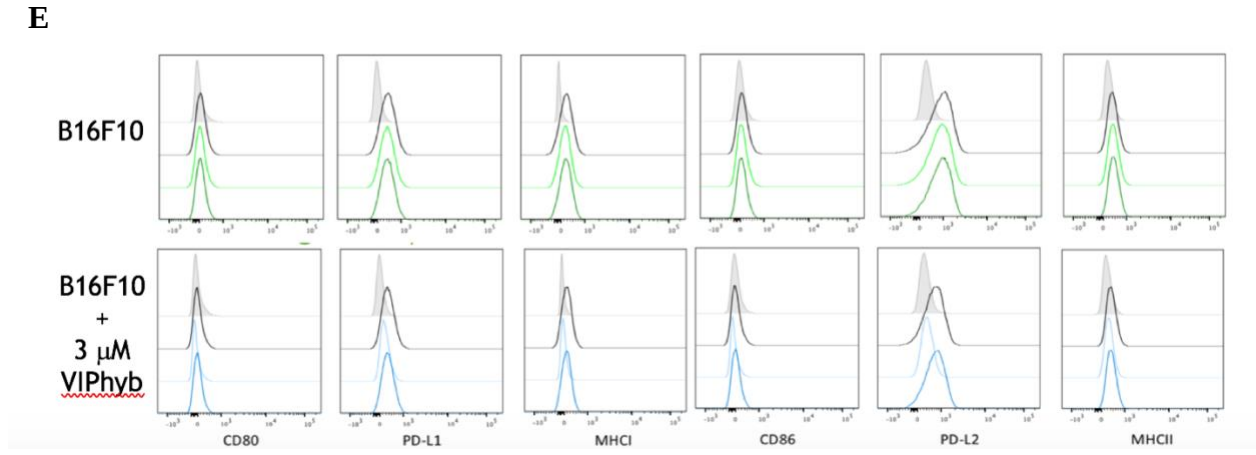


Figure 8. B16 Melanoma Expresses VPAC2 but is not Directly Affected by VIPhyb. (A) Image of Western blot probed for VPAC1 and VPAC2. (B) Quantification of absorbance of B16 cells administered various concentrations of VIPhyb. (C) Image of migrated cells through a matrigel layer treated with either VIPhyb or a combination of VIPhyb and an anti-PD-1 antibody. (D) Expression of CD80, PD-L1, MHCI, CD86, PD-L2, and MHCII on live B16 cells cultured for 48 hr in the presence of IFNγ and 3 μM VIPhyb.

Discussion

Numerous studies indicate the immunosuppressive activity of VIP in a variety of cell types and cancers. Therefore, antagonism of the VIP receptors should result in a potent immunostimulatory effect. Previous research in the Waller Lab explored the use of VIPhyb as an immunotherapeutic to promote T cell responses, particularly in the CD8+ compartment and dendritic cell maturation. This work into VIPhyb has corroborated its therapeutic benefit on murine cytomegalovirus, graft vs. leukemia effect in murine allo-transplantation, and murine C1498 leukemia[35,36,55]. In these settings, VIPhyb effectively increased production of inflammatory cytokines and modulated the expression of immune checkpoint molecules, pointing to a possible benefit for the highly immunogenic melanoma. Based on these findings, evaluated whether VIPhyb administration would have an anti-tumor effect on melanoma growth in mice based upon the hypothesis that it would induce T cell proliferation and synergize with a PD-1 checkpoint inhibitor.

Melanoma has proven to be a particularly immunogenic tumor type, with a high number of infiltrating leukocytes and abundant presentation of melanocyte associated antigens[56]. Further, numerous melanomas upregulate the T cell checkpoint molecules CTLA-4 and PD-L1, rendering this tumor type a prime target for immunotherapeutics, particularly checkpoint inhibitors[57]. The data presented here provide evidence that VIPhyb, a peptide antagonist to the vasoactive intestinal peptide signaling pathway increases T cell proliferation, and synergizes with an anti-PD-1 antibody. Although VIPhyb treatment as a single agent failed to reduce tumor burden in B16F1-bearing mice, it did significantly increase the amount of T cells, specifically CD8+ T cells.

The greatest effects of VIPhyb, however, were seen when administered in mice in combination with a PD-1 inhibitor. Using monoclonal antibodies to reduce PD-1 signaling has

profound effects on exhausted T cells in cancer and is currently FDA approved for treatment against melanoma in the clinic[58]. PD-1 checkpoint blockade is known to enhance inflammatory cytokine secretion and T cell proliferation, which may be why VIPhyb synergizes with PD-1 inhibition to create robust T cell expansion *in vitro* and *in vivo*. This combination most likely affects CD8+ T cells most, as CD8+ T cells were increased both in a splenocyte co culture and in vivo experiments. Because PD-L1 expression on melanoma cells has been proven to be sufficient for immune evasion[59], T cells in mice treated only with VIPhyb may become anergic due to the upregulation of expression of PD-L1 on melanoma cells[60]. Since CD8+ cytotoxic T cells are the primary effectors of an anti-tumor response, this validates the reduced tumor growth in combination treated mice[61]. This concept would also explain the lack of splenocyte proliferation *in vitro* when VIPhyb is administered alone. As a result, when administered together, VIPhyb synergizes with PD-1 inhibition. Taken together, these data indicate that VIPhyb treatment alone does not affect tumor size, but bolsters CD8+ proliferation, an effect that is augmented in combination with a PD-1 inhibitor to reduce tumor burden and enhance survival.

The anticancer activity of treatment with VIPhyb appears to be strictly T cell mediated. VIPhyb reversed exogenous VIP mediated splenic T cell suppression *in vitro*, suggesting that it may have the same effect on stimulating T cell expansion by antagonizing endogenous VIP *in vivo*. Further evidence for a T cell-mediated mechanism-of-action for VIPhyb is the absence of a direct effect on melanoma growth, invasiveness, and modulation of inhibitory surface molecules despite expression of VPAC2. Interestingly, a nanoparticle designed to target the tumor draining lymph nodes (TDLN) in mice, was able to stabilize the peptide *in vitro* leading to increased potency. Appropriately, the T cell stimulatory effect of VIPhyb was conserved with a VIPhyb-NP formulation, and was able to block the effects of exogenous VIP at higher concentrations.

Thus, VIPhyb-NP may be more effective in activating an adaptive immune response *in vivo* than the free drug formulation.

In conclusion, we show here that antagonizing the VIP pathway with VIPhyb results in significant enhancement of T cell growth *in vitro* and reduction of tumor growth while increasing survival as a combination therapy with a PD-1 monoclonal antibody. While the mechanism of how these drugs interact is a topic for future research, this synergistic effect supports the idea of blocking the VIP pathway as a novel therapeutic method in cancer immunotherapy.

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