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Antagonism of the vasoactive intestinal peptide and PI3K δ signaling pathways in T cell-based therapies for leukemia and lymphoma

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By

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An abstract of a dissertation 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 Doctor of Philosophy in Immunology and Molecular Pathogenesis
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Abstract

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By Christopher Thomas Petersen

The critical role of T cells in tumor surveillance and eradication in a variety of cancers has been established by recent advances in the understanding of T cell-tumor interactions. These advances have led to the development of a wide array of immunotherapeutic strategies that have seen varying degrees of clinical success. The use of T cell-based immunotherapies has largely been explored in settings of solid tumors whose high mutational burden provide a large amount of neo-antigenic targets. Unlike solid tumors, hematological malignancies such as leukemia and lymphoma have a relatively low mutational burden that severely limits their potential to be recognized and eliminated by autologous T cells. In addition to problems with tumor cell recognition, T cell responses to both solid and hematological malignancies are also inhibited by active and passive immune evasion strategies. While T cell responses to solid tumors largely fail as a result of exhaustion and inhibitory signals in the tumor microenvironment, endogenous antigen-specific T cells recognizing leukemia and lymphoma cells may be limited by the failure of antigen-specific T cells to fully activate *in vivo* leading to their subsequent deletion. This failure of primary T cell activation is attributed to the disseminated nature of hematological malignancies that results in decreased antigen and danger signal densities. Thus, the failure of T cells to eliminate solid tumors is an issue of exhaustion while the failure to eliminate leukemia and lymphoma cells is an issue of immunological ignorance or anergy. Despite these fundamental differences, similar strategies to enhance the anti-tumor T cell response have been explored in both disease settings. Hematological malignancies served as the prototypical target of immunotherapies with the advent of stem cell transplants and monoclonal antibodies. While these therapies remain powerful tools for the treatments of these diseases, many emerging therapies focus on enhancing autologous T cell responses. Some of these treatments include checkpoint blockade, cytokine therapies, and therapeutic and prophylactic vaccinations. The results of immunotherapies designed to bolster autologous anti-tumor T cell responses have been less promising in hematological malignancies due in large part to the reduced immunogenicity of the malignant cells which renders T cells anergic or senescent. In spite of this large obstacle, T cell-based therapies hold tremendous promise for leukemia and lymphoma treatment, and as such, it is critical to explore additional avenues for strengthening the anti-tumor response in these settings.

We addressed the issues of T cell anergy and senescence in settings of leukemia and lymphoma by using antagonists to the vasoactive intestinal peptide (VIP) and PI3K δ pathways to improve T cell activation and reverse or prevent senescence in settings of murine model systems of leukemia and lymphoma and pre-clinical studies of T cell expansion and CAR T cell manufacturing using clinical samples obtained from

lymphoma patients. Using a peptide VIP antagonist, VIPhyb, as a therapeutic in leukemic mice we found that small subcutaneous doses led to a significant reduction in tumor burden and a significant survival benefit. The effect of VIPhyb was T cell-mediated as VIPhyb-treated mice had increased frequencies of effector CD8 T cells with reduced expression of PD-1 at later time points during tumor development, and the anti-tumor promoting activity of VIPhyb required the presence of CD8+ T cells in treated mice. Furthermore, T cells from VIPhyb-treated mice conferred protection from acute myeloid leukemia challenge following adoptive transfer to Rag1 knockout recipients. Based on these results, we explored the use of VIPhyb in combination with the PI3K δ inhibitor Idelalisib in the setting of *ex vivo* anti-CD3/CD28-mediated T cell expansion for chimeric antigen receptor T cell manufacture. Expansion of T cells from heavily treated DLBCL patients in the presence of VIPhyb and Idelalisib resulted in significantly increased yields as well as a preservation of the naïve and central memory compartments. T cells expanded in the presence of Idelalisib and VIPhyb had the greatest *in vivo* persistence in murine xenografts and significantly enhanced antigen-specific cytotoxic activity in an OVA-expressing murine tumor model. The results of our murine and human studies highlight the role of the VIP and PI3K δ pathways in T cell dysfunction in settings of leukemia and lymphoma. The addition of these inhibitors to clinical expansion cultures of T cells for adoptive cell therapies may provide a much-needed tool that is currently missing in immunotherapeutic strategies for the treatment and management of hematological malignancies.

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Chapter 1:

Introduction

1-1-0 Hematological Malignancies and the Immune System

1-1-1 Overview of Hematological Malignancies

Hematological malignancies are a diverse group of cancers and neoplasms that arise from precursors and mature cells of the hematopoietic system. Two of the most common hematological malignancies are leukemia and lymphoma which each consists of a large variety of diseases that are classified according to the transformed cell type and anatomical origin. Leukemias develop in the bone marrow and are present in the peripheral blood while lymphomas develop in the lymphatic system with a low abundance of tumor cells in the peripheral blood.[1, 2] In the United States, hematological malignancies account for 10.2% of total new cancer cases in 2017 with lymphoma accounting for the greatest number of new blood cancer diagnoses at 47%.[3] Treatment advances over the last several decades have significantly improved 5-year survival rates in the United States for all forms of blood cancers with the survival rate for leukemias being the lowest at 69%.[4] The introduction and refinements of allo and auto stem cell transplants since the first transplant was performed in 1957 has contributed to these survival increases.[5] In addition to stem cell transplants, clinicians also have a wide variety of chemotherapeutics at their disposal including alkylators and hypomethylating agents.[6, 7] Despite numerous highly efficacious treatment options, there remains a significant number of patients who succumb to their disease. Treatment failures and transplant-associated complications in the form of chronic and acute graft-versus-host disease have prompted the investigation into alternative therapies.

These investigations have ushered in a new era of therapies for hematological malignancies of which many are immune based and do not involve stem cell transplants. These immunotherapies are designed to bolster a patient's anti-tumor response as the natural anti-tumor response in leukemia and lymphoma patients fails to sufficiently eliminate malignant cells.[8] Exploiting a patient's own immune system to eliminate cancer avoids chemotherapy-associated toxicities and transplant-associated complications potentially offering a safer alternative. Despite the tremendous promise of immunotherapies in the treatment of hematological malignancies, several biological properties of T cells obtained from heavily pre-treated cancer patients and manufacturing have held them back from their full potential. Thus, it is imperative to devise strategies that circumvent these obstacles in order to increase the efficacy of cellular immunotherapies used to treat leukemia and lymphoma.

1-1-2 History of Immunotherapies for Leukemia and Lymphoma

Immunotherapeutic approaches to the treatment of cancer data back to the 19th century following observations of cancer regression in patients injected with bacterial cultures.[9] Progress was slow in the tumor immunology field until the mid 20th century when Macfarlane Burnet developed his theory of cancer immunosurveillance.[10] This theory remained controversial with immunologists due to conflicting reports and was not widely accepted until a key study was performed in 2001 that strongly supported it.[11-13] Despite a lack of scientific

consensus on the subject, the immune system was being used in a variety of ways since the 1950's in the treatment of cancer. Hematological malignancies served as the main target of these therapies with the most common being allogeneic stem cell transplant. While the first transplant was performed in 1957 using an identical twin donor, the first successful allotransplant from an unrelated donor was performed in 1980 greatly increasing the feasibility of the procedure.[5, 14] In addition to various transplantation methods, several other breakthrough immunotherapies were developed as a result of research on treatments for hematological malignancies.

Malignancies of the hematopoietic system are unique in that the tumor cells often derive from cells that are non-essential for survival. As such, elimination of tumor cells along with their healthy counterparts is possible as a treatment approach. This strategy led directly to the development of monoclonal antibodies as therapeutics designed to target specific subsets of cells. The first monoclonal antibody therapies were successfully used to treat B cell lymphoma and were targeted to the idiotype of the immunoglobulin expressed by mutated clones.[15] This strategy was severely limited as the murine origin of the therapeutic antibodies rendered them targets for anti-mouse antibodies produced by the patients.[15] This issue was addressed a few years later with the production of humanized murine antibodies with reduced potential for antibody-mediated clearance.[16] The targeting of lineage markers expressed by normal and malignant B cells followed soon after with the discovery of CD20 and CD19 in the

early 1980's.[17, 18] This strategy enabled the specific elimination of malignant and normal cells belonging to the same lineage and has become a standard in the care of patients with B cell lymphomas since the first successful clinical use of the anti-CD20 monoclonal antibody Rituximab in the early 2000's.[19, 20]

Vaccination has long been a focus of immunological research with most efforts aimed at eradicating infectious diseases. A large body of work on T cell-tumor cell interactions revealed dynamics and mechanisms with striking similarities to those of T cells and their targets in settings of bacterial and viral infections. These studies formed the rationale behind efforts to develop therapeutic and prophylactic vaccines for various cancers. Hematological malignancies served as one of the earliest candidates for anti-cancer vaccines due in large part to knowledge of a specific antigenic target. Similar to monoclonal anti-idiotype antibodies, the idiotype of malignant B cell clones was used as a target in early attempts at vaccination for lymphoma.[21] Since then a wide variety of tumor antigens have been identified that are differentially expressed across the various forms of leukemia and lymphoma and have been considered as targets for vaccination.[22] Additional vaccination strategies include the use of irradiated tumor cells in combination with various antigen presenting cells (APC) and/or effector lymphocytes.[23, 24] Regardless of the strategy used, clinical trials using vaccines for hematological malignancies have seen limited success. This is largely due to the suppressive tumor microenvironment preventing a robust T cell response.

1-1-3 Emerging Immunotherapies for Leukemia and Lymphoma

Rapid advancements in molecular genetic techniques have enabled the development of a new generation of targeted immunotherapies for hematological malignancies. The expression of hematopoietic lineage markers on leukemia and lymphoma cells makes them prime targets for these therapies. Additionally, a large body of work detailing the mechanisms and dynamics of T cell activation in response to antigen recognition has led to a new class of therapeutic monoclonal antibodies. These antibodies have shown tremendous promise in cancer treatment as well as a wide variety of additional clinical settings.

Bispecific T cell engagers (BiTEs) are dual-specificity antibody fragments with separate idiotypes that recognize two different antigens.[25] BiTEs typically consist of two single chain variable fragments (scFv) with one being specific for the CD3 complex and the other with specificity for a target expressed on a tumor cell.[25] Use of antibody fragments rather than intact antibodies reduces production difficulty and prevents binding to and activation of cells expressing F_c receptors. The purpose of these antibody fragments is to physically couple a T cell with a tumor cell to trigger its killing. Binding of the CD3-specific scFv triggers T cell activation and cytotoxic functions only when the other scFV is bound to the target antigen on a tumor cell.[26] As such, BiTEs have the potential to specifically kill target cells with minimal off-target effects to cells that do not express either target

antigen. The first demonstration of BiTE tumoricidal activity *in vitro* came in 1995 with a BiTE directed to CD3 and the epithelial antigen 17-A.[27] Since then, clinical use of BiTEs has largely been restricted to treatment of acute lymphoblastic leukemia (ALL). Blinatumomab is a fully FDA-approved BiTE with specificity for CD3 and CD19 and has shown increased efficacy over standard chemotherapy in adult ALL patients.[28] Of note, loss of CD19 on tumor cells in relapsed cases occurred following blinatumomab treatment highlighting the need for additional target antigens.[29] Additional BiTEs are currently in development with a wide variety of specificities for extra and intracellular tumor antigens.[25, 30]

T cell activation involves the dynamic expression of co-stimulatory and co-inhibitory molecules for achieving sufficient response strength while minimizing inflammatory damage to local tissues. Investigation into the temporal expression and function of these “immune checkpoint molecules” was jump-started with the discovery of CTLA-4 in the 1980’s and PD-1 in the 1990’s.[31-33] The observation that the function of exhausted virus-specific T cells could be restored via blockade of PD-1 prompted numerous studies on the role of PD-1-PDL-1 interactions in cancer.[34, 35] These and numerous other basic studies led to the development of an entire class of monoclonal antibodies termed “checkpoint blockers” which function to prevent negative signals to T cells. The anti-CTLA-4 antibody ipilimumab was tested in non-Hodgkin lymphoma patients with minimal efficacy.[36] In contrast to this study, a landmark clinical trial testing ipilimumab in metastatic melanoma patients found that CTLA-4 blockade significantly

prolonged survival.[37] This study provided proof-of-concept data that significantly increased interest in this area. Currently in development and clinical practice for the treatment of hematological malignancies are numerous monoclonal antibodies directed towards CTLA-4, PD-1, and PD-L1/L2.[38, 39]

Immunotherapeutic treatment approaches have their roots in cellular-based therapies such as stem cell transplant and lymphocyte infusions. Much of the focus on cellular therapies was shifted to immune modulatory drugs and biologicals in the 2000's after clinical success with those agents. More recently, however, much of the focus has shifted back to cellular therapies with the advent of T cells engineered to recognize specific targets. Chimeric antigen receptor T cell (CAR T) technology involves the use of autologous T cells that are retrovirally transduced to express a chimeric receptor consisting of one or more co-stimulatory domains and a single chain variable fragment that determines specificity.[40] Binding of the scFv to its target triggers T cell activation and subsequent killing of the target cell.[40] All cells expressing the target antigen are potentially eliminated, and as such CAR T therapies have largely been limited to hematological malignancies that express a lineage-specific antigen associated with the malignant clone and a dispensable population of non-malignant counterparts. CAR T cells have two major benefits over standard cellular therapies for hematological malignancies: 1. The use of autologous cells eliminates the need for a suitable donor and any potential consequences of alloreactivity and 2. The technology allows selection of a specific antigenic target.

Clinically, CAR T cells have been tested in a multitude of trials for pediatric and adult B cell malignancies with common antigenic targets being CD19 and CD20.[41] These therapies have seen tremendous success for the treatment of leukemia with complete response rates in some trials being greater 90%.[41] Preliminary results of trials using CAR T cells for the treatment of B cell lymphomas have been promising but less impressive.[42] Supportive therapies consisting of checkpoint blockade agents may help to improve outcomes as significant upregulation of co-inhibitory molecules occurs early following CAR T administration (Figure 1).

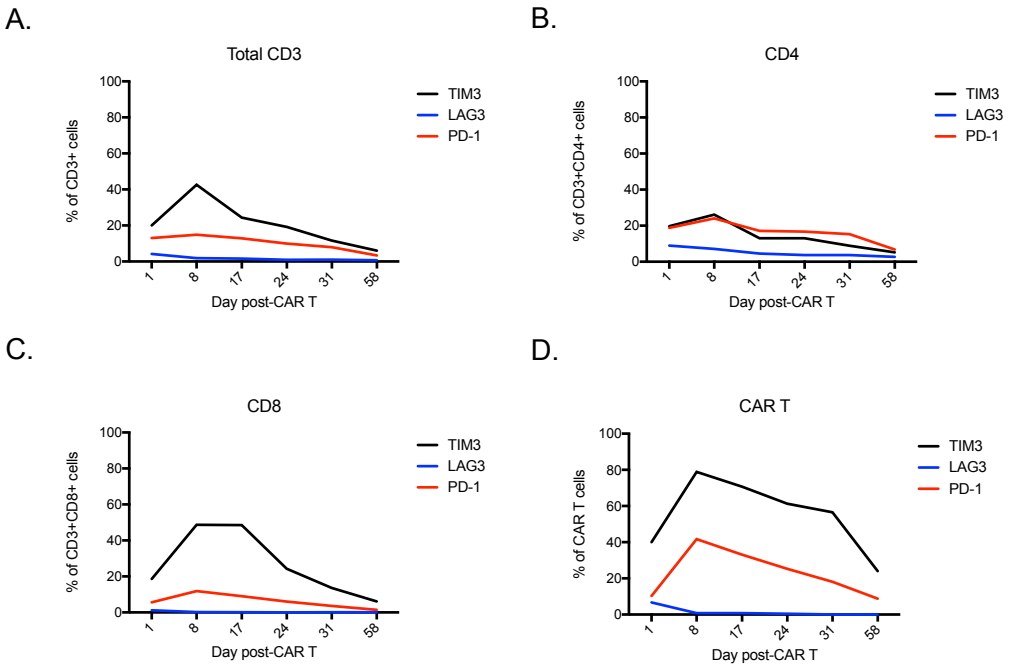


Figure 1: Significant upregulation of co-inhibitory molecules on T cells following CAR T infusion. Peripheral blood mononuclear cell samples from a

DLBCL patient were phenotyped by flow cytometry periodically over the first two months after CD19 CAR T infusion.

1-2-0 T Cell Responses and Tumor Immune Evasion Strategies

1-2-1 Tumor Antigen Recognition

CD4 and CD8 T cells recognize their antigenic targets as a peptide bound to a major histocompatibility (MHC) molecule presented on the surface of an antigen presenting cell. In settings of infection, these peptides are derived from bacterial or viral proteins that are mainly processed by one of two pathways: cytoplasmic proteins are processed and loaded onto MHC class I molecules while endocytosed proteins are processed and loaded onto MHC class II.[43] Cytotoxic CD8 T cells are restricted to peptides presented on MHC class I while CD4 T cells are restricted to peptides presented on MHC class II.[44] Processing and presentation of tumor antigens involve the same pathways as antigens derived from infectious microorganisms. Some of the antigens expressed by virally induced tumor cells are derived from viral proteins while others are derived from self-proteins. Tumor antigens expressed by leukemia and lymphoma cells that can elicit spontaneous responses and are derived from self-proteins fall into one of three major classes: 1. Mutated/modified proteins, 2. Overexpressed normal proteins, and 3. Proteins not normally expressed by the hematopoietic cells from which the tumor cells develop.[45]

Central tolerance via thymic selection helps prevent T cell responses to normal self-proteins through deletion of self-reactive thymocytes.[46] Despite this highly efficient mechanism to prevent T cell-mediated destruction of healthy cells and tissues, spontaneous responses to tumor cells, which are derived from normal cells, occurs. One of the enabling features of malignant cells is genetic instability that leads to the expression of mutated forms of normal proteins. These mutant proteins are processed by cytoplasmic proteasomes to generate neoantigens that consist of short peptides that can be loaded onto MHC class I molecules. Additionally, post-translational modifications of normal proteins can lead to the production of neoantigens. These antigens may then be recognized by antigen-specific CD8 T cells.[45] A single point mutation in a normal parental gene is sufficient to produce a mutated peptide that can trigger a response by an antigen-specific CD8 T cell.[47] The immunogenicity of a particular cancer can largely depend its mutational burden as cells with a high mutational burden are more likely to process and present peptides derived from mutant proteins. Solid malignancies such as melanoma have the highest mutational burden while hematological malignancies have the lowest.[48] As such, the likelihood of a spontaneous anti-leukemia or lymphoma T cell response may be lower due to the production of fewer neoantigens. In addition to mutated proteins, T cells may also be used to target wild type proteins that are overexpressed in leukemia and lymphoma cells.[22]

One of the greatest breakthroughs in identifying tumor antigens was the discovery of a protein expressed by a murine cell line that elicited CD8 T cell responses *in vitro*. This study found that the protein was expressed at high levels in mastocytoma cell lines but not in normal tissues, and that transfer of the protein to resistant tumor cells made them susceptible to lysis.[49] Van den Eynde and colleagues had unknowingly described a germline antigen, expression of which is restricted to a small subset of cells and tissues excluding most adult cells. The first germline antigens discovered in human tumors were the MAGE antigens followed by the testicular antigen NY-ESO-1.[50, 51] Expression of MAGE and NY-ESO-1 have been described in leukemia and lymphoma and have been explored as targets for immunotherapeutic treatment approaches.[52, 53] While the majority of tumor antigens that have been described-to-date are recognized by MHC class I-restricted CD8 T cells, antigens recognized by CD4 T cells have also been described.[54]

1-2-2 T Cell Activation and Checkpoint Molecules

Following the recognition of antigen presented by an APC or tumor cell, T cells undergo a wide variety of biochemical changes that endow them with effector functions. Leukemia and lymphoma cells, being derived from cells of the hematopoietic lineage, are often antigen presenting cells themselves. Binding of the T cell receptor (TCR) and the CD4 or CD8 co-receptor to the peptide-MHC complex triggers numerous signaling cascades that culminate in the transcription

of immune response genes. T cell activation involves the orchestrated up and down-regulation of a number of molecules that either promote or inhibit the response. The dynamic expression of these “checkpoint molecules” is tightly regulated in order to give a response of sufficient strength and appropriate duration.

In addition to signals emanating from the TCR, signals downstream of co-stimulatory receptors are required for optimal T cell activation.[55] Ligation of CD28 with either CD80 or CD86 increases autocrine production of IL-2 to enhance proliferation and promotes T cell survival through upregulation of the anti-apoptotic protein Bcl-X_L.[56, 57] While expression of CD28 is constitutive on the majority of naïve T cells, the expression of other co-stimulatory molecules is limited to activated T cells. One of these molecules, the inducible T cell co-stimulator (ICOS), is expressed following ligation of the TCR; binding to its ligand enhances T cell proliferation and effector functions.[58] Additional inducible co-stimulatory molecules include 4-1BB and OX-40 which belong to the tumor necrosis factor receptor family and have been shown to be important in anti-tumor T cell responses.[55, 59, 60]

Following initial activation of a T cell in response to tumor antigen presented by an APC and/or tumor cell, T cells enter an effector phase in which they produce cytokines, chemokines, and cytotoxic molecules. These immune mediators function to recruit and promote maturation of additional immune effector cells

and/or directly kill target cells. Co-culture of antigen-specific T cells with hematopoietic tumor cells induces the secretion of IFN-γ and granzyme B, polyfunctional immune effector molecules that function to increase antigen presentation and promote apoptosis, respectively.[61-63] CD8 T cells are the predominant effector T cells in anti-leukemia and lymphoma responses, but CD4 T cells also play a major role through their production of pro-inflammatory cytokines that enhance CD8 T cell cytotoxic activities.[64]

While co-stimulatory molecule expression is required for optimal T cell activation, the expression of co-inhibitory molecules is necessary to initiate the contraction phase that prevents inflammatory tissue damage. These molecules are induced at later stages during T cell activation and function to negatively regulate T cell signaling via phosphatase recruitment or phosphorylation of inhibitory residues on signaling proteins. Two of the most crucial co-inhibitory molecules are CTLA-4 and PD-1.[55] High avidity binding of CTLA-4 to CD80 and CD86 on antigen presenting cells greatly reduces CD28-mediated positive signals.[65] Binding of PD-1 on T cells to its ligands PD-L1 or PD-L2 on APC or tumor targets recruits the phosphatases SHP-1 and SHP-2 which dephosphorylate the activating kinases Lck and ZAP70.[66, 67] The results of this dephosphorylation are reduced proliferation and cytokine secretion.[67] The overall effect of co-inhibitory molecule ligation is a dampening of the response that ultimately leads to its termination.

1-2-3 Immune Evasion by Leukemia and Lymphoma Cells

T cell-mediated elimination of established leukemia and lymphoma and resistance to further tumor challenge were described in the 1970's in murine and rat models.[68-70] The role of T cells in the prevention of tumor development was not well-understood until the early 2000's when the requirement for T cell-produced IFN-γ and perforin for solid tumor immunosurveillance was described.[13] A greater understanding of the role of T cells in the prevention of hematological malignancies was gained later when the requirement of T cells and Fas-FasL interactions was described for the prevention of B cell lymphomas.[71] These and other studies solidified a crucial role for T cells in both the prevention of tumor growth and the elimination of established tumors. Despite these essential functions, immunocompetent humans and animals still develop hematological malignancies. Undermining robust anti-leukemia and lymphoma T cell responses are a wide array of active and passive immune evasion strategies employed by malignant cells to subvert their elimination. This immune evasion enables growth and development of tumor cells that outpace the ability of T cells to respond.

Hematological malignancies employ many of the same immune evasion strategies as solid malignancies, but their unique development and distribution endows them with unique immune evasion mechanisms.

Many leukemias and lymphomas present as widely disseminated diseases and as such do not form a microenvironment that is typical of solid tumors. This

dissemination leads to a great reduction in danger associated molecular patterns (DAMPs), molecules expressed by stressed cells that can activate CD8 T cells. A study using a murine model showed that T cell dysfunction in acute leukemia was largely due to its wide dissemination as T cells only mounted an anti-tumor response when a solid tumor was formed through subcutaneous inoculation as opposed to intravenous inoculation.[72] The likely mechanism behind this failure was elucidated in another study that observed deletion of antigen-specific T cells via bim-dependent apoptosis following encounter with disseminated leukemia cells.[73] Disseminated lymphomas also inhibit T cell responses with one underlying mechanism being cross presentation of antigen by APC in the absence of sufficient co-stimulation.[74]

Leukemia and lymphoma cells exploit co-inhibitory pathways through the overexpression of co-inhibitory receptor ligands. Overexpression of PD-L1 has been observed on many leukemia and lymphoma cells. Notable examples include constitutive expression on Hodgkin lymphoma Reed-Sternberg cells and overexpression on mediastinal B cell lymphoma cells.[75, 76] Additionally, the interaction of PD-1 on CD8 T cells and regulatory T cells with PD-L1 expressed on acute myeloid leukemia cells have been shown to be important in leukemia immune evasion.[77, 78] Additional evidence of the crucial role of co-inhibitory receptors in leukemia immune evasion came from the observation that anergy and deletion of adoptively transferred antigen-specific CD8 T cells could be prevented by simultaneous blockade of CTLA-4, LAG3, and PD-1.[73]

B cell malignancies are unique in that the tumor cells are derived from professional antigen presenting cells. As such, they have the capacity to present antigen directly to CD4 T cells through expression of MHC class II. This antigen presenting function of B cell lymphoma cells is often abrogated, however, due to mutations in or loss of genes involved in class II antigen processing. Loss of the CIITA transcription factor has been observed in diffuse large B cell lymphoma (DLBCL) leading to significantly reduced expression of surface MHC class II on DLBCL cells.[79] Reduced MHC class II expression on tumor cells has also been observed in patients with follicular B cell lymphoma that was attributed to mutations in CREB binding protein.[80] In addition to MHC class II, impairments in the MHC class I presentation pathway have also been observed in B cell leukemia as primary CLL cells showed a reduced ability to upregulate MHC class I in response to IFN-γ stimulation.[81]

Taken together, the failure of T cell responses to leukemia and lymphoma cells is largely due to deletion of antigen specific cells and induction of anergy via alterations in the function and activation status of antigen presenting cells and defects in the antigen-presenting properties of the tumor cells themselves. Therapeutic interventions designed to exploit these evasion mechanisms have been utilized clinically with promising results. However, there is still a need to develop new strategies that circumvent the inhibitory processes that prevent T cells from successfully eradicating leukemia and lymphoma cells.

1-3-0 Vasoactive Intestinal Peptide

1-3-1 Receptors, Signaling, and Non-Immune Functions

A 1970 article published in *Science* by Said and Mutt described a novel peptide isolated from porcine small intestine. This peptide had diverse functions in research animals including vasodilation, increased cardiac output, and increased blood glucose levels.[82] Eventually, the peptide would be named vasoactive intestinal peptide (VIP) and defined as a highly conserved 28 amino acid peptide with an alpha helical structure and N-terminal tail. [83] VIP is a member of the heavily conserved secretin superfamily with common VIP peptide sequences found among mammals and is predominately secreted by neurons of the central and peripheral nervous systems including neurons innervating the gut, the genitourinary tract, and the upper respiratory tract.[82, 84-87] VIP is also secreted by several different types of immune cells.[88, 89] In addition to the physiological functions initially described by Said and Mutt, VIP also plays a large role in the relaxation of smooth muscle in the respiratory tract.[90] Disruption of the VIP gene in mice revealed an additional function of VIP in the regulation of circadian rhythms.[91]

VIP binds to three receptors which each belong to the type B G protein coupled receptor (GPCR) family: VPAC1, VPAC2, and PAC1.[92] Characteristic of these receptors is a structure consisting of seven transmembrane subunits and a large N-terminal ectodomain. The affinity of VPAC1 for VIP is equal to the affinity for VPAC2 while the affinity of PAC1 for VIP is significantly lower.[93] Mutational studies revealed that binding of VIP involves the C-terminal alpha helix with residues Asp^{3}, Phe^{6}, $Thr^{7,}$ Tyr^{10}, Tyr^{22}, and Leu^{23} being critical for binding while residues in the N-terminal tail are necessary for the initiation of VIP signaling.[93, 94] Binding of VIP results in internalization of its receptors which desensitizes cells to further VIP stimulation.[95, 96] VPAC1 and VPAC2 receptors are expressed widely throughout the body with expression in neurons and tissues of the central nervous system, organs of the gastrointestinal tract, breast, kidney, pancreas, smooth and cardiac muscle, thyroid, lymphoid organs, and leukocytes.[97-99]

Binding of full length VIP involves N-terminal tail insertion into the membrane where it contacts transmembrane helices 2, 3, and 7 of the receptor.[100-102] The predominant signaling pathway activated by VIP is the cyclic AMP (cAMP)-protein kinase-A pathway. VIP stimulation of cells expressing VIP receptors results in a large accumulation of intracellular cAMP that leads to activation of protein kinase A through Gαs and subsequent MAP kinase and CREB signaling.[103, 104] An additional study observed that VPAC2 can also associate

with Gαi or Gαq to activate a lipid-mediated pathway involving activation of PLC-γ.[105]

1-3-2 Effects of VIP on T Cell Function

Early studies of VIP following its initial discovery largely focused on its neurological, gastrointestinal, and respiratory functions. Its functions in the immune system were described later when it was observed that VIP has a wide variety of effects on both the innate and adaptive arms of the immune system.[99] In particular, VIP signaling has been found to have profound anti-inflammatory effects on T cell responses in mice and humans with influences on T cell maturation, proliferation, and effector functions. Further supporting a role for VIP in T cell immunomodulation are clinical observations that low concentrations of VIP in serum correlated with disease severity in two forms of autoimmune arthritis.[106, 107]

Expression of VIP receptors on T cells is dynamically regulated with constitutive expression of VPAC1 on naïve CD4 and CD8 T cells. VPAC1 is expressed at significantly higher levels on CD4 T cells than CD8 T cells, and the receptors are downregulated in response to stimulation of the TCR/CD3 complex.[108, 109] There is conflicting evidence for the expression of VPAC2 on T cells with one report claiming that T cells do not express VPAC2 either at rest or post-stimulation and

others claiming that VPAC2 expression is inducible.[108, 110, 111] The systems used to examine VPAC2 expression in these studies varied dramatically which likely accounts for this discrepancy. While the expression of VPAC2 on mature T cells is unclear, VPAC2 has been shown to be expressed on human and mouse thymocytes with a transition to predominant expression of VPAC1 late during maturation.[112, 113] The functional consequences of this reversal remain unclear as VPAC2 knockout mice showed normal distributions of thymocyte populations.[113] The transcription factor Ikaros is largely attributed as the main regulator of VPAC1 expression in T cells as evidenced by studies in primary T cells and T cell lines.[114] T cells respond to VIP by increasing levels of intracellular cyclic AMP and activating the CREB pathway. Phosphorylation of CREB enables CREB binding protein (CBP) binding and promoter engagement. Sequestration of CBP via CREB-CBP binding mediates many of the immunological effects in T cells following VIP stimulation, as CBP is also a binding partner for NF-κB.[99] CD8 T cells require a higher concentration of VIP in order to initiate a response while CD4 T cells are responsive to VIP concentrations as low as 100 pM in culture.[115]

Early reports detailing the responsiveness of T cells to VIP largely focused on the peptide's anti-proliferative effects. Stimulation of unfractionated and purified CD4 and CD8 murine T cells in the presence of VIP resulted in reduced proliferation and a decreased capacity to secrete the cytokine IL-2.[116, 117] Later, it was discovered that T cells secrete VIP *in vitro* in response to stimulation with a wide array of pro-inflammatory factors including anti-TCR antibodies.[118] These results

revealed another source of VIP in addition to VIPergic neurons innervating peripheral lymphoid organs. Studies of the effects of VIP on T cells are predominantly focused on the CD4 subset as these cells express high levels of VPAC1. *In vitro* and *in vivo* studies have highlighted a significant role for VIP in the modulation of CD4 T cell function and maturation. Presentation of antigen in combination with VIP *in vivo* results in the differentiation of CD4 T cells to CD25+FoxP3+ T cells with the capacity to suppress T cell activation.[119] Indirectly, co-culture of CD4 T cells with monocytic dendritic cells generated in the presence of VIP results in the generation of regulatory T cells. This was demonstrated in human and murine studies and was due to failure of dendritic cells to upregulate CD80 and CD86, high secretion of IL-10, and reduced nuclear translocation of NF-κB prior to interacting with T cells.[120, 121] In addition to the promotion of Treg development, VIP has also been shown to play a large role in the promotion of Th2 responses. Interaction of CD4 T cells with VIP-stimulated macrophages results in increased secretion of IL-4, reduced secretion of IFN-γ, and differentiation of CD4 T cells into Th2 effectors through macrophage-expressed CD86.[122] Additionally, the presence of VIP has been shown to promote the differentiation of Th2 effectors into memory cells via reduced Fas-FasL signaling.[123-125] Further supporting a role for VIP in Th2 responses were observations that the absence of VIP signaling enhanced Th1 responses in a model of viral infection.[126]

While the majority of studies on VIP-mediated T cell suppression have focused on the CD4 subset, effects of VIP on CD8 T cell function have also been described. One of the earliest studies that focused on CD8 responses described VIP-mediated inhibition of CD8 cytotoxicity in a granzyme/perforin independent manner via reduced expression of FasL.[127] Later studies focused on CD8 T cells in the context of murine cytomegalovirus infection (mCMV) and found that the absence of VIP signaling either through genetic knockout or treatment with a peptide antagonist significantly enhanced CD8 T cell anti-viral functions. VIP knockout mice or antagonist-treated mice had greater numbers of antigen-specific CD8 T cells which expressed reduced levels of the co-inhibitory receptor PD-1 and secreted significantly more IFN-γ and TNF-α. The functional consequences of these effects were reduced viral loads and resistance to lethal viral challenge.[126, 128, 129] Similar effects of absent or reduced VIP signaling on CD8 T cell function were also observed in murine models of allogeneic bone marrow transplantation and acute leukemia.[115, 130]

1-3-3 VIP and Cancer

The near ubiquitous expression of VIP receptors in the body means that they are also expressed on tumor cells derived from many different cell types. Expression and overexpression of these receptors on tumor cells has shown to have numerous consequences on the proliferation and survival of many tumor cell types *in vitro* and *in vivo*. Studies examining malignant cells and tissues have

determined that VPAC1 and VPAC2 proteins are expressed on carcinomas, pancreatic tumors, lung tumors, glioblastomas, and many other tumor types.[98, 131] VIP receptors are also expressed on malignant cells of hematopoietic origin as observed in studies on T and B lymphoblast and myeloma cell lines and patient samples.[132-135] Similar to VIP receptor expression profiles of normal cells, VPAC1 is the predominant VIP receptor expressed by tumor cells.[136] One exception is that some T cell lines express high levels of VPAC2 unlike their cells of origin.[134] In addition to expression of VIP receptors at normal levels, many tumors have been found to overexpress either VPAC1 or VPAC2 leading some to propose the use of radiolabeled VIP as a cancer diagnostic.[137]

The functional consequences of VIP receptor expression by tumor cells have not been determined for all tumors examined to date. However, studies on select cell lines have revealed that VIP signaling can promote proliferation of tumor cells. For example, addition of exogenous VIP to cultures of non-small cell lung cancer (NSCLC) cells promoted the growth of human NSCLC cells *in vitro.* Further, xenograft formation was inhibited by up to 80% through the subcutaneous administration of a vasoactive intestinal peptide antagonist.[138] Similar results were observed in a breast cancer xenograft model.[139] In addition to these studies, growth inhibition with VIP antagonists has been observed in numerous tumor cell types including colon cancer, melanoma, and leukemia.[140] The results of these studies highlight the potential of VIP antagonists as treatments for a wide variety of cancer. VIP does not act as a trophic factor for all VIP receptor-

expressing tumor cells, however, as demonstrated in specific myeloid and lymphoid leukemia cell lines.[115, 130] Elucidating the functional consequences of VIP signaling in these tumor cells will require further study.

1-4-0 Phosphoinositide 3-Kinase δ (PI3K δ)

1-4-1 Overview of PI3K δ

Phosphoinositide 3-kinases (PI3K) comprise a family of lipid kinases that phosphorylate the inositol ring component of inositol-containing lipids in the inner leaflet of the plasma membrane.[141] Of the 3 classes of PI3K, class I PI3K members have the most significant influence on immune cells. Class I PI3K consists of two subclasses of heterodimeric kinases: Class 1A (α, β, and δ) and 1B (γ) that differ in their use of regulatory and catalytic subunits and activation mechanisms. Class 1A PI3K associate with the p85 regulatory subunit while the single Class 1B member associates with p101.[142]

Phosphorylation of receptor tyrosine kinase YXXM motifs recruits the regulatory subunit of Class 1A PI3K, p85.[141] Phosphorylation of p85 by tyrosine kinases such as Lck relieves its inhibition of the p110 catalytic subunit so that it can phosphorylate phosphoinositide (3,4) bisphosphate (PIP2) to generate phosphoinositide (3,4,5) trisphosphate (PIP3).[143] In contrast, the Class 1B member p110 γ is predominantly activated by the βγ subunit of GPCRs as

initially described using a lymphoma cell line.[144] Proteins containing pleckstrin homology (PH) domains are then recruited to the inner leaflet via PH-PIP3 binding and become activated to transmit downstream signals.[145] Due to the diversity of proteins that contain PH domains, PI3K signaling leads to a large variety of cellular effects including growth, maturation, changes in metabolism, and survival.[146] Activation of the Akt and mTOR pathways downstream of both PI3K classes is responsible for many of these effects in immune cells.[147] Additionally, initial activation of B and T cells through the BCR and TCR, respectively, involves recruitment of Tec kinases through PI3K-mediated PIP3 generation.[148] Lipid phosphatases shut off PI3K signaling through removal of phosphates on the inositol ring. One of the most important negative regulators of PI3K signaling in immune cells is the phosphatase and tensin homolog (PTEN), a protein that is often mutated in malignant cells enhancing their tumorigenicity.[149]

Particular attention has been given to the role of the class 1A PI3K catalytic subunit p110 δ (PI3K δ) in immune cells. The p110 δ subunit was discovered in the late 1990's in human and mouse tissues, and among the tissues tested, its mRNA expression was found to be limited to immune cells and testes.[150] The specific expression of PI3K δ in immune cells was deduced later where it was found in lymphoid and myeloid cells including T cells, B cells, NK cells, neutrophils, and macrophages.[151-154] The diverse functions of PI3K δ signaling in these cells were elucidated through genetic techniques and pharmacological inhibition. The overall consensus reached by these studies is that PI3K δ

signaling in immune cells is a major contributor to inflammatory processes including cytokine secretion, cytoskeletal rearrangements, and cell survival.[142]

1-4-2 Role of PI3K δ in T Cell Development and Function

The generation of p110 δ knockout mice and the development of highly specific p110 δ inhibitors have revealed significant insight into the role of PI3K δ in T cell development and function. Numerous studies have highlighted the crucial roles of this specific PI3K isoform both in thymocytes and mature T cells. Additionally, rare mutations in the genes encoding p110 δ or one of the three genes encoding the p85 regulatory subunit in humans have revealed further insight into the functions of PI3K δ as well as the consequences of unrestrained signaling from this enzyme.

Mice with inactive p110 δ catalytic domains generated via point mutation (p110 δ^{D910A}) have given tremendous insight into the role of PI3K δ in T cell development. Studies of these mice have shown that thymic development is normal with frequencies and absolute numbers of thymocytes similar to wild type levels.[155] Accounting for this normal development in p110 δ^{D910A} mice is functional redundancy with p110 γ as studies using dual p110 γ/δ knockout mice observed profound deficiencies in thymocyte development with arrest at the pre-

TCR checkpoint.[156, 157] These effects were not observed in single knockouts of either p110 isoform. Interestingly, despite severe lymphopenia in double knockout mice, multi-organ inflammation was observed that was associated with eosinophilic infiltration.[157] Taken together, the combined actions of p110 δ and p110 γ are required for normal T cell development.

PI3K δ activates several signaling pathways downstream of the TCR and CD28. As such, loss of PI3K δ has a multitude of negative functional consequences in mature T cells responding to antigen or CD3 cross-linking. In mice bearing the p110 δ^{D910A} point mutation, abnormal T cell phenotypes and functions are observed only in mature T cells. CD4 T cells from p110 δ^{D910A} mice show reduced CD44 expression, reduced proliferation, and reduced IL-2 secretion in response to antigen-specific and anti-CD3 stimulation, but not in response to anti-CD3/CD28 stimulation.[155] Similarly, general pharmacological inhibition of PI3K with the antagonist LY294002 results in reduced T cell proliferation in response to CD3 with or without IL-2.[158] Additional experiments showed that mouse T cells lacking p110 δ catalytic activity showed reduced development towards either Th1 or Th2 subsets when stimulated with their cognate antigen as measured by production of IFN-γ and IL-4. In this study, impairment of T cell function was not observed when CD28 co-stimulation was provided to cells *in vitro* suggesting that CD28 signaling does not fully rely on PI3K.[159] A study using CD28 molecules with mutated YXXM motifs helped to further support this assertion and found divergent roles for CD28 and PI3K in transmitting survival

and proliferative signals.[160] In addition to its role in T helper cell differentiation, PI3K δ has also been shown to be a critical factor in the development and function of regulatory T cells (Treg). Mice bearing the p110 δ^{D910A} mutation show reduced frequencies and absolute numbers of Tregs in peripheral lymphoid organs despite enhanced selection in the thymus. Tregs from p110 δ^{D910A} mice displayed impaired inhibition of T cell proliferation and lacked the ability to produce IL-10.[161] Further supporting a role for PI3K δ in Treg development and function was a study that examined p110 δ^{D910A} mice in settings of solid tumors. The authors observed reduced Treg numbers and impaired function in p110 δ^{D910A} and p110 δ antagonist-treated mice leading to reduced tumor growth and enhanced survival.[162]

Studies on the role of PI3K δ have primarily focused on CD4 T cells, but its role in CD8 T cell function has also been described. In one study, pharmacological inhibition of p110 δ reduced proliferation and secretion of IFN-γ by OT-I T cells stimulated by peptide-loaded APCs *in vitro*.[163] Using a *Listeria monocytogenes* infection model, another study found that the CD8 T cell response in infected p110 δ^{D910A} mice was impaired with fewer antigen-specific T cells in the blood and peripheral lymphoid organs during both primary and secondary challenges. Intriguingly, late memory responses were not impaired due to normal accumulation of memory precursors.[164] Additional insights into the role of PI3K δ have been attained through the study of activating mutations found in human subjects. Patients with a heterozygous gain-of-function mutation in the *PIK3CD*

gene have decreased CD4:CD8 ratios and reduced frequencies of CD45RA+CCR7+ CD8 T cells indicating a skewing towards short-lived effector cells. While TCR signaling in these patients is normal, hyperphosphorylation of Akt and chronic activation of the mTOR pathway results in increased susceptibility to infection and cancer.[165] Similarly, patients with a heterozygous mutation in any of the three p85 regulatory subunits have an overrepresentation of terminally differentiated, senescent CD8 T cells that express high levels of CD57.[166] In both patient cohorts, T cell abnormalities and associated-symptoms could be reduced through administration of rapamycin.[165, 166] The effects of unrestrained PI3K δ signaling on CD8 T cell phenotype and function highlight its role in the generation of short-lived effector cells and terminal differentiation. In further support of this is the reduction in tetramer+ cells and minimal effect on the generation of memory precursors in p110 δ^{D910A} mice during the acute phase of *Listeria* infection.[164]

1-4-3 Idelalisib: a PI3K δ-Specific Inhibitor

Intense interest in the PI3K pathway as a target in several diseases settings has been sparked by the elucidation of its numerous roles in leukocyte biology and cancer biology. In particular, the PI3K pathway has been found to be of tremendous relevance in cancer as mutations in the phosphatase PTEN occur in a large variety of malignancies.[167] Also, as signals through the B cell receptor (BCR) are transduced through PI3K δ, BTK, and Akt, the PI3K pathway is particularly relevant in B cell malignancies such as CLL in which chronic BCR

activation is a strong promoter of tumor cell survival via unrestrained production of PIP3 and activation of downstream kinases.[168] Numerous PI3K antagonists have been developed and tested in clinical trials with different antagonists having specifities for one or more of the Class 1A PI3K isoforms.[169] Idelalisib (CAL-101) is an FDA-approved chemical PI3K antagonist with strict specificity for the δ isoform that is currently used in chronic lymphocytic leukemia (CLL) treatment.[170] Studies on CLL patient samples and cell lines showed that specific inhibition of PI3K δ with Idelalisib was more effective than pan PI3K inhibition, did not result in significant normal cell death, and that cell death in CLL blasts occurred via caspase-dependent apoptosis.[171, 172]

The effects of Idelalisib on healthy leukocytes are less well characterized, but some studies indicate that Idelalisib alters functional responses in activated T cells. In one study, T cells activated with anti-CD3 monoclonal antibodies showed reduced secretion of IFN-γ, IL-6, and IL-10 and reduced expression of CTLA-4 transcript indicating an inhibitory effect during primary activation.[172] Further insight into the effects of Idelalisib on T cells has been obtained from studies of adverse events in CLL and Non-Hodgkin lymphoma (NHL) patients taking Idelalisib. One study described an NHL patient with severe enterocolitis which was thought to be due to CD8+ T cell infiltration.[173] An additional study in CLL patients found severe hepatotoxicity that was associated with increased lymphocyte infiltration and a reduction in peripheral blood Tregs.[174] Murine tumor models have further highlighted possible pro-inflammatory effects of Idelalisib

administration. In one study, administration of Idelalisib to tumor-bearing mice decreased the frequency of regulatory T cells and increased the frequency of granzyme-B-producing CD8 T cells following vaccination resulting in a reduction in tumor burden and significant survival enhancement.[175] The results of this study together with the observation of Idelalisib-related adverse events support a role for Idelalisib in the enhancement of pro-inflammatory responses. This is in direct conflict, however, with the suppression observed in T cells activated *in vitro* either in the aforementioned study on T cells activated in the presence of Idelalisib or in *in vitro* studies using mice deficient in p110 δ catalytic activity. These discrepancies will require further investigation in order to determine the context-specific activities of Idelalisib and their underlying mechanisms.

Chapter 2:

Administration of a Vasoactive Intestinal Peptide Antagonist Significantly Enhances the Autologous Anti-Leukemia Response in Murine Models of Acute Leukemia

2-1-0 Abstract

Vasoactive intestinal peptide (VIP) is a neuroendocrine peptide hormone that has potent anti-inflammatory activities. VIP signaling through its receptor VPAC1 on T cells leads to reduced proliferation and a reduction in pro-inflammatory cytokine secretion. We report here that inhibition of the VIP pathway with a peptide antagonist significantly enhances a T-cell-dependent, autologous anti-leukemia response in murine models of acute myeloid leukemia and T lymphoblastic leukemia. Subcutaneous administration of the VIP antagonist, VIPhyb, resulted in reduced tumor burden and significantly enhanced survival (30-50% survival) over vehicle-treated controls (0-20% survival). The T cells in mice treated with VIPhyb expressed lower levels of the co-inhibitory PD-1 and secreted higher levels of IFN-γ. Further, T cells from VIPhyb-treated survivors were protective against C1498 following adoptive transfer. These data highlight the potential for the VIP pathway as a novel target for immunomodulation in settings of hematological malignancies.

2-2-0 Introduction

Strategies to enhance the autologous anti-cancer response have been under intense study following the success of clinical trials involving patients with solid malignancies. [176] A variety of approaches have been explored to overcome the suppressive tumor microenvironment and circumvent tumor cell immune evasion.

More specifically, many studies have aimed at enhancing anti-tumor T cell responses via a checkpoint blockade strategy that modulates signaling from co-inhibitory and co-stimulatory molecules. For example, treatment with a monoclonal antibody that blocks signaling through the co-inhibitory molecule CTLA-4 significantly improved survival in patients with metastatic melanoma. [37]

Hematological malignancies have traditionally been treated with chemotherapeutics alone or in conjunction with autologous or allogeneic hematopoietic stem cell transplantation (HSCT). [177] The anti-tumor activities of allogeneic HSCT come in the form of graft-versus-leukemia effects in which donor T cells directly kill transformed recipient cells due to differences in minor and/or major histocompatibility molecules. [177] Despite the widespread use and success of allogeneic transplant, the associated morbidity and mortality of the procedure has prompted investigations into therapies such as checkpoint blockade that provide an enhancement of autologous anti-leukemia immune responses.

Previous work has highlighted a role for vasoactive intestinal peptide in the modulation of T cell responses in the setting of antiviral immunity. [128, 129] Vasoactive intestinal peptide (VIP) is a 28 amino acid neuropeptide that is predominantly secreted from nerve terminals and the gastrointestinal tract from which it derives its name. [82, 99] VIP is also secreted from a wide variety of cells in the immune system including cells of both the myeloid and lymphoid lineages. [88,

[99] The VIP receptors VPAC1 and VPAC2 are widely expressed throughout the body with differentially regulated expression on the surface of immune cells. [99, 109] VPAC1 is constitutively expressed on lymphocytes while VPAC2 is considered to be inducible following an inflammatory stimulus. [108, 109, 111] Signaling through these receptors involves activation of adenylyl cyclase via $G\alpha_s$ resulting in increased levels of intracellular cyclic AMP. [178] These events lead to activation of protein kinase A followed by activation of the p38 and CREB pathway. [178, 179] VIP signaling in the immune system has potent anti-inflammatory effects on cells of both the innate and adaptive arms. Effects on T cells include a skewing of responses towards Th2 effector functions as well as the promotion of T_{reg} development through effects on dendritic cells. [119, 120, 122, 180, 181] Additionally, the presence of VIP inhibits T cell proliferation *in vitro.* [116] One of the largest physiological effects of VIP in myeloid and lymphoid cells is a marked reduction in a variety of pro-inflammatory cytokines including type 1 and 2 interferons, TNF-α, IL-1, and IL-2. [88, 99, 116] Additionally, the presence of VIP leads to increases in the Th2 cytokines IL-4, IL-5, IL-13 and the anti-inflammatory IL-10. [88, 99]

Using the C1498 model of acute myeloid leukemia and the LBRM model of acute T lymphoblastic leukemia, we investigated the immunological effects of pharmacological VIP signaling blockade and their subsequent effects on tumor burden and survival. Treatment of mice with seven small subcutaneous doses of the small peptide antagonist VIPhyb resulted in significantly enhanced T cell

immunity with increased numbers of CD8 T cells, reduced PD-1 expression, and enhanced secretion of the pro-inflammatory cytokines TNF-α and IFN-γ. Mice treated with VIPhyb had a significantly reduced tumor burden, which translated into significantly enhanced survival over vehicle-treated controls. Further supporting a role for T cells in the survival benefits of VIPhyb treatment was the transfer of tumor protection to Rag 1 knockout recipients receiving T cells from VIPhyb-treated donors. The data presented here highlight the potential for VIP antagonism in the treatment of hematological malignancies. In addition, VIP antagonism may hold promise as a treatment for a variety of other cancers and viral infections in which suboptimal T cell responses contribute to disease progression, morbidity, and mortality.

2-3-0 Materials and Methods

Mice

Six to eight week old B10.BR mice were used for the LBRM model and were bred at the Emory University Animal Care Facility (Atlanta, GA). For the C1498 model, six to eight week old male albino B6 mice (B6(Cg)-*Tyr*$^{c\text{-}2J}$/J) were purchased from Jackson Laboratories. Six to eight week old male *Rag 1* knockout (B6.129S7-*Rag1*$^{tm1Mom/J}$) were purchased from Jackson laboratories. VIP-PHI knockout mice were bred at Emory University [91]. 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.*

Cell Lines

The dsRed and luciferase-transfected acute myeloid leukemia cell line C1498 was a generous gift from Dr. Bruce Blazar. [182] The T lymphoblastic leukemia cell line LBRM was obtained from American Type Culture Collection (ATCC) and transfected with luciferase as previously described. [183] C1498 and LBRM cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 50 µM 2-mercaptoethanol. All cell cultures were kept in a 37 degree Celsius 5% CO_2 humidified incubator. Cell viability was assessed before injection via trypan blue exclusion and was always greater than 95%. Cells were injected in PBS intravenously via the lateral tail vein.

VIPhyb Treatment

VIPhyb peptide was purchased from New England Peptide. The desiccated peptide was reconstituted in sterile molecular grade water and diluted in sterile PBS. A 10 µg peptide dose in a 200 µL injection volume was delivered to each mouse subcutaneously. Treatment began the day before or the day of tumor injection and continued for seven days. In some experiments, treatment was

delayed until day 8 post-tumor cell injection. An equal volume of PBS was injected in control mice.

Bioluminescent Imaging

Tumor burden was assessed using bioluminescent imaging. Animals were first anesthetized using a cocktail of ketamine (80 mg/kg) and xylazine (8 mg/kg). Prior to imaging, the abdomens of B10.BR mice were shaved with an electric razor. D-luciferin was injected via the intraperitoneal route at a dose of 150 mg/kg (Perkin Elmer-122799). Images were acquired on an IVIS spectrum imager (Perkin Elmer, Waltham, MA). Quantification of tumor burden was performed using Living Image software (Perkin Elmer) by creating a region of interest over each mouse. Measurements are reported as photons/s/cm^2.

Adoptive Transfer

Splenic T cells from VIPhyb-treated tumor survivors or naïve B6 mice were purified using the EasySep T Cell Isolation Kit (StemCell Technologies-19851). 5 x 10^6 cells were adoptively transferred via tail vein injection into naïve Rag1 knockout mice. Rag1 knockout mice receiving cells from naïve donors served as controls. One week post-adoptive transfer, 1 x 10^6 C1498 were injected intravenously into both groups of mice. Mice were then challenged with a second

dose of tumor cells and monitored for survival, tumor burden, and T cell phenotype.

Flow Cytometry

Peripheral blood (cheek bleeds) and/or spleen samples were collected from mice at the time points indicated and then prepared for flow cytometric analysis. Blood samples were collected in EDTA-coated tubes. Spleens were flushed with complete media using a syringe, and clumps were removed by passing the cells through a 70 μm strainer. Red blood cells were lysed in ammonium chloride buffer. Samples were then incubated in the presence of an anti-CD16/32 Fc receptor-blocking antibody following by staining with FITC CD3, PerCP CD8, Alexa Fluor 700 CD4, PE-Cy7 CD69, APC-Cy7 CD25, PE CD62L, V450 CD44, and APC PD-1 (Pharmingen-553141, 561801, 561092, 557956, 552879, 561038, 553151, 560451, 562761). Intracellular cytokine staining was performed on splenocytes using Leukocyte Activation Cocktail, BD Cytofix/Cytoperm kit, and antibodies towards CD3, CD4, CD8, IFN-gamma and TNF-α (Pharmingen-554714, 554411, 560658). Samples were run on a FACS Aria flow cytometer (BD Biosciences). List mode files were analyzed using FlowJo software ver 9.8.5 (Tree Star, Inc). Analysis of T cell markers was performed as previously described. [128]

VIP Receptor Expression

VPAC1 and VPAC2 protein levels were determined by Western blot. Protein lysates were prepared from C1498 cells, and 15 µg were loaded onto a 4-20% gradient gel (BioRad- 4561096). Protein samples were then transferred to a nitrocellulose membrane and probed with 1:2000 dilutions of anti-VPAC1 and anti-VPAC2 polyclonal antibodies (Santa Cruz Biotechnologies- sc-30019, sc-30020). Expression of VPAC1 and VPAC2 mRNA was determined by semi-quantitative RT-PCR. Briefly, RNA was extracted using the RNeasy Mini Kit, and 1 µg of RNA was converted to cDNA using the Quantitect Reverse Transcription Kit (Qiagen- 74104, 205310). The following primers were used in the PCR reactions: *VPAC1* forward GATATGGCCCTCTTCAACAACG reverse GAAGTTGGCCATGACGCAAT *VPAC2* forward CCAGATGTTGGTGGCAATGC reverse GTATGTGGATGAGATGCCAATAGG *18s rRNA* forward CGGCTACCACATCCAAGGAA reverse GCTGGAATTACCGCGGCT. Products were run on a 1% agarose gel and imaged using a GelDoc XR+ system (Biorad).

CREB Signaling

Phosphorylation of CREB was determined by flow cytometry and Western blot. Briefly, splenic murine T cells were isolated using the EasySep T Cell Isolation Kit (StemCell Technologies- 19851) and then cultured in complete RPMI containing 0.5% fetal bovine serum overnight. Cells were then incubated at 37 degrees Celsius in the presence of VIPhyb for 30 minutes followed by stimulation

with VIP for 15 minutes. Flow cytometry was performed using BD Phosflow reagents (BD Biosciences-558052) according to the manufacturer's protocol. Antibodies used were Alexa-488 CD3, PE-Cy7 CD4, APC CD8, and PE pS133 CREB (Pharmingen- 557666, 552775, 553035, 558436). Samples were run on a FACS Aria flow cytometer (Becton Dickson, San Jose, CA). Western blotting was performed under the same stimulation conditions using rabbit polyclonal antibodies to pS133 CREB and CREB at a 1:1000 dilution (Cell Signaling Technology- 9191, 9197).

T Cell Proliferation Assay

Purified splenic T cells from B6 mice were labeled with 1 μM CFSE (Thermo Fisher- C34554) and incubated in 96 well flat-bottom plates pre-coated with functional anti-CD3 antibody (eBioscience- 16-0032-81). Cells were stimulated for 48 hours and stained for CD3, CD4, and CD8. Samples were run on a FACS Aria (Becton Dickinson), and proliferation was assessed by dilution of CFSE.

Apoptosis Assay

Apoptosis in C1498 was measured *in vitro* by culturing cells with VIPhyb or daunorubicin HCl (Sigma Aldrich- 30450). The extent of cell death was measured with an Annexin V Apoptosis Detection Kit (eBioscience- 88-8007-72). Sytox blue

(Life Technologies- S34857) was used in lieu of propidium iodide due to the dsRed expression in C1498. Data were acquired using a FACS Aria flow cytometer (Becton Dickson).

Statistical Analysis

Data were analyzed for statistical significance using GraphPad Prism version 5.0d for Mac (GraphPad Software). Each group under study contained at least 5 mice with each experiment being repeated at least twice. Data are presented as ± SD. Differences in survival were calculated using the Kaplan-Meier log-rank test. Comparison of two groups was performed using an unpaired student's T-test while comparison of three or more groups was performed using one-way ANOVA followed by a Tukey post-test for multiple comparisons. BLI imaging data was analyzed using a non-parametric test. A p-value of less than 0.05 was considered statistically significant.

2-4-0 Results

Early and late administration of VIPhyb significantly enhanced survival in two models of acute leukemia

In order to evaluate VIPhyb as an immunotherapy for leukemia, we injected leukemia cells into syngeneic recipients and administered seven doses of VIPhyb

early or late during the course of leukemia development and monitored mice for survival. Figure 1A shows the treatment and inoculation timelines. B10.BR mice bearing LBRM T cell lymphoblastic leukemia showed a significant enhancement of survival when treated early with VIPhyb (Figure 1B). Similarly, VIPhyb-treated C57Bl/6 mice bearing C1498 AML also showed significantly increased survival when VIPhyb was administered daily from the day preceding leukemic challenge to 5 days afterward (Figure 1C). In order to determine whether the effects of VIPhyb were due directly to antagonism of VIP signaling, we inoculated VIP wild type and VIP knockout mice with C1498 and monitored survival. As shown in Figure 1D, mice that lacked VIP survived significantly longer than mice with functional VIP. The overall survival of these mice was lower than mice treated with VIPhyb, however. Next, we sought to determine whether VIPhyb was efficacious when administered late during tumor development. To test this, we administered 7 daily doses of VIPhyb or PBS in C1498-bearing mice from days 8-14 post-tumor inoculation. As shown in Figure 1E, there was a significant enhancement of survival following late VIPhyb administration.

Early administration of VIPhyb lowered tumor burden in a lymphocyte-dependent manner

Based on the improvement in survival obtained with early VIPhyb administration, we examined the tumor burden in treated mice vs PBS-treated controls. We used bioluminescent imaging to quantify the overall tumor burden. Mice treated with an

early course of VIPhyb had significantly lower tumor burden 26 days after inoculation with leukemia compared to PBS-treated controls (Figures 2A and 2B). To determine whether the reduced tumor burden was the result of immunological action, we repeated the experiment with *Rag 1* knockout mice, which lack functional T and B cells. VIPhyb treatment was not effective at protecting *Rag 1* knockout mice from C1498 tumor-associated death (Figure 2C).

VIPhyb reduces CREB phosphorylation in CD4 and CD8 T cells following VIP stimulation and enhances T cell proliferation in vitro

To determine whether administration of VIPhyb prevents signaling from VIP receptors in response to exogenous VIP, T cells from B6 mice were purified and cultured in low serum medium overnight followed by VIP stimulation in the presence or absence of VIPhyb. Levels of CREB phosphorylation were then measured by phospho-flow cytometry and Western blot. CD4 and CD8 T cells responded differently to high and low concentrations of VIP with CD8 T cells failing to signal from concentrations as low as 100 pM (Figure 3A). Pre-incubation with VIPhyb was able to block CREB phosphorylation in both CD4+ and CD8+ T cells, however, only CD8 T cells had reduced levels of CREB phosphorylation at a higher concentration of VIP indicating increased sensitivity of CD8+ T cells to VIPhyb (Figure 3A). Figure 3B shows reduced CREB phosphorylation in the total T cell compartment following incubation with VIPhyb prior to VIP stimulation. Next, we determined the functional effect of VIPhyb-

mediated VIP signaling blockade on T cells *in vitro*. We cultured CFSE-labeled B6 T cells in 96 well plates pre-coated with anti-CD3 antibody for 48 hours and assessed proliferation by flow cytometry. Addition of VIP to cultures resulted in a modest reduction of proliferation while addition of VIPhyb enhanced proliferation in response to CD3 stimulation (Figure 3C). Although not significant, addition of VIPhyb to cultures containing exogenous VIP partially reversed VIP-mediated suppression of T cell proliferation (Figure 3D).

C1498 cells express the VPAC2 receptor and PD-L1 but do not respond to VIPhyb

Due to the possibility of VIPhyb-mediated direct tumor growth inhibition, we next tested whether the peptide affected C1498 growth and survival. Using a semi-quantitative PCR approach we determined that C1498 cells express mRNA for the VPAC2 receptor (Figure 4A). Quantitative RT-PCR confirmed these findings as the VPAC2 sequence could be successfully amplified while the VPAC1 sequence could not (data not shown). In order to determine whether the gene expression results directly translated to protein expression we performed Western blots using polyclonal antibodies to VPAC1 and VPAC2. As shown in Figure 4B, no bands were detected when membranes were probed for VPAC1, but probing for VPAC2 yielded a single band. These results demonstrate that C1498 cells express only the VPAC2 receptor.

Since blockade of this receptor with VIPhyb could potentially lead to inhibition of tumor cell growth and survival, we next examined the growth and survival properties of C1498 cells in culture in the presence or absence of VIP, VIPhyb, or the chemotherapeutic daunorubicin. The growth properties of C1498 cells did not differ significantly among VIP, VIPhyb, combination, and negative control cultures as measured by BLI (Figure 4C). Further, cultures containing either VIP or VIPhyb did not show significant differences in survival *in vitro* compared to control cultures (Figures 4D and 4E). As expected, cultures containing daunorubicin showed significant cell death beginning at 24 hours (Figures 4D and 4E).

C1498 cells are a well-established PD-L1-expressing cell line [77]. Upregulation of PD-L1 on the surface of tumor cells in response to pro-inflammatory cytokines is a major mechanism of tumor immune evasion. As such, we wished to determine whether culture with VIPhyb would lead to reduced expression of PD-L1 following stimulation with IFN-γ. As shown in Figure 4D, upregulation of PD-L1 in response to IFN- γ was not prevented at any of the VIPhyb concentrations tested indicating that the potential to signal through the PD-1 pathway was not inhibited in the tumor cells themselves.

Adoptive transfer of T cells from VIPhyb-treated tumor survivors protect Rag 1 knockout mice from tumor-associated death

In order to determine whether the enhanced survival following VIPhyb treatment was T cell-mediated, we purified splenic T cells from either leukemia-naïve B6 mice or VIPhyb-treated C1498 survivors and adoptively transferred them to *Rag 1* knockout recipients. Seven days post-transfer, *Rag 1* knockout mice were injected with C1498 and subsequently monitored for survival and T cell phenotype. As shown in Figure 5A, the T cell frequency in peripheral blood was not significantly different between groups. However, the frequency of T cell subsets differed significantly with the majority of T cells from VIPhyb-treated donors being CD8+ T cells (Figure 5B). Additionally, the T cells from VIPhyb-treated donors were predominately CD44+CD62L- effectors indicating increased levels of activation (Figure 5C and D). Tumor burden was lower in mice receiving T cells from VIPhyb-treated donors 21 days post-injection (Figure 5E), which translated to a significant survival benefit (Figure 5F).

Administration of VIPhyb reduced PD-1 expression and enhanced pro-inflammatory cytokine secretion in CD4+ and CD8+ T cells

To examine the underlying mechanism whereby T cells enhance the anti-leukemia effect following VIPhyb treatment, peripheral blood was collected from PBS and VIPhyb-treated mice 15 and 30 days post-C1498 inoculation and phenotyped using flow cytometry. Of particular interest was the expression of the inhibitory molecule PD-1. The frequency of PD-1 expressing CD4+ T cells in C1498-bearing mice was significantly lower in mice that received VIPhyb

treatment at day 15 post-tumor cell injection (Figure 6A). This effect was transient, however, as no significant difference was observed 30 days post-tumor injection (Figure 6A). In contrast, the frequency of PD-1-expressing CD8+ T cells from VIPhyb-treated mice trended towards significance on day 15 post-leukemia inoculation but was significantly lower on day 30 (Figure 6A). With an increased frequency of CD44+CD62L- CD8+ effectors in VIPhyb-treated mice (Figure 6B) we examined the expression of PD-1 on CD4+ and CD8+ T cells with different activation statuses 15 days post-tumor inoculation. PD-1 expression on naïve CD4 T cells was not significantly lower following either 1 or 7 doses of VIPhyb in leukemia-bearing mice (Figure 6C). However, expression of PD-1 on naïve CD8+ T cells was significantly lower with either schedule of VIPhyb administration (Figure 6C). Expression of PD-1 on central memory phenotype CD4+ T cells in VIPhyb-treated mice was not significantly lower than PBS control but was significantly lower on CD8+ T cells from mice treated with 7 doses of VIPhyb (Figure 6C). PD-1 expression on CD4+ and CD8+ T effector cells was only significantly lower than PBS control with 7 doses of VIPhyb (Figure 6C).

We next examined the functional capacity of T cells from the spleens of VIPhyb and PBS-treated C1498-bearing mice by intracellular cytokine staining. The frequency of IFN-γ-producing cells in both the CD4 and CD8 compartments was significantly higher in VIPhyb-treated mice 15 days post-tumor injection (Figure 6D). In contrast to IFN-γ production, there was no significant difference in the frequency of TNF-α-producing CD4+ or CD8+ T cells (Figure 6D).

2-5-0 Discussion

A large body of literature exists that highlights the potent anti-inflammatory actions of VIP in a wide variety of cell types. Due to these anti-inflammatory effects, VIP itself has been explored as a potential therapeutic for a variety of autoimmune disorders. [184] As such, it stands to reason that blockade of endogenous VIP signaling would result in stronger inflammatory responses. Previous work performed by our lab has explored the VIP pathway as a therapeutic target using the VIPhyb antagonist to strengthen T cell responses and promote dendritic cell maturation in settings of antiviral immune responses and allogeneic transplant. [128-130] Based on the observed effects of VIPhyb on antiviral CD8+ T cell responses in particular, we sought to determine whether VIPhyb would have a therapeutic effect buy inducing autologous T cells with anti-leukemia activity. Adaptive immune responses to transformed myeloid and lymphoid cells rely largely on the CD8+ compartment, and thus we hypothesized that VIPhyb could help overcome the immunosuppressive environment created by leukemia cells. Previous work has demonstrated the therapeutic benefit of VIPhyb on the graft-versus-leukemia effect in murine models of allo-transplantation. [130] Administration of VIPhyb in this setting modulated the expression of immune checkpoint molecules on donor T cells which enhanced their anti-leukemia effects. [130] The effects of VIP signaling blockade on host T

cells was not addressed by this study, and as such we examined the effects of VIPhyb on the autologous anti-leukemia response in the absence of transplant.

Acute myeloid leukemia blasts have been shown to create an immunosuppressive environment through a wide variety of mechanisms including upregulation of IDO and secretion of factors that inhibit T cell proliferation. [185] The active subversion of T cell responses by AML blasts renders these immunosuppressive activities a prime target for immunotherapeutics. The data presented here provide evidence that the use of a peptide antagonist to the vasoactive intestinal peptide signaling pathway enhances survival in murine models of both T cell lymphoma and acute myeloid leukemia. The survival benefits were T cell-mediated as transfer of T cells from VIPhyb-treated mice to Rag1 knockout recipients were protected from C1498 challenge. The greatest effects of VIPhyb were seen in CD8 T cells, which failed to respond to lower concentrations of VIP and showed a greater reduction in VIP signaling in response to VIPhyb when exposed to higher VIP concentrations. This is most likely due to lower expression of the VPAC1 receptor on CD8+ T cells in comparison to CD4+ T cells. [109] Lower expression of the receptors would result in quicker saturation following the introduction of a peptide antagonist. This enhanced blockade accounts for the greater frequencies of CD8+ T cells in treated mice as well as the increased frequencies of effector cells. Due to the fact that CD8+ T cells are the primary effectors in anti-tumor immune responses this helps to explain the reduced tumor burden in treated mice. [186] Further supporting

a T cell-mediated mechanism-of-action for VIPhyb is the lack of an effect on either growth or survival of the tumor cells *in vitro* despite expression of VPAC2. It has been established that many human and murine tumor cell lines overexpress VPAC1 or VPAC2, and the growth of some cell lines is supported by VIP and can be directly inhibited by VIP antagonists.[114, 138, 187, 188] We did not observe similar results which supports an off-target mechanism.

The enhanced *in vitro* proliferation and enhanced cytokine secretion in tumor-bearing mice can be explained in part by the VIPhyb-mediated reduction in CREB signaling. Following phosphorylation of CREB by protein kinase A, CREB binds its co-activator CBP. [189] CBP binds to the same site on phosphorylated CREB as the RelA subunit of NF-κB, and thus phosphorylation of CREB sequesters one half of the NF-κB dimer that is required for its transcriptional activity. [189, 190] Downstream of NF-κB are a variety of pro-inflammatory genes including IL-2 and IFN-γ, which are necessary for T cell proliferation and functional activity. [191] This helps to explain the enhanced proliferation of T cells as well as the increased IFN-γ secretion as VIPhyb-mediated reduction of CREB phosphorylation would enhance NF-κB activity. Stimulation through the TCR leads to secretion of VIP by T cells *in vitro,* and as such, stimulation with anti-CD3 would increase endogenous levels of VIP.[118] VIPhyb was able to significantly reduce endogenous VIP-mediated T cell suppression, but was not able to significantly prevent suppression from both endogenous and exogenous VIP. Since the source of VIP in tumor-bearing mice would be endogenous VIP it

can be reasoned that VIPhyb would significantly enhance T cell proliferation *in vivo.* Taken together, VIPhyb treatment in tumor-bearing mice blocked CREB signaling from endogenous VIP and lead to enhanced proliferation and cytokine secretion in T cells. These effects were strongest in the CD8 compartment contributing to reduced tumor burden and enhanced survival.

Among the observed effects of VIPhyb on T cells, the reduced expression of PD-1 on CD4 and CD8 subsets would have been a large contributor to the reduced tumor burden in treated mice. Several studies have demonstrated the importance of the PD-1-PD-L1 signaling axis in AML progression and anti-AML T cell responses. [192-194] It has been established that AML blasts upregulate PD-L1 in response to pro-inflammatory cytokines. [194] Additionally, the PD-1-PD-L1 signaling pathway has been shown to be a major contributor to immune evasion by C1498 *in vivo.* [77] Reduction of PD-1 signaling through the use of monoclonal antibodies has demonstrated to have profound effects on exhausted T cells in models of chronic infection and cancer and have proven highly efficacious in many clinical trials. [176, 195, 196] The functional effects of PD-1 signaling blockade include enhanced pro-inflammatory cytokine secretion, increased proliferation, and enhanced production of cytotoxic molecules. [195] The reduced expression of PD-1 on T cells following VIPhyb treatment along with increased T cell proliferation and secretion of IFN- γ highlight the relevance of the PD-1 pathway in the survival effects and reduced tumor burden observed in treated mice. The lack of an effect of VIPhyb on the expression of PD-L1 on the tumor cells

following stimulation with IFN- γ points towards the interference of this pathway being specifically on the T cell axis.

We show here that blockade of VIP receptors on T cells using a peptide antagonist functions in a similar manner to currently used checkpoint blockade methods with significant enhancement of T cell function, reduction of tumor burden, and enhanced survival in two models of acute leukemia. Interestingly, late administration of VIPhyb led to an equivalent net increase in the fraction of surviving mice (compared with saline injection) as injections of VIPhyb starting 1 day prior to inoculation with leukemia, supporting the potency of VIP receptor blockade as an anti-cancer immunotherapeutic and the clinical relevance of this approach. Whether simultaneous blockade of co-inhibitory receptors and VIP receptors would result in additive or synergistic effects is a topic of future investigation. Taken together, the results presented here highlight the potential for VIP signaling blockade as an immunotherapeutic strategy in the treatment of hematological malignancies.

2-6-0 Figure Legends

Figure 2-7-1. Early and late administration of VIPhyb improves survival in AML and T cell leukemia-bearing mice. Albino B6 mice were administered 10 μg VIPhyb subcutaneously for 7 consecutive days beginning either the day

before or one week after receiving 10^6 C1498 or 2 x 10^6 LBRM cells intravenously. **(A)** Timeline of tumor injection and VIPhyb treatment. **(B)** Survival of LBRM-bearing mice receiving either PBS or VIPhyb. (n =10 PBS, n =10 VIPhyb) **(C)** Survival of C1498-bearing mice receiving either PBS or VIPhyb. (n = 16 PBS, n = 14 VIPhyb) **(D)** Survival of WT and VIP KO mice following C1498 inoculation. (n = 10 WT, n = 10 VIP KO) **(E)** Survival data from two experiments of C1498-bearing mice receiving late treatment with PBS or VIPhyb. (n = 20 PBS, n = 20 VIPhyb) *p<0.05 and ***p<0.001 indicate significant differences between the control and treated groups.

Figure 2-7-2. VIPhyb treatment led to reduced tumor burden in mice, which required the presence of lymphocytes. C1498-bearing mice were injected i.p with luciferin, anesthetized, and imaged in an IVIS spectrum imager. Rag1 knockout mice and wild type albino B6 mice were injected with 10^6 C1498 cells i.v and treated with an early course of VIPhyb or PBS. **(A)** Representative BLI image of late stage C1498-bearing albino B6 mice treated with an early course of either PBS or VIPhyb. The scale indicates the intensity of the signal emitted from C1498 cells. **(B)** Quantification of tumor burden reported as average flux emitted from each mouse's entire body. **(C)** Survival of C1498-bearing, VIPhyb-treated Rag1 knockout mice compared to wild type C1498-bearing B6 albino mice treated with either PBS or VIPhyb. (n = 9 PBS, n = 10 VIPhyb, n = 4 Rag1 KO PBS, n=8 Rag1 KO VIPhyb) The red box on the x-axis indicates the treatment

period. *p<0.05, **p<0.01, ***p<0.001 indicate significant differences between or among groups of mice.

Figure 2-7-3. VIPhyb reduced CREB phosphorylation in T cells following VIP stimulation and enhanced proliferation *in vitro*. Splenic T cells were purified by negative selection and cultured in complete media containing 0.5% fetal bovine serum overnight to reduce basal CREB phosphorylation levels. Cells were then incubated in the presence of 10 μM VIPhyb prior to stimulation with 1 nM or 100 pM VIP. Levels of CREB phosphorylation were then assessed by Phosflow and Western blot. The effect of VIPhyb on the *in vitro* proliferation of T cells was assessed by culturing CFSE-labeled splenic T cells in a 96 well plate pre-coated with anti-CD3 antibody. CFSE dilution was measured 48 hours later by flow cytometric analysis of live CD3+ cells. **(A)** Representative histograms indicating levels of CREB phosphorylation in VIP-stimulated CD4 and CD8 T cells. The solid gray histogram represents the isotype, the black histogram represents unstimulated samples, the red histogram represents samples stimulated with VIP, and the blue histogram represents samples pre-incubated with VIPhyb prior to VIP stimulation. **(B)** Western blot showing CREB phosphorylation in splenic T cells treated with 1 nM VIP or 10 μM VIPhyb + 1 nM VIP. **(C)** Histogram indicating the level of CFSE dilution in control wells and wells containing 1 nM VIP with or without 10 μM VIPhyb. The solid gray histogram represents T cells cultured in wells without anti-CD3, the black histogram represents control wells not containing peptide, the red histogram represents

wells containing VIP, the blue histogram represents wells containing VIPhyb alone, and the green histogram represents wells containing VIP and VIPhyb. **(D)** Quantification of CFSE dilution normalized to control wells for three independent experiments. *p<0.05, **p<0.01 indicate significant differences among culture conditions.

Figure 2-7-4. C1498 cells express VPAC2 but are not directly affected by VIPhyb treatment. RNA and protein from cultured C1498 cells were extracted, and expression of VPAC1 and VPAC2 was determined by RT-PCR and Western blot. C1498 cells were cultured in the presence or absence of VIPhyb or daunorubicin as positive control. Survival of cells was measured by annexin V-sytox blue staining and flow cytometry while growth of the cells was measured by BLI. Expression of PD-L1 was determined by flow cytometry following 48-hour culture in the presence of 10 ng/mL IFN-γ and various concentrations of VIPhyb. **(A)** Image of PCR products run on a 1% agarose gel with 18s rRNA as control. **(B)** Image of Western blot probed for VPAC1 and VPAC2. **(C)** Growth curves of C1498 cells under various culture conditions as measured by BLI. **(D)** Flow cytometry plots of C1498 cells cultured with 1 μM VIPhyb, 1 μM daunorubicin, 1 nM VIP, or no drug as control. Cells in the lower-right quadrant are in early apoptosis while cells in the upper right quadrant are dead. **(E)** Expression of PD-L1 on live C1498 cells cultured for 48 hours in the presence of IFN- γ and VIPhyb.

Figure 2-7-5. Adoptive transfer of T cells from VIPhyb-treated tumor survivors conferred protection to Rag1 knockout mice. Splenic T cells from VIPhyb-treated mice that survived C1498 were purified by negative selection. 5 x 10^6 T cells were adoptively transferred to Rag 1 knockout recipients followed by injection of 10^6 C1498 cells one week later. Peripheral blood samples were analyzed 14 days post-tumor inoculation for the frequencies of T cell subsets. Tumor burden was assessed 21 days post-tumor inoculation by BLI. **(A)** T cell frequencies in peripheral blood of tumor-bearing Rag 1 KO mice receiving naïve T cells or T cells from VIPhyb-treated mice. **(B)** Frequencies of CD4 and CD8 T cells in tumor-bearing Rag 1 KO mice receiving naïve T cells or T cells from VIPhyb-treated mice. **(C)** Flow cytometry plots showing differential expression of the markers CD44 and CD62L on peripheral blood T cells from tumor-bearing Rag 1 KO mice receiving naïve T cells or T cells from VIPhyb-treated mice. **(D)** Quantification of T cell subsets as defined by the expression of CD44 and CD62L. **(E)** Comparison of tumor burden as assessed by BLI between mice receiving naïve T cells and mice receiving T cells from VIPhyb-treated donors. **(F)** Survival of mice receiving naïve T cells vs mice receiving T cells from VIPhyb-treated donors following a second challenge of 10^6 tumor cells. (n = 10 naïve T cells, n = 6 VIPhyb-treated T cells) *p<0.05, **p<0.01, ***p<0.001 indicate significant differences between groups.

Figure 2-7-6. VIPhyb treatment reduced PD-1 expression in CD4 and CD8 T cells and enhanced the production of pro-inflammatory cytokines.

Peripheral blood and spleen samples were collected on days 15 and 30 post-tumor cell injection and analyzed by flow cytometry. **(A)** PD-1 expression on total peripheral blood CD4 and CD8 T cells at the indicated time points. **(B)** Frequency of CD44+CD62L- effector CD4 and CD8 T cells in peripheral blood 15 days post-tumor cell inoculation. **(C)** Expression of PD-1 on subsets of peripheral blood CD4 and CD8 T cells 15 days post-tumor cell injection as defined by the expression of CD44 and CD62L. Graphs compare the expression of PD-1 in mice treated with PBS, 1 single dose of VIPhyb, or seven consecutive daily doses of VIPhyb. **(D)** Expression of IFN-γ and TNF-α in splenic T cells from C1498-bearing mice treated with PBS or 7 doses of VIPhyb. *$p<0.05$, *$p<0.01$, and ***$p<0.001$ indicate significant differences between groups.

2-7-0 Figures

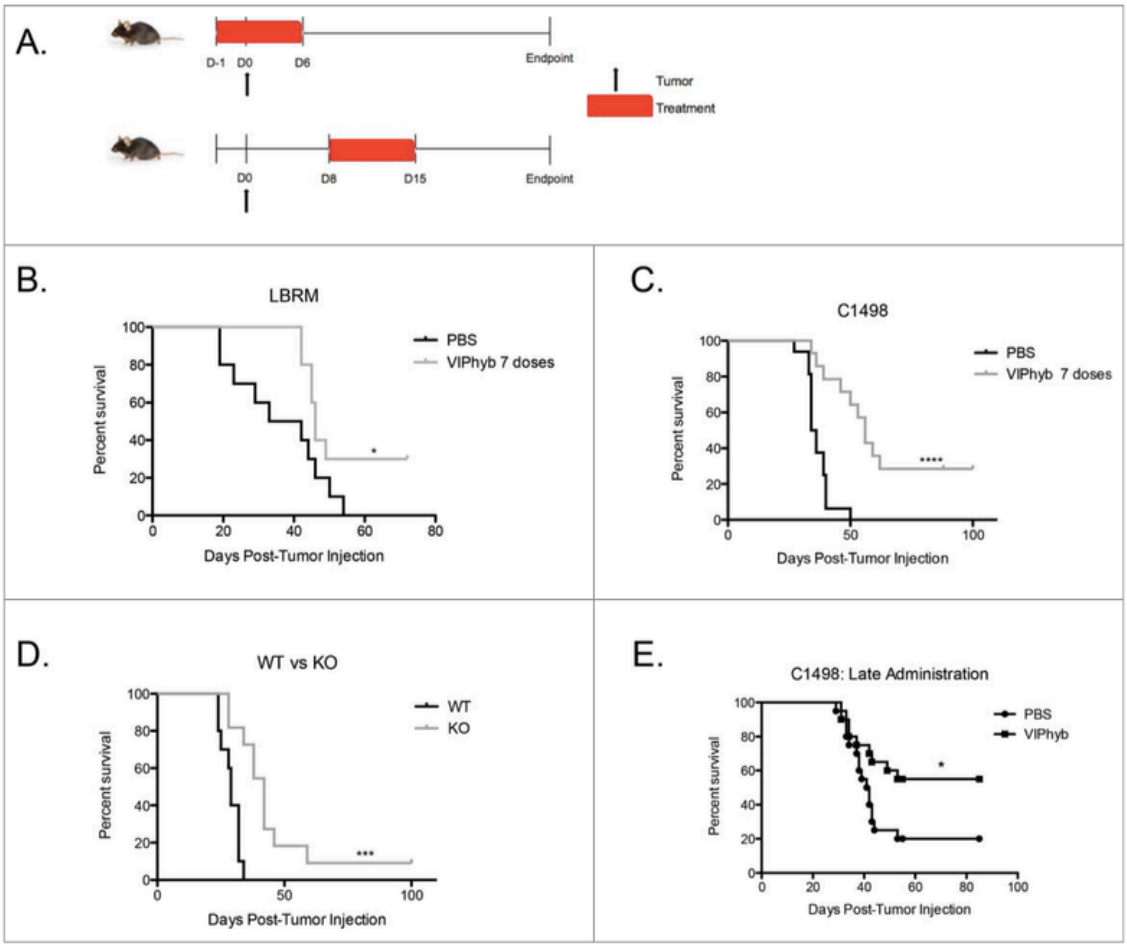


Figure 2-7-1

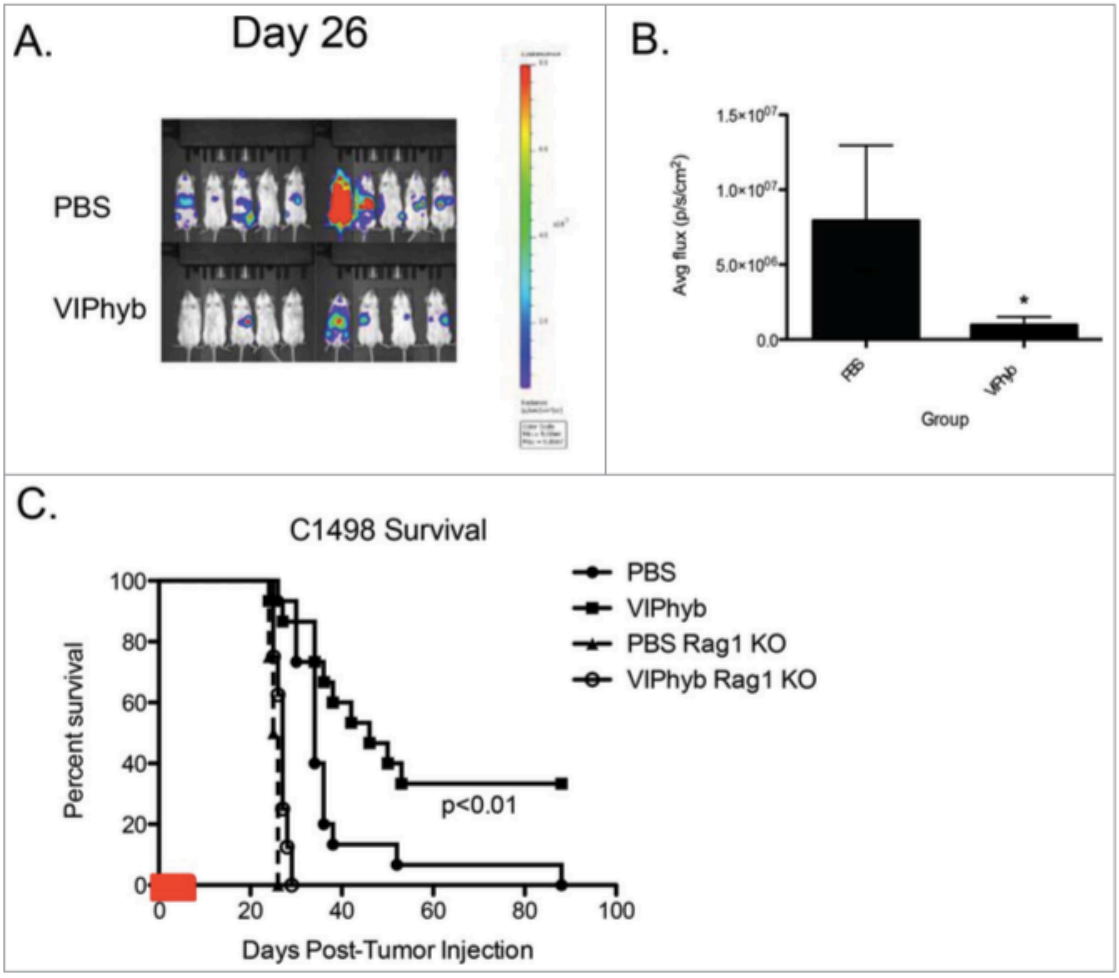

A.
Day 26
PBS
VIPhyb
B.
Avg flux (p/s/cm²)
1.5×10^07
1.0×10^07
5.0×10^06
0.0
*
PBS
VIPhyb
Group
C.
C1498 Survival
Percent survival
100
80
60
40
20
0
0
20
40
60
80
100
Days Post-Tumor Injection
p<0.01
PBS
VIPhyb
PBS Rag1 KO
VIPhyb Rag1 KO

Figure 2-7-2

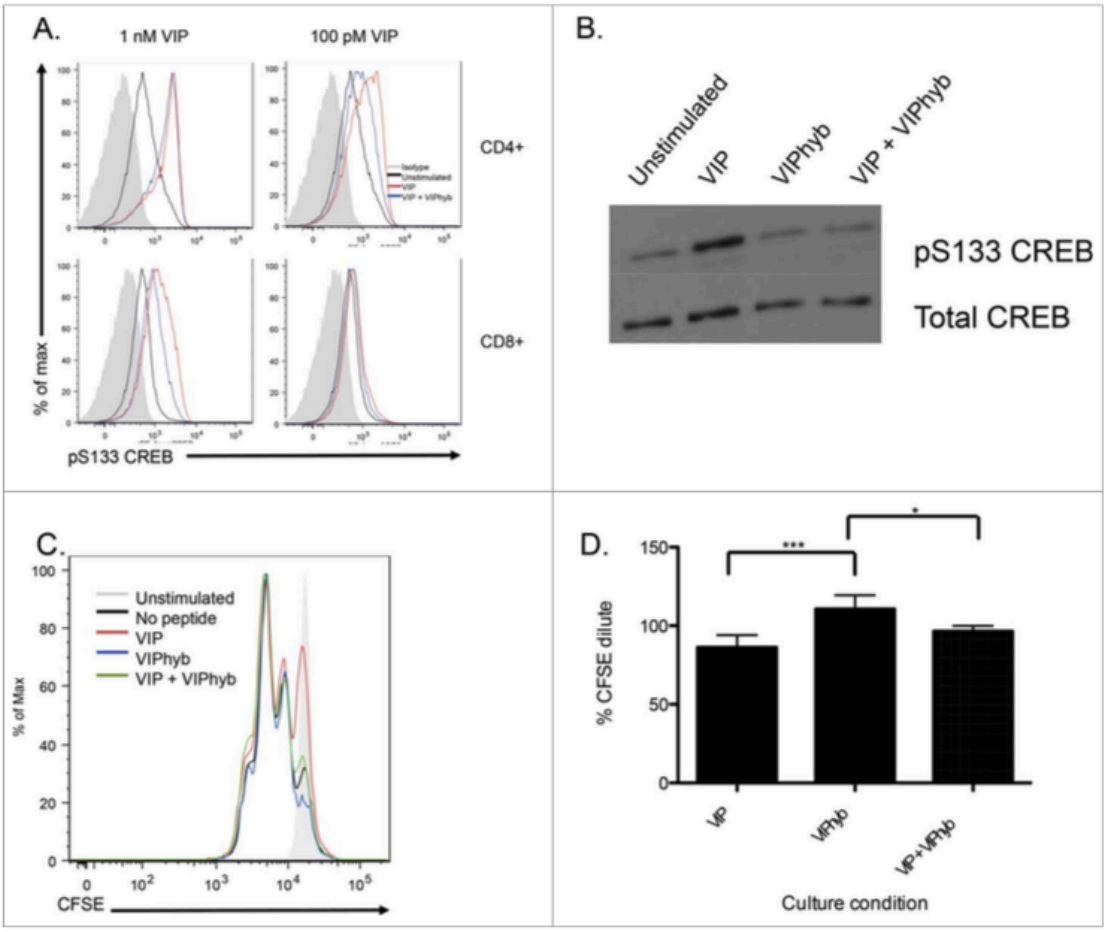

A.
1 nM VIP
100 pM VIP
CD4+
CD8+
% of max
pS133 CREB
B.
Unstimulated
VIP
VIPhyb
VIP + VIPhyb
pS133 CREB
Total CREB
C.
Unstimulated
No peptide
VIP
VIPhyb
VIP + VIPhyb
% of Max
CFSE
D.
% CFSE dilute
***
*
VIP
VIPhyb
VIP+VIPhyb
Culture condition

Figure 2-7-3

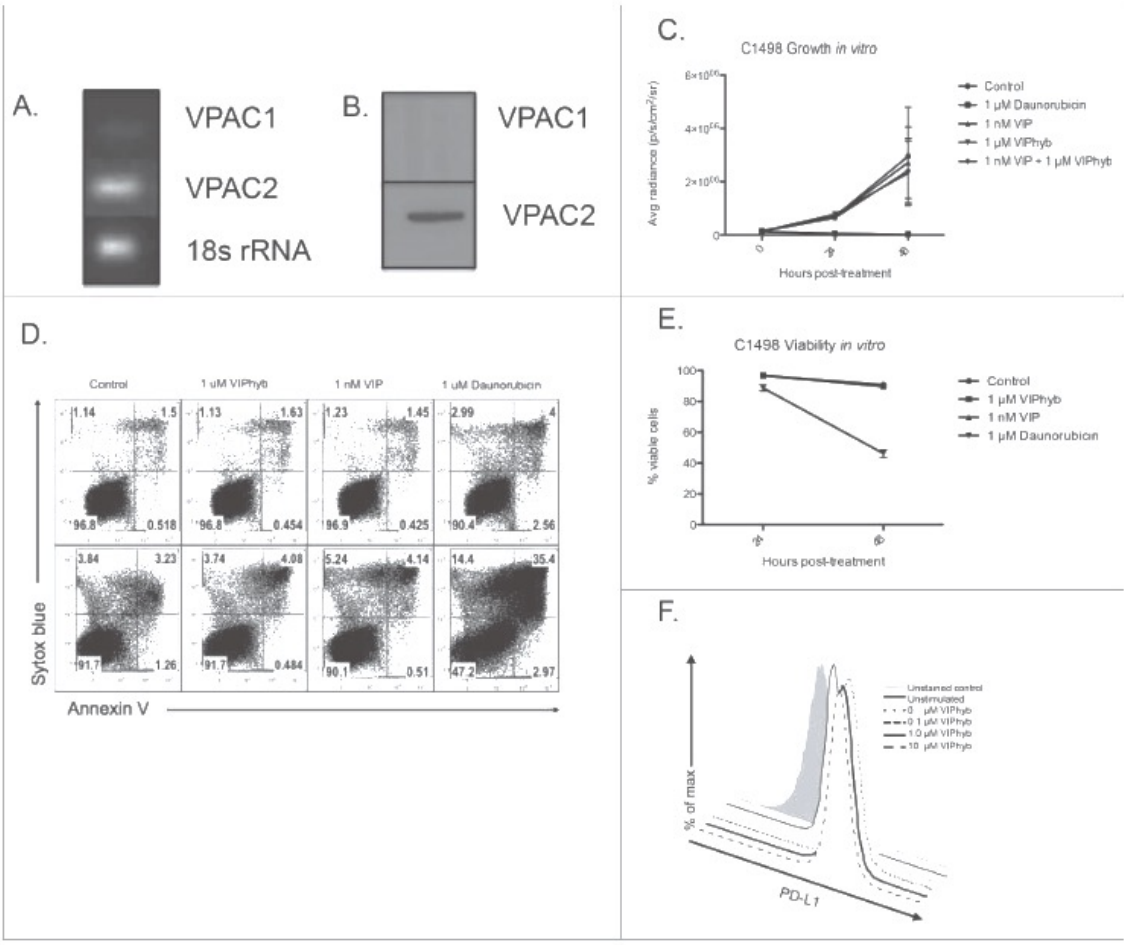

A.
VPAC1
VPAC2
18s rRNA
B.
VPAC1
VPAC2
C.
C1498 Growth in vitro
Avg radiance (p/s/cm²/sr)
Hours post-treatment
Control
1 μM Daunorubicin
1 nM VIP
1 μM VIPhyb
1 nM VIP + 1 μM VIPhyb
D.
Control
1 uM VIPhyb
1 nM VIP
1 uM Daunorubicin
Sytox blue
Annexin V
E.
C1498 Viability in vitro
% viable cells
Hours post-treatment
Control
1 μM VIPhyb
1 nM VIP
1 μM Daunorubicin
F.
% of max
PD-L1

Figure 2-7-4

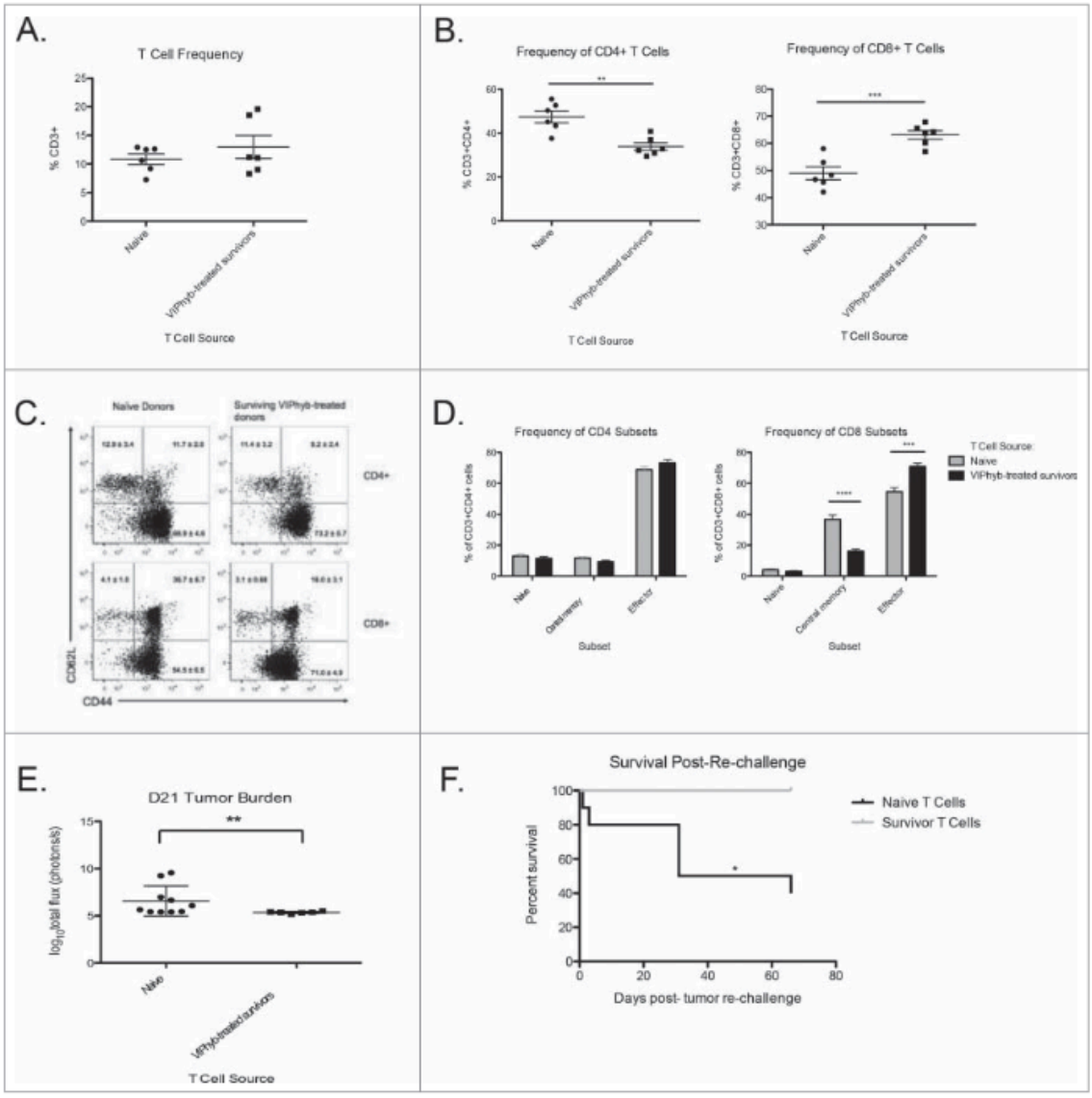

A.
T Cell Frequency
% CD3+
Naive
ViPhyb-treated survivors
T Cell Source
B.
Frequency of CD4+ T Cells
% CD3+CD4+
Frequency of CD8+ T Cells
% CD3+CD8+
C.
Naive Donors
Surviving ViPhyb-treated donors
CD4+
CD8+
CD62L
CD44
D.
Frequency of CD4 Subsets
% of CD3+CD4+ cells
Frequency of CD8 Subsets
% of CD3+CD8+ cells
T Cell Source:
Naive
ViPhyb-treated survivors
Subset
Naive
Central memory
Effector
E.
D21 Tumor Burden
log10total flux (photons/s)
F.
Survival Post-Re-challenge
Percent survival
Naive T Cells
Survivor T Cells
Days post- tumor re-challenge

Figure 2-7-5

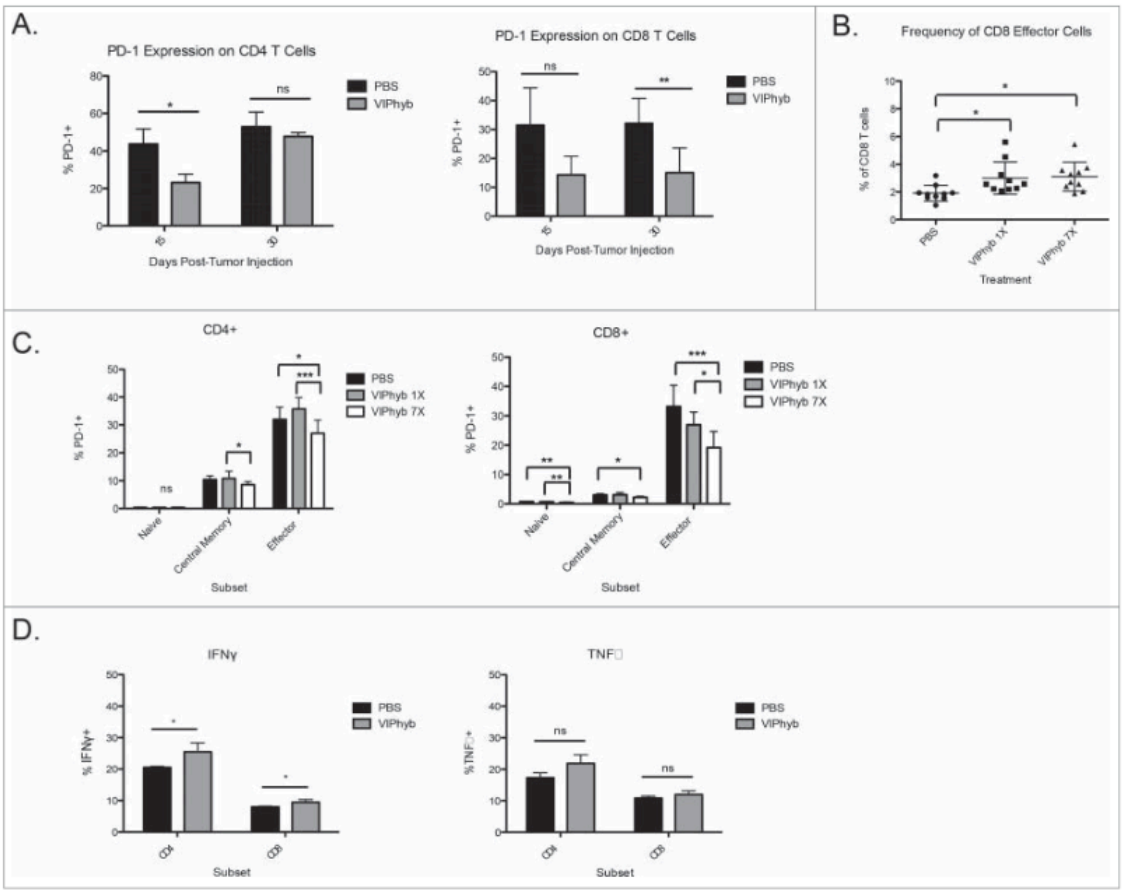

A.
PD-1 Expression on CD4 T Cells
% PD-1+
Days Post-Tumor Injection
PBS
VIPhyb
PD-1 Expression on CD8 T Cells
B.
Frequency of CD8 Effector Cells
% of CD8 T cells
PBS
VIPhyb 1X
VIPhyb 7X
Treatment
C.
CD4+
CD8+
VIPhyb 1X
VIPhyb 7X
Naive
Central Memory
Effector
Subset
D.
IFNγ
TNF
% IFNγ+
% TNF+
CD4
CD8

Figure 2-7-6

Chapter 3:

Improving *ex vivo* T Cell Expansion from DLBCL Patients for T Cell Therapies via Antagonism of PI3K δ and VIP

3-1-0 Abstract

Chimeric antigen receptor (CAR) T cell technology offers great promise for improving the treatment of patients with hematological malignancies including aggressive B cell lymphomas. The success of this therapy is currently restricted by the variable success of expanding patients' T cells *ex vivo* and limited persistence of cytotoxic T cells in CAR T cell-treated patients. These problems are particularly relevant to lymphoma patients previously treated with numerous cycles of lymphocyte-damaging chemotherapies, and motivate the development of novel strategies to enhance *ex vivo* T cell expansion and their persistence *in vivo*. We demonstrate that blockade of PI3K δ and antagonism of vasoactive intestinal peptide (VIP) signaling partially inhibits terminal differentiation (mean frequency 54.4% CD27+CD28+ T cells vs 27.4% in control cultures, $p<0.05$) during anti-CD3/CD28 bead-mediated expansion. This culture strategy increased yields of T cells cultured from lymphoma patients with a median of 83.7% more T cells when expanded in the presence of PI3K δ and VIP antagonists, increased survival of human T cell from a lymphoma patients in a murine xenograft model, and enhanced cytotoxic anti-lymphoma activity of antigen-specific murine T cells in a murine lymphoma model. Associated with improved biologic properties of PI3K δ and VIP antagonist-expanded T cells from lymphoma patients are increased expression of the anti-apoptotic Bcl-2 protein, reduced terminal differentiation of T cells, and preservation of co-stimulatory molecule expression. Taken together, synergistic blockade of these pathways is an attractive strategy to enhance the expansion and functional capacity of T cells during CAR T manufacture.

3-2-0 Introduction

The early success of chimeric antigen receptor (CAR) T cell therapy has been greatest in the treatment of B cell leukemias, most notably pediatric B-cell lymphoblastic leukemia (ALL) treated with anti-CD19 CAR T.[197] Diffuse large B cell lymphoma (DLBCL) is a CD19 positive non-Hodgkin B cell lymphoma for which the use of anti-CD19 CAR T cell therapy is currently being evaluated.[198, 199] The efficacy of anti-CD19 CAR T in the treatment of adult B cell lymphoma patients has been less than what has been observed in pediatric ALL patients, due in part to T cell quality. Adult lymphoma patients have been exposed to multiple rounds of cytotoxic therapies prior to the attempted manufacture of CAR T cells.[199] Importantly, one of the major off-target effects of these therapies is damage to the patient's healthy T cells.[200] The end result of cell-intrinsic deficits in T cell function in heavily pre-treated patients has been increased incidence of inadequate *ex vivo* T cell expansion leading to manufacturing failure, and lack of adequate expansion of re-infused CAR T cells *in vivo*.[201] Many lymphoma patients are thus unable to obtain durable remissions with CAR T cell treatment. As such, it is imperative to devise methods that improve the overall quality and yield of T cells expanded from lymphoma patient apheresis products. Since the net expansion of unfractionated T cells expanded in culture with anti-CD3/CD28 beads for 10-14 days is much less than what would be predicted based upon the cell cycle length of optimally activated T cells expanding in vivo to antigen, we hypothesized that adding agents that decrease activation-induced terminal

differentiation and cell death could have a favorable effect on net T cell expansion.[202-204]

To test this hypothesis, we studied blood samples from DLBCL patients prior to treatment and samples from DLBCL patients who had received multiple treatment lines. Of note, lymphoma patients who had received prior treatment had a significantly higher proportion of CD27-CD28- T cells when compared to either healthy controls or newly-diagnosed DLBCL patients. The over-abundance of these cells was associated with failure of *in vitro* T cell expansion as loss of CD28 results in inadequate survival and expansion in cultures with anti-CD3/CD28 beads.[56, 57, 205] In order to improve the quality and overall yield of T cells obtained from pre-treated DLBCL patients, we explored the use of a PI3K δ inhibitor (idelalisib) and a peptide antagonist to vasoactive intestinal peptide (VIPhyb) added during the expansion period. Our rationale for using these agents was previous data showing enhanced anti-cancer immunity VIPhyb-treated mice and reports of autoimmunity after stopping drug in patients treated with idelalisib.[115, 130, 173, 174, 206] Low concentrations of idelalisib resulted in significantly increased yield as a well as a preservation of less terminally-differentiated cells. Further, the frequency of CD27+CD28+ T cells was significantly increased at the end of the expansion period. The phenotypic effects observed in samples expanded with idelalisib were enhanced by addition of VIPhyb suggesting an additive or synergistic relationship between these two signaling pathways. Additionally, the T cells expanded in both idelalisib and

VIPhyb had the greatest *in vivo* persistence when adoptively transferred to NSG mice. Interestingly, the *in vivo* anti-tumor activity of T cells expanded in idelalisib, VIPhyb, or a combination was significantly greater than that of T cells from control cultures. These results suggest that antagonism of the PI3K δ and VIP signaling pathways may be a promising approach to limit T cell exhaustion during CAR T manufacturing. By improving the quality and yield of T cells generated during manufacture, the outcomes of CAR T therapy for lymphoma patients could be significantly improved.

3-3-0 Materials and Methods

Peripheral Blood Mononuclear Cell Samples

Peripheral blood mononuclear cells were obtained from consenting DLBCL patients and healthy controls via apheresis or phlebotomy and ficoll-hypaque separation of peripheral blood. All samples were processed immediately after collection and phenotyped by flow cytometry. An additional aliquot of mononuclear cells was frozen in Cryostor CS10 (StemCell Technologies, Vancouver, CA) and stored in liquid nitrogen until use. The study was approved by Emory University's Institutional Review Board (Approval IDs IRB80716 and IRB60350).

Mice

Six to eight week old male luciferase-expressing C57Bl/6 and male NSG (NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ) mice were bred at the Emory University Animal Care Facility (Atlanta, GA). Male C57/Bl6 SJL (PepBoy) and TCR transgenic OT-I and OT-II strains were purchased from the Jackson Laboratory. 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.*

Compounds

Idelalisib (CAL-101) was purchased from BocSci (Shirley, NY) and stored as a 10 mM stock solution in DMSO at -20 degrees Celsius. Duvelisib was purchased from SelleckChem (Houston, TX) and was provided as a 10 mM stock solution in DMSO. Dilutions of idelalisib and duvelisib were performed in DMSO prior to addition to cell cultures. Mast cell chymase was purchased from Sigma Aldrich (St. Louis, MO) and diluted in sterile PBS. VIPhyb (KPRRPYTDNYRELRKQMAVKKYLNSILN) was purchased from New England Peptide (Gardner, MA) and reconstituted in sterile molecular grade water. VIPhyb dilutions were made in sterile PBS prior to addition to cell cultures.

Human T Cell Cultures

Frozen PBMC were thawed and rested overnight at 37 degrees in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, and 50 µM 2-mercaptoethanol (complete media). Cells were then cultured in 96 well flat-bottom plates in complete media containing 30 U/mL IL-2 (Peprotech, Rocky Hill, NJ) and anti-CD3/CD28 beads (Life Technologies, Carlsbad, CA) at a 1:1 bead to cell ratio. Idelalisib or duvelisib were added at culture initiation, and VIPhyb was added daily. The final DMSO concentration was 0.1% in all wells. Cells were counted and subcultured on day 7 with the addition of fresh beads, IL-2, and compounds. The cells were then allowed to expand for an additional 3 or 7 days. Cultures were split during the expansion period as needed. Cells were counted and phenotyped on day 7 and on day 10 or 14. Cell numbers and fold expansion are reported as the final yield back-calculated to a single well accounting for dilution and subculture.

Mouse T Cell Expansion and Bioluminescent Imaging

Splenic T cells from luciferase+, C57/Bl6, OT-I, and OT-II mice were purified using the EasySep T Cell Isolation Kit (StemCell Technologies, Vancouver, BC). Cells were cultured in 96 well flat-bottom plates in complete RPMI 1640 containing 30 U/mL IL-2 (R&D Systems, Minneapolis, MN). T cells were stimulated with anti-CD3/CD28 beads (Life Technologies, Carlsbad, CA) at a 1:1 bead to cell ratio for 3 days then analyzed using bioluminescent imaging. D-luciferin (Perkin Elmer, Waltham, MA) was added to each well at 150 µg/mL and

luminescence in each well was measured using an IVIS spectrum imager (Perkin Elmer, Waltham, MA). Quantification of relative cell numbers was performed by generating a region of interest over each well and measuring the luminescence. Measurements were given as photons/s/cm^2 and are reported as normalized to stimulated DMSO control. For experiments examining proliferation, T cells were labeled with 1 μM CFSE (Life Technologies, Carlsbad, CA) prior to stimulation.

Phenotypic Analysis

Analysis of samples from patients, healthy volunteers, and mice prior to and during expansion was performed by flow cytometry. Anti-human CD3 PE-CF594, CD4 APC-Cy7, CD8 FITC, and CD27 PE were purchased from BD Biosciences (San Jose, CA). Anti-human CD28 Alexa-Fluor 700 was purchased from BioLegend (San Diego, CA). Anti-mouse CD3 PE-Cy7, CD8 PerCP, CD44 APC, IL-2 PE, and IFN-γ FITC were purchased from BD Biosciences (San Jose, CA). Sytox blue was used for live cell discrimination (Life Technologies, Carlsbad, CA).

T cell Xenografts

T cells from one DLBCL patient apheresis sample (38.7% CD27-CD28-) were expanded over the course of 14 days as described above. On the final day of expansion, the cells were thoroughly washed, counted, and re-suspended in

sterile PBS. 3 x 10^6 cells were injected intravenously into NSG mice via the lateral tail vein. Fourteen days following adoptive transfer, blood was collected and the frequencies, absolute numbers, and phenotypes of persisting human T cells were determined by flow cytometry using CD45 APC and CD3 PE-CF594 (BD Biosciences, San Jose, CA).

Western Blot

Protein lysates were prepared from newly diagnosed DLBCL patient T cells on day 14 of expansion. Lysates (30 µg) were run on a 12% agarose gel and transferred to a nitrocellulose membrane (BioRad, Hercules, CA). Membranes were probed with a 1:1000 dilution of hamster anti-human Bcl-2 (Pharmingen) overnight at 4°C. Expression of β-actin was used as a loading control.

Tumor Challenge and Adoptive Transfer

E.G7 OVA tumor cells were purchased from the American Tissue Type Culture Collection and cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 4 mg/mL G418, 100 U/mL penicillin, 100 µg/mL streptomycin, and 50 µM 2-mercaptoethanol. To establish tumors, 5 x 10^5 E.G7 OVA cells were injected in sterile PBS subcutaneously into the right flank of C57Bl/6 SJL (PepBoy) mice. Seven days later, 3-day-expanded C57Bl/6, OT-I, and OT-II T cells (2 x 10^6 C57Bl/6, 2 x 10^6 OT-I, and 1 x 10^6 OT-II) were injected i.v. with measurable

tumors. Tumor growth was monitored using calipers. Tumor volume was calculated as (length x width^2)/2. Mice were sacrificed once the tumors reached IACUC guidelines.

Statistical Analysis

Statistical analysis was performed using Prism version 5d for Mac (GraphPad, La Jolla, CA). Significant differences between two groups were determined using a two-tailed Student's t test. Statistical significance among 3 or more groups was determined using a one-way ANOVA with a Tukey or Dunnet post-test. Significant survival differences were calculated in a pairwise fashion comparing each group to a control. Each experiment was repeated at least twice, and p values less than 0.05 were considered significant.

3-4-0 Results

T cells from heavily treated DLBCL patients show loss of CD27 and CD28 and fail to expand ex vivo

We studied a cohort of 5 DLBCL patients identified at diagnosis, prior to cytotoxic chemotherapy, and 10 patients who had received prior chemotherapy, including patients relapsed after autologous stem cell transplantation (ASCT) (Table 1). Phenotypic analysis of patients' T cells by flow cytometry showed several

abnormalities from chemotherapy-treated DLBCL patients. Most striking was the high frequency of CD27-CD28- T cells (median 36.8%, range 4.58-77.3), which was not observed in either healthy controls (median 5.8%, range 1.7-12.4) or untreated DLBCL patients (median 3.9%, range 0.5-23.1) (Figure 1A). The frequency of CD27-CD28- T cells correlated significantly to the total number of chemotherapy cycles with a significantly higher frequency in patients previously treated with greater than 11 cycles when compared to healthy controls and newly diagnosed DLBCL patient samples (Figure 1B and 1C). T cells from DLBCL patients' blood with a high frequency of CD27-CD28- T cells failed to expand in cultures with anti-CD3/CD28 beads as previously described.[207] To determine whether the presence of CD27-CD28- (double negative) T cells limited expansion of CD27+CD28+ T cells, we FACS-purified T cells from an extreme case of heavily pre-treated lymphoma into two populations (CD27-CD28- or T cells expressing CD27 and/or CD28) and expanded them alongside unsorted cells for 14 days using anti-CD3/CD28 beads and low dose IL-2 (Figure 2A). Both the unsorted total T cell population and the sorted double negative population failed to expand in culture (Figure 2B). In contrast, removing the double negative population by FACS enabled 20-fold expansion of the remaining single positive CD27/CD28 and double positive CD27+CD28+ T cells with increased viability at the end of the expansion period (Figure 2B and 2C). These results are consistent with the fratricide of naïve T cells by T effector memory cells that has been recently described.[208]

Addition of idelalisib to mouse T cell expansion cultures increases yield and does not inhibit cytokine production

We next examined the effect of idelalisib on mouse T cells expanded with anti-CD3/CD28 beads with low dose IL-2 in order to determine whether the addition of a PI3K δ inhibitor could augment T cell yield and function. We observed significantly higher expression of CD25 and CD44 on both CD4 and CD8 murine T cells expanded in the presence of 100 nM idelalisib (Figure 3A). This phenotype is consistent with delayed downregulation of early activation markers. Based on previous work, we tested the vasoactive intestinal peptide antagonist VIPhyb alone or in combination with idelalisib and examined the effect on polyfunctional cytokine production. IL-2 and IFN-γ secretion in cultures with idelalisib alone was not significantly different from control while addition of VIPhyb alone or in combination with idelalisib resulted in slightly higher cytokine secretion (Figure 3B). We next examined the effect of idelalisib on proliferation rates using CFSE-labeled B6 T cells cultured for 3 days with anti-CD3/CD28 beads and measuring dye dilution. T cells cultured in the presence of idelalisib show reduced, but not statistically significant, proliferation in response to anti-CD3/CD28 stimulation (Figure 3C and 3D). Despite this, bioluminescent imaging of luciferase+ mouse T cells showed that concentrations of 100 nM to 10 μM idelalisib significantly increased net expansion after three days of culture (Figure 3E). These results suggested that idelalisib limited cell death rather than increased proliferation rates.

Idelalisib and significantly improved yield and delayed terminal differentiation during ex vivo T cell expansion

Based on the results obtained with murine T cells, we tested the effects of idelalisib and VIPhyb on the expansion and phenotype of T cells from DLBCL patients and healthy volunteers. T cells were expanded for 10 or 14 days with CD3/CD28 beads and low dose IL-2 in the presence or absence of idelalisib and/or VIPhyb. T cell expansion from healthy volunteers was significantly increased with the addition of idelalisib (Figure 4A). Additionally, the inclusion of idelalisib significantly increased T cell yield in cultures from lymphoma patients (Figure 4B and 4C). Cell yields from cultures containing a combination of idelalisib and VIPhyb did not differ significantly from cultures containing idelalisib alone (Figure 4C), but the addition of Idelalsib or a combination of idelalisib and VIPhyb resulted in significantly increased the frequencies of CD27+CD28+ T cells and reduced the proportion of CD27-CD28- T cells (Figure 4D and 4E). Similar results were obtained with the addition of the dual PI3K δ/γ inhibitor duvelisib (Supplementary Figure 2). Expression of PD-1 on T cells expanded in the presence of idelalisib or a combination of idelalisib and VIPhyb was lower after 10 days of culture while addition of VIPhyb alone resulted in lower expression of PD-1 during short-term expansion (Supplementary Figure 3). In order to determine a possible mechanism underlying the increased cell yields, we examined levels of Bcl-2 by Western blot in T-cells from blood samples of newly

diagnosed lymphoma patients cultured under various conditions. As shown in Figure 4F, levels of Bcl-2 were highest in T cells expanded in the presence of idelalisib or a combination of idelalisib and VIPhyb suggesting that blocking the PI3K and VIP signaling pathways leads to an increase in T cell survival in culture.

DLBCL patient T cells expanded in the presence of idelalisib and VIPhyb had the greatest in vivo persistence in a murine xenograft model

To determine whether the preservation of less terminally-differentiated T cells in cultures expanded in the presence of idelalisib and VIPhyb had an effect on T cell persistence *in vivo*, we expanded T cells from a DLBCL patient and injected them into irradiated NSG mice. Blood was collected 14 days post-injection and analyzed for the presence of human CD45+CD3+ cells by flow cytometry (Figure 5A). Prior to injection, the expression of CD27 and CD28 was highest on T cells expanded in the presence of idelalisib and VIPhyb (Figure 5B). Addition of VIPhyb alone did not have a significant effect on T cell recovery, while the addition of idelalisib significantly increased the frequency of human T cells in mouse peripheral blood (Figure 5C and 5D). Interestingly, human T cells were highest in mice that received T cells expanded in both idelalisib and VIPhyb than in mice injected with T cells expanded in the presence of idelalisib alone (Figure 5C and 5D). Taken together, the addition of both idelalisib and VIPhyb significantly improved the *in vivo* persistence of DLBCL patient T cells expanded over a 14-day culture period.

Addition of either VIPhyb or idelalisib to T cell expansion cultures significantly enhances antigen-specific anti-tumor activity in a murine lymphoma model

Having established an effect on the *in vivo* persistence of T cells expanded in the presence of idelalisib and/or VIPhyb, we next determined the effect of these culture conditions on anti-tumor activity *in vivo*. CD45 congenic B6 SJL mice were subcutaneously injected with OVA-expressing E.G7 lymphoma cells in the right flank. Tumors were allowed to grow for seven days upon which a mixture of *ex* vivo-expanded OT-I (CD8), OT-II (CD4), and non-specific B6 T cells (in a 2:1:2 ratio; 5 x 10^6 total T cells) were injected intravenously (Figure 6A). The mixture of antigen-specific transgenic CD8, CD4, and wild type (non-specific) T cells were used in these experiments to mimic CAR T cell preparations following transduction with CAR-containing lentivirus in which a portion of the T cells are antigen-specific and a portion are polyclonal. Mice receiving no T cells or only cultured wild type B6 T cells were used as negative controls. T cells were expanded for 3 days in the presence of DMSO, idelalisib, VIPhyb, or a combination of Idelalisib and VIPhyb prior to injection. The phenotype of the cells just prior to injection is shown in Figure 6B and 6C. Measured tumor growth was slower in mice receiving T cells expanded in the presence of idelalisib, VIPhyb, or a combination of both (Figure 6D), and tumors removed at necropsy were smaller (Figure 6E). Mice that received T cells expanded in idelalisib, VIPhyb, or a combination of both survived significantly longer than mice that received no T

cells, B6 T cells alone, or control mixtures of OT-I, OT-II, and B6 cells expanded in DMSO (Figure 6F). In summary, addition of VIPhyb and idelalisib either alone or in combination to T cell expansion cultures significantly impacted the antigen-specific T cell response following adoptive transfer to lymphoma-bearing mice.

3-5-0 Discussion

The most common initial treatment for DLBCL patients involves the combination of rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHIOP).[198, 209] Cyclophosphamide functions as an alyklator with potent effects on immune cells including damage and death to both malignant and healthy cells when administered at high doses.[6] Although many DLBCL respond to R-CHOP and similar therapies, a substantial portion of patients experience relapse. Patients with highly chemorefractory disease, including those patients relapsed after ASCT, are the initial patient population for whom CAR T cell therapy is currently being explored.[201, 210] Multiple rounds of cytotoxic chemotherapies causes extensive damage to T cells which may result in insufficient yield or poor quality products during CAR T manufacturing.[211, 212] We observed a significant increase in T cells lacking expression of CD27 and CD28 in previously-treated DLBCL patients which significantly correlated with the number of prior chemotherapy cycles. Loss of CD27 and CD28 has been observed in patients as they age, but age was not a significant contributing factor to the frequency of CD27-CD28- T cells in our subjects as untreated lymphoma patients (median

age- 67) had similar frequencies of CD27-CD28- T cells as untreated healthy controls (Figure 1A and 1C).[213] The loss of CD28 from T cells is particularly important as many of the currently used manufacturing methods utilize anti-CD3/CD28 bead-mediated stimulation.[213] Signaling through CD3 in the absence of a co-stimulatory signal such as CD28 leads to anergy and an inability to proliferate.[56, 214] Additionally, T cells that lack both CD27 and CD28 co-stimulatory domains may have immunosuppressive properties as their removal enabled expansion of T cells that otherwise failed to proliferate (Figure 2C).[42]

The lymphoid-lineage specific PI3K δ inhibitor idelalisib is currently in use as a therapeutic for CLL and functions by blocking downstream survival and differentiation signals in malignant B cells.[170, 215, 216] In addition to B cells, the δ isoform of PI3K is also expressed in T cells and NK cells.[217] PI3K δ signaling has been shown to be crucial for CD8 T cell-mediated anti-listeria responses and also in the function of Treg in murine solid tumor models.[162, 164] The differentiation signals downstream of PI3K δ including AKT make it an attractive target during anti-CD3/CD28 bead stimulation as strong TCR stimulation leads to exhaustion and terminal differentiation of T cells.[218] T cells of the naïve and central memory compartments are the most effective in T cell-based therapies, and partially blocking terminal differentiation is an attractive strategy to use during *ex vivo* T cell expansion.[208] Addition of idelalisib to T cell expansion cultures resulted in significantly increased frequencies of CD27 and CD28 co-expressing cells which are characteristic markers of less terminally differentiated cells.[219] This is likely

due to a partial blockade of activation signals through the TCR that are mediated by PI3K.[142] A similar block or delay in differentiation was observed in p110δ knockout murine T cells stimulated with anti-CD3 antibodies *in vitro.*[164] Importantly, patients with non-Hodgkin lymphoma have been shown to have an overabundance of memory T cells when compared to patients with other malignancies, presumably due to prior exposure to alkylator or alkylator-like chemotherapies.[208] These memory populations could potentially interfere with expansion of naïve T cells through fratricide limiting the efficacy of therapies that utilize autologous T cells.[208] Thus, preserving naïve T cells that begin in low abundance in lymphoma patient samples and would otherwise be lost during CD3/C28 bead-mediated expansion would help to improve the quality of the product.

Strong *in vitro* stimulation of the TCR leads to activation induced cell death (AICD), the extent of which can be reduced by the anti-apoptotic protein Bcl-2.[220] PI3K δ inhibition resulted in increased Bcl-2 levels suggesting a reduction in AICD. Higher frequencies of less terminally differentiated cells suggest that addition of idelalisib helped to partially block terminal differentiation. Increased signaling through CD28 cannot account for the increased levels of Bcl-2 as CD28 signaling was shown not to have an effect on protein levels in T cells,[57] thus the mechanisms underlying increased Bcl-2 expression need further exploration. Increased cell viability helps explain increased yields as there was no significant

difference in CFSE dilution. Taken together, blockade of PI3K δ likely reduced TCR signal strength reducing terminal differentiation and AICD.

VIP is a neuropeptide that signals through a cyclic AMP-PKA pathway and has a large variety of anti-inflammatory effects.[88, 99] Among the observed effects of VIP on T cells is a reduction in pro-inflammatory cytokine secretion, reduced proliferation, reduced cytotoxicity, and Treg generation making the pathway an attractive target for T cell-based therapies.[116, 119, 127, 181, 221] Addition of VIPhyb did not significantly affect total T cell yield, but did increase the frequency of CD27+CD28+ T cells when added with idelalisib and had an additive positive effect on the *in vivo* persistence of human T cells. Utilizing an alternative approach to block VIP signaling through cleavage of endogenously-produced VIP peptide with mast cell chymase gave similar results (Supplementary Figure 3).[222] One of the mechanisms by which VIP exerts its anti-inflammatory effects is through a reduction in NF-κB signaling through phosphorylation and activation of CREB.[115, 223] Signaling through the NF-κB pathway enhances T cell survival so relieving this inhibition would increase the survival of T cells activated *in vitro*.[224] Interestingly, T cells expanded in VIPhyb alone had significantly increased anti-tumor activity. VIPhyb enhances both autologous anti-tumor T cell responses as well as graft-versus-leukemia responses in murine leukemia models.[115, 130] Therefore, we postulate that addition of idelalisib helps to partially block terminal differentiation while addition of VIPhyb further helps to increase persistence and enhances cytotoxicity.

We show here that addition of idelalisib in combination with VIPhyb significantly increases yield and produces better quality T cell products that have greater *in* vivo persistence and anti-lymphoma activity. We propose that antagonism of these two pathways is an attractive strategy to expand polyfunctional T cells with favorable *in vivo* persistence for leukemia and lymphoma patients planned to receive T cell therapies. Utilizing this or similar strategies to support autologous T cell manufacturing may increase the potential application of CAR and other T cell therapies for heavily treated patients with hematological malignancies.

3-6-0 Figure Legends

Table 3-7-1: DLBCL patient demographics and treatment information. Information on untreated DLBCL patients is given in the top portion of the table while treated patient information is contained in the bottom portion. All procedures were approved by the Institutional Review Board of Emory University (Approval ID IRB0716).

Figure 3-7-1: T cells from DLBCL patients who have received multiple rounds of chemotherapy show extensive loss of CD27 and CD28. Peripheral blood samples from healthy controls, untreated DLBCL patients, and heavily-treated DLBCL patients were examined for the expression of CD27 and CD28 by flow cytometry. (A) Representative flow plots of CD27 and CD28 expression

gated on CD3+ cells. (B) Linear regression showing the correlation between number of total chemotherapy cycles and frequency of CD27-CD28- cells in DLBCL patient samples. (C) Quantification of CD27-CD28- cells from healthy controls (n=4), untreated DLBCL patients (n=5), and DLBCL patients who received greater than or equal to the median number (10.5) of chemotherapy cycles (n=5 of 10 samples from treated DLBCL patients).

Figure 3-7-2: Depletion of CD27-CD28- cells enables the expansion of heavily damaged DLBCL patient T cells. A heavily treated DLBCL patient sample was rested overnight followed by FACS sorting to separate CD27-CD28- cells from the remaining populations. Double negative T cells, T cells depleted of double negative cells, and unsorted T cells were then stimulated with anti-CD3/CD28 beads with IL-2 for 14 days. (A) Flow plots showing the sorting strategy and post-sort purity. (B) Cell counts over the course of expansion. (C) T cell viability at the end of the expansion period.

Figure 3-7-3: Murine T cells activated in the presence of Idelalisib and/or VIPhyb retain their cytokine secreting capacity and give an increased yield at the end of the expansion period. CFSE-labeled or unlabeled murine T cells were stimulated with anti-CD3/CD28 beads +/- Idelalisib and/or VIPhyb for 3 days followed by flow cytometry analysis. (A) Expression of CD25 and CD44 on total T cells at the end of the expansion period. (B) Production of IFN-γ and IL-2 after re-stimulation on day 3. (C) Representative histograms showing CFSE

dilution of T cells stimulated in the presence or absence of Idelalisib. (D) Quantification of the frequency of CFSE dilute T cells from 3 independent experiments. (E) Fold expansion of T cells relative to controls as assessed by bioluminescent imaging. **p<0.01, ***p<0.001

Figure 3-7-4: Pharmacological blockade of PI3K δ and VIP signaling results in increased cell yield and preservation of CD27+CD28+ cells during *in vitro* T cell expansion. T cells from healthy donors and DLBCL patients were expanded *in vivo* in the presence or absence of Idelalisib and VIPhyb for fourteen days. Cells were counted on days 7 and 14 of expansion and phenotyped by flow cytometry on day 14. (A) Expansion profiles of healthy controls. (B) Representative expansion curve from heavily-treated DLCBL patient T cells. (C) Waterfall plot showing the expansion of DLBCL patient samples relative to DMSO controls. The numbers above the bars indicate specific patient samples. (D) Representative flow plots showing the expression of CD27 and CD28 on the total T cell population. (E) Quantification of double positive and double negative T cells from 10 lymphoma patients. (F) Western blot of Bcl-2 levels in a lymphoma patient's T cells on day 14 of expansion under the indicated culture conditions. *p<0.05

Figure 3-7-5: Expansion of DLBCL patient T cells in the presence of Idelalisib and VIPhyb significantly enhances their *in vivo* persistence following adoptive transfer to NSG mice. T cells from a heavily-treated DLBCL

patient were expanded in the presence or absence of Idelalisib and/or VIPhyb for 14 days followed by transfer to irradiated NSG mice. Blood was analyzed 14 days post-transfer for the presence of human CD45+CD3+ cells. (A) Experimental outline. (B) Flow plots showing the phenotype of T cells on day 14 of expansion just prior to adoptive transfer. (C) Representative flow plots from the blood of NSG mice showing the frequency of human T cells. (D) Quantification of T cell frequencies in NSG mice 14 days post-adoptive transfer from one of 3 independent experiments. ** $p<0.01$, *** $p<0.001$

Figure 3-7-6: Expansion of T cells in the presence of Idelalisib and/or VIPhyb significantly enhances their anti-tumor activity in a murine lymphoma model. OT-I, OT-II, and B6 T cells were expanded for 3 days using anti-CD3/CD28 beads in the presence or absence of Idelalisib and/or VIPhyb. Cells were then mixed (2 OT-I: 1 OT-II: 2 B6 ratio) and injected into B6 SJL mice bearing subcutaneous E.G7 OVA tumors. Growth of tumors was monitored by caliper measurement with volume calculated as (length x width^2)/2. (A) Experimental outline. (B) Flow plots showing the phenotype of T cells on day 3 of expansion just prior to injection into tumor-bearing mice. (C) Histogram showing the expression of CD62L on T cells on day 3 of expansion. Black = DMSO, Gray = 100 nM Idelalisib, Green = 3 μM VIPhyb, Red = Idelalisib + VIPhyb. (D) Tumor growth curve and quantification of tumor volume on the final day of allowable growth. (E) Images of tumors removed from sacrificed mice on day 18 of growth. Note that the tumor from one mouse from the Idelalisib + VIPhyb group

regressed and was undetectable. (F) Survival curve of tumor-bearing mice receiving T cells expanded under the indicated conditions. **p<0.01 n=4 or 5 mice per group from 2 independent experiments.

Supplementary Figure 3-7-1: Idelalisib and VIPhyb reduce PD-1 expression on DLBCL patient T cells stimulated with CD3/CD28 beads. DLBCL patient T cells were stimulated with anti-CD3/CD28 beads in the presence of IL-2 and then phenotyped by flow cytometry for the expression of PD-1. (A) PD-1 expression on DLBCL patient T cells on day 10 of expansion. (B) PD-1 expression on FACS-sorted T cell subsets that were stimulated for 4 days with CD3/CD28 beads in the presence or absence of VIPhyb.

Supplementary Figure 3-7-2: The dual PI3K δ/γ inhibitor Duvelisib also increases DLBCL patient T cell yield and preserves expression of CD27 and CD28 at the end of the expansion period. DLBCL patient T cells were expanded for 14 days with CD3/CD28 beads in the presence or absence of Duvelisib. T cells were counted on days 7 and 14 and phenotyped by flow cytometry on day 14 to examine expression of the co-stimulatory molecules CD27 and CD28. (A) Expansion curve of DLBCL patient T cells. (B) Flow cytometry plots showing increased expression of CD27 and CD28 on DLBCL patient T cells expanded in the presence of Duvelisib.

Supplementary Figure 3-7-3: Antagonism of VIP signaling by either addition of VIPhyb or cleavage by mast cell chymase results in similar effects on DLBCL patient T cell phenotype at the end of expansion. DLBCL patient T cells were expanded for 10 days with CD3/CD28 beads in the presence or absence of Idelalisib, VIPhyb, and/or mast cell chymase. Cells were analyzed for expression of CD27 and CD28 at the end of the expansion period by flow cytometry. Flow cytometry plots of CD27 and CD28 expression are shown.

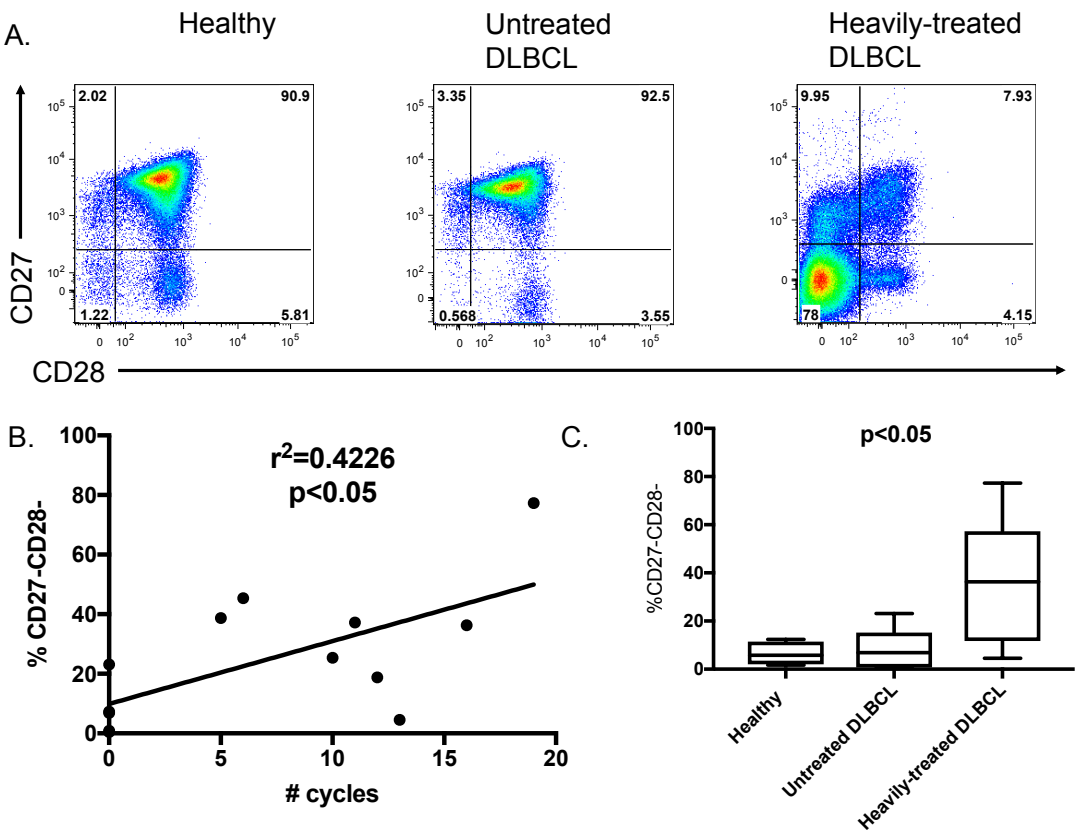
Supplementary Figure 3-7-4: Expansion of healthy T cells in the presence of Idelalisib increases the frequency of TNF-α and IL-2 dual-secreting CD8 T cells. Healthy control T cells were expanded for 14 days in the presence of the indicated compounds followed by re-stimulation with PMA and ionomycin. Intracellular staining was then performed to assess cytokine-secreting capacity. (A) Representative dot plots showing secretion of IL-2 and TNF-α in CD4 and CD8 T cells. (B) Representative dot plots showing secretion of IFN-γ and TNF-α in CD4 and CD8 T cells. (C-D) Quantification from 3 healthy controls.

3-7-0 Figures

Patient	Gender	Age	Total cycles of chemotherapy	WBC (x 10^3/μL)	ALC (x 10^3/μL)	Absolute CD3 (per μL)	Absolute CD4 (per μL)	Absolute CD8 (per μL)	Absolute CD19 (per μL)	Absolute CD56 (per μL)	Absolute CD16 (per μL)
1	*M*	*33*	*12*	*6.1*	*1.281*	*343*	*99*	*233*	*0*	*206*	*214*
2	*M*	*61*	*19*	*11.6*	*1.856*	*1013*	*440*	*512*	*156*	*219*	*256*
3	*M*	*64*	*7*	*6.5*	*.455*	*259*	*110*	*149*	*0*	*89*	*92*
4	*F*	*52*	*5*	*5.4*	*1.728*	*1030*	*366*	*355*	*326*	*124*	*214*
5	*F*	*64*	*11*	*3.4*	*1.650*	*1576*	*423*	*1091*	*0*	*40*	*55*
6	*F*	*33*	*21*	*2.7*	*.648*	*706*	*70*	*636*	*0*	*94*	*118*
7	*M*	*56*	*10*	*6.6*	*1.888*	*1940*	*809*	*264*	*N/A*	*N/A*	*N/A*
8	*M*	*41*	*12*	*3.1*	*.690*	*202*	*112*	*87*	*0*	*147*	*133*
9	*M*	*37*	*5*	*8.8*	*.880*	*832*	*379*	*450*	*0*	*15*	*34*
10	*F*	*38*	*12*	*5*	*1.100*	*792*	*261*	*496*	*0*	*176*	*211*
		Median-46.5	**Median-10.5**	**Median-5.75**	**Median-1.1905**	**Median-812**	**Median-313.5**	**Median-402.5**	**Median-0**	**Median-124**	**Median-133**

Patient	Gender	Age	Total cycles of chemotherapy	WBC (x 10^3/μL)	ALC (x 10^3/μL)	Absolute CD3 (per μL)	Absolute CD4 (per μL)	Absolute CD8 (per μL)	Absolute CD19 (per μL)	Absolute CD56 (per μL)	Absolute CD16 (per μL)
1	*M*	*55*	*0*	*8.1*	*2.660*	*1085*	*933*	*107*	*N/A*	*N/A*	*N/A*
2	*F*	*51*	*0*	*8.8*	*2.675*	*2517*	*1734*	*604*	*N/A*	*N/A*	*N/A*
3	*F*	*75*	*0*	*8.5*	*1.173*	*652*	*538*	*91*	*N/A*	*N/A*	*N/A*
4	*M*	*70*	*0*	*15.1*	*6.886*	*4968*	*4645*	*247*	*N/A*	*N/A*	*N/A*
5	*F*	*67*	*0*	*8.5*	*3.332*	*2465*	*1038*	*789*	*N/A*	*N/A*	*N/A*
		Median-67	**Median-0**	**Median-8.5**	**Median-2.675**	**Median-2465**	**Median-1038**	**Median-247**	**Median-0**	**Median-124**	**Median-133**

Table 3-7-1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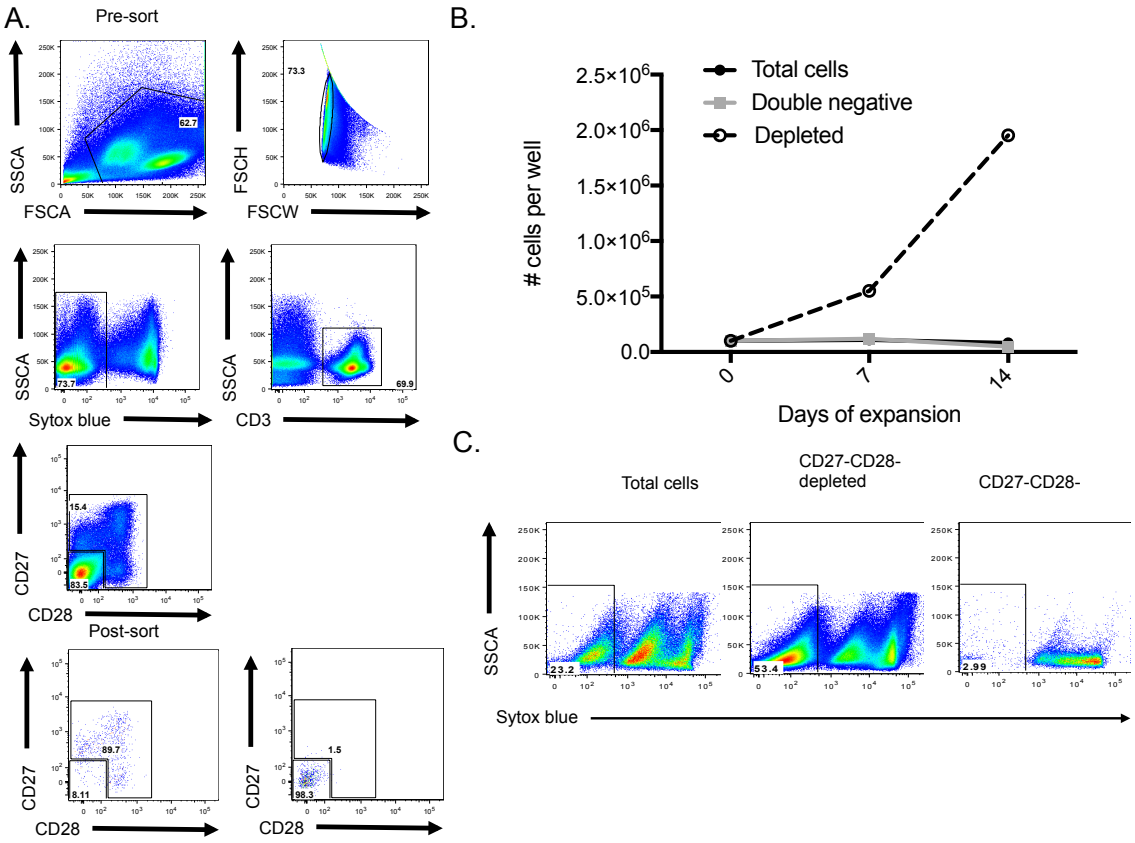
Healthy
Untreated DLBCL
Heavily-treated DLBCL

Figure 3-7-1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15.4
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2.5×10^6
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14
Days of expansion
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Total cells
CD27-CD28-depleted
CD27-CD28-
SSCA
Sytox blue
23.2
53.4
2.99

Figure 3-7-2

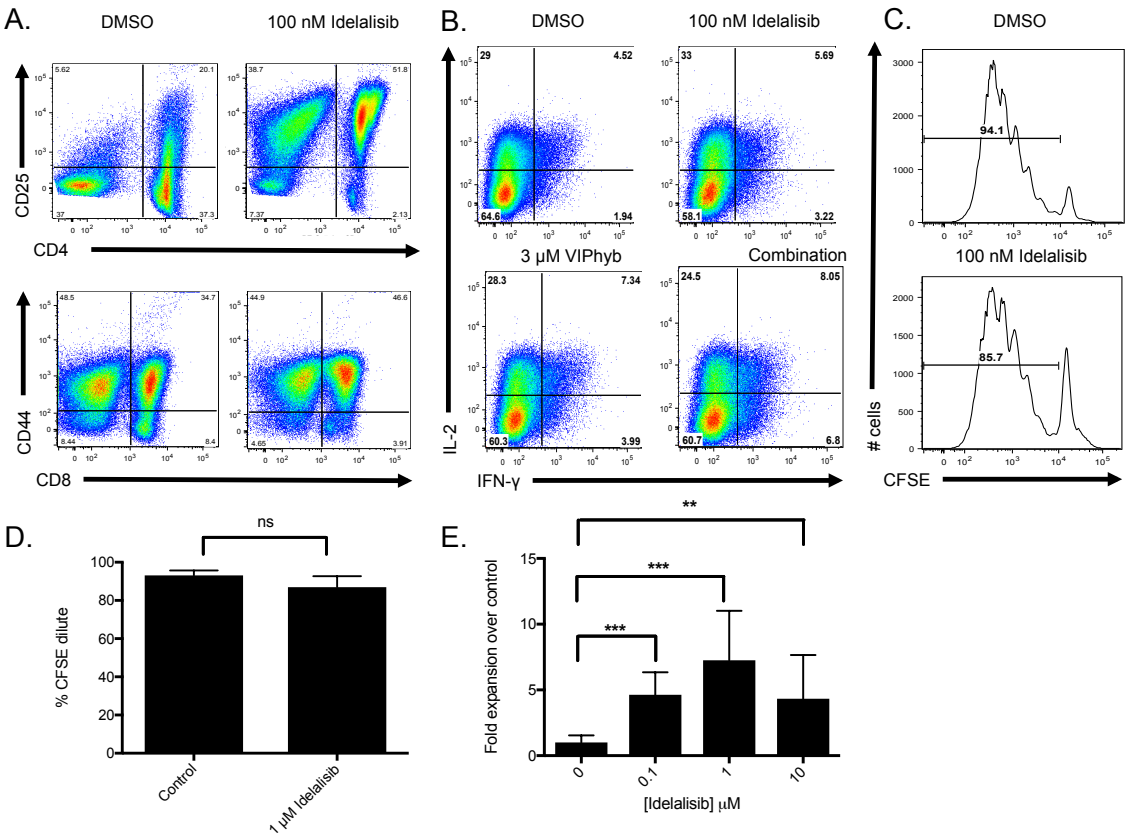

A.
DMSO
100 nM Idelalisib
CD25
CD4
CD44
CD8
B.
DMSO
100 nM Idelalisib
3 μM VIPhyb
Combination
IL-2
IFN-γ
C.
DMSO
94.1
100 nM Idelalisib
85.7
cells
CFSE
D.
ns
% CFSE dilute
Control
1 μM Idelalisib
E.
**
***
***
Fold expansion over control
[Idelalisib] μM

Figure 3-7-3

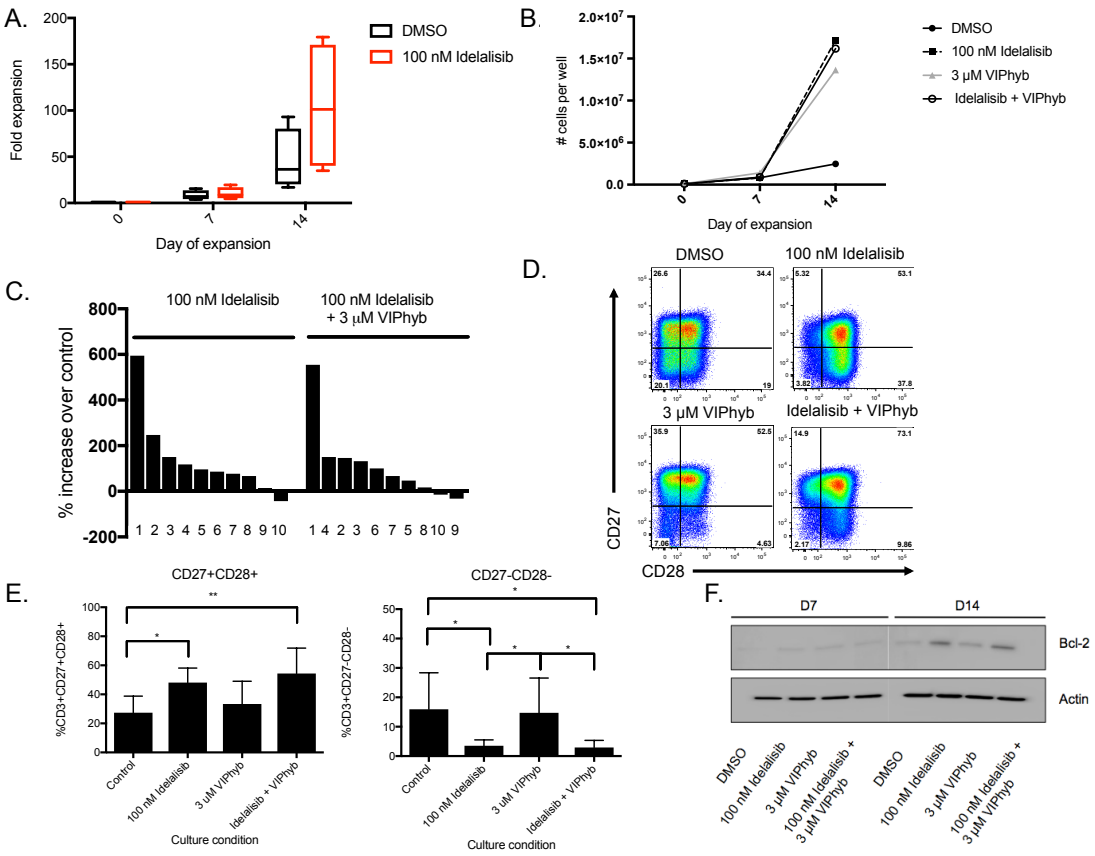

A.
Fold expansion
DMSO
100 nM Idelalisib
Day of expansion
B.
cells per well
DMSO
100 nM Idelalisib
3 μM VIPhyb
Idelalisib + VIPhyb
Day of expansion
C.
100 nM Idelalisib
100 nM Idelalisib + 3 μM VIPhyb
% increase over control
D.
DMSO
100 nM Idelalisib
3 μM VIPhyb
Idelalisib + VIPhyb
CD27
CD28
E.
CD27+CD28+
CD27-CD28-
%CD3+CD27+CD28+
%CD3+CD27-CD28-
Control
100 nM Idelalisib
3 μM VIPhyb
Idelalisib + VIPhyb
Culture condition
F.
D7
D14
Bcl-2
Actin
DMSO
100 nM Idelalisib
3 μM VIPhyb
100 nM Idelalisib + 3 μM VIPhyb

Figure 3-7-4

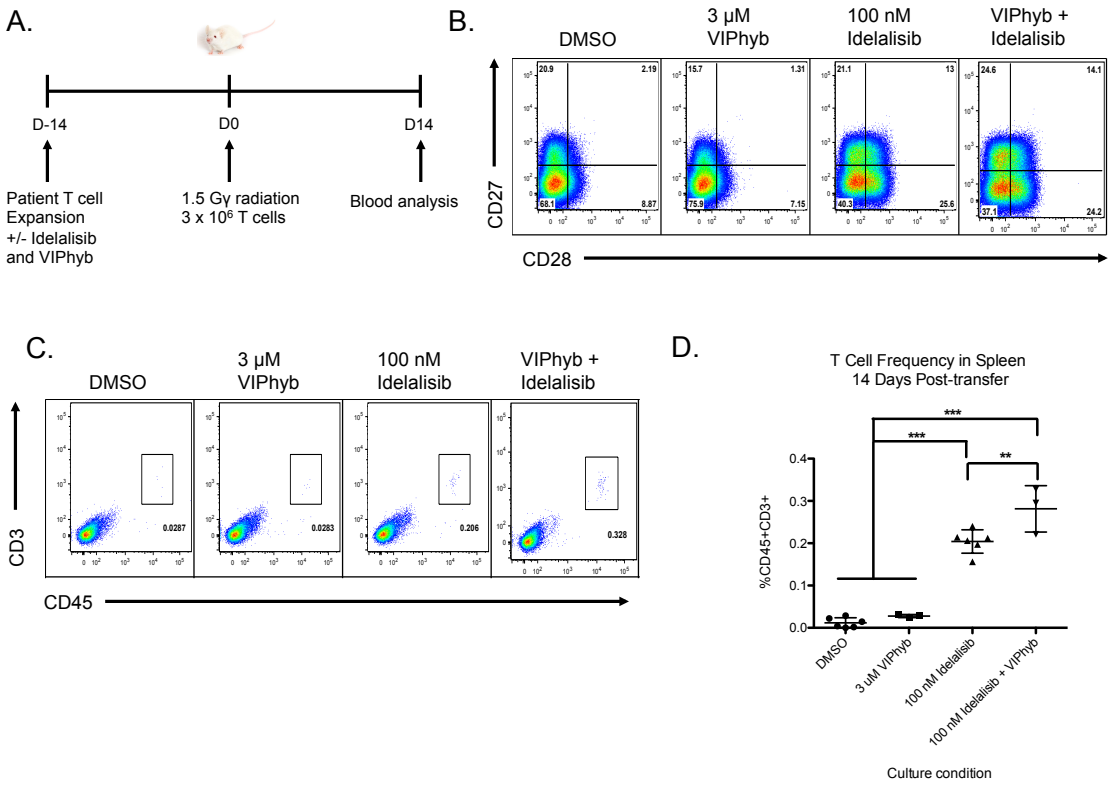

A.
D-14
D0
D14
Patient T cell Expansion +/- Idelalisib and VIPhyb
1.5 Gy radiation 3 x 10^6 T cells
Blood analysis
B.
DMSO
3 μM VIPhyb
100 nM Idelalisib
VIPhyb + Idelalisib
CD27
CD28
C.
DMSO
3 μM VIPhyb
100 nM Idelalisib
VIPhyb + Idelalisib
CD3
CD45
D.
T Cell Frequency in Spleen
14 Days Post-transfer
%CD45+CD3+
DMSO
3 uM VIPhyb
100 nM Idelalisib
100 nM Idelalisib + VIPhyb
Culture condition

Figure 3-7-5

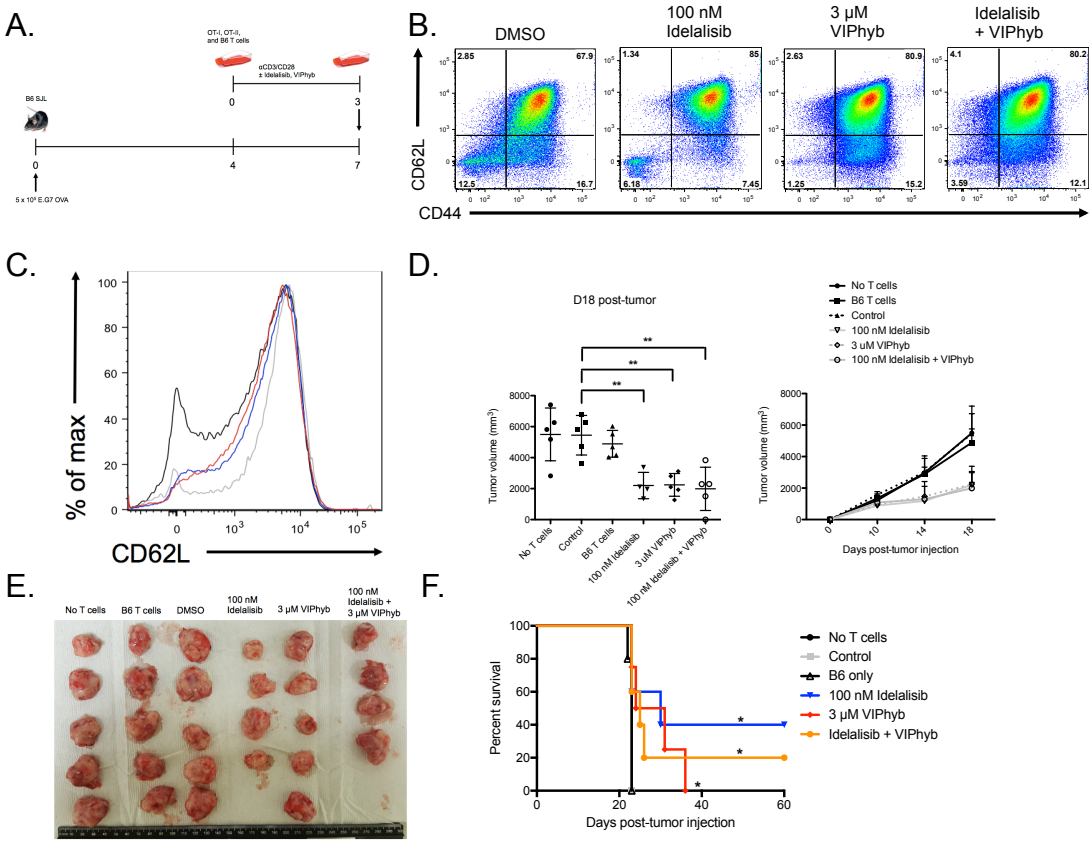
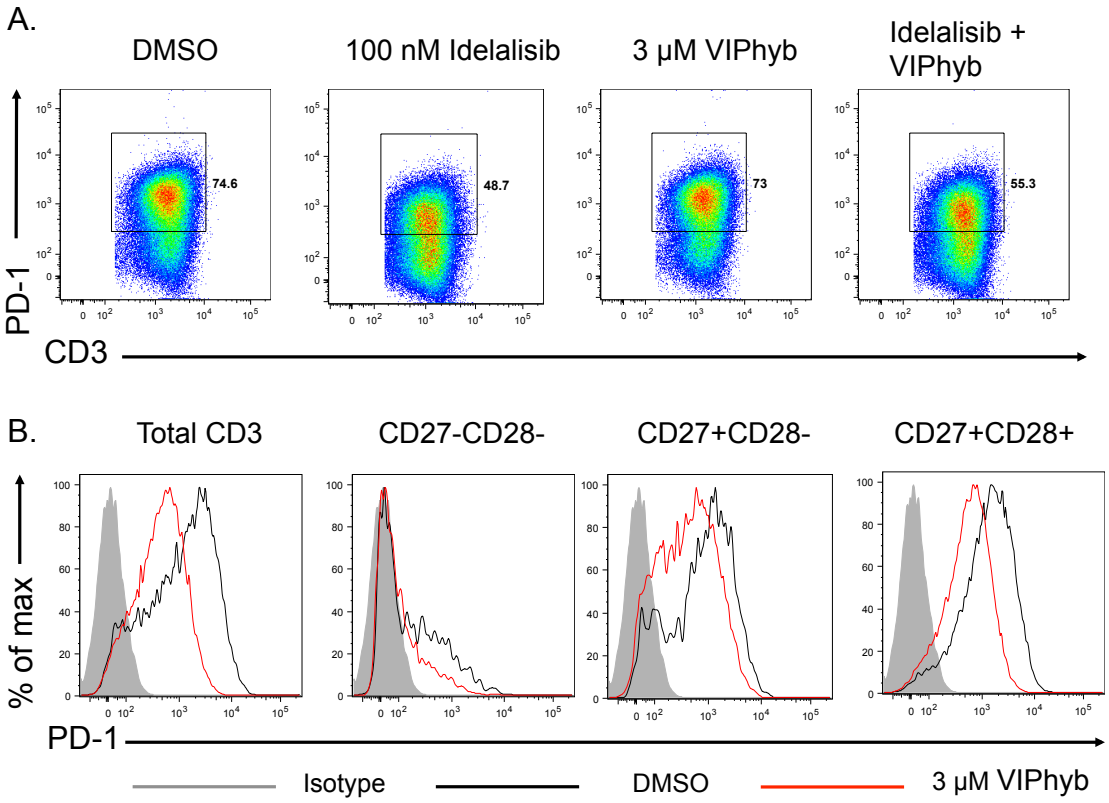
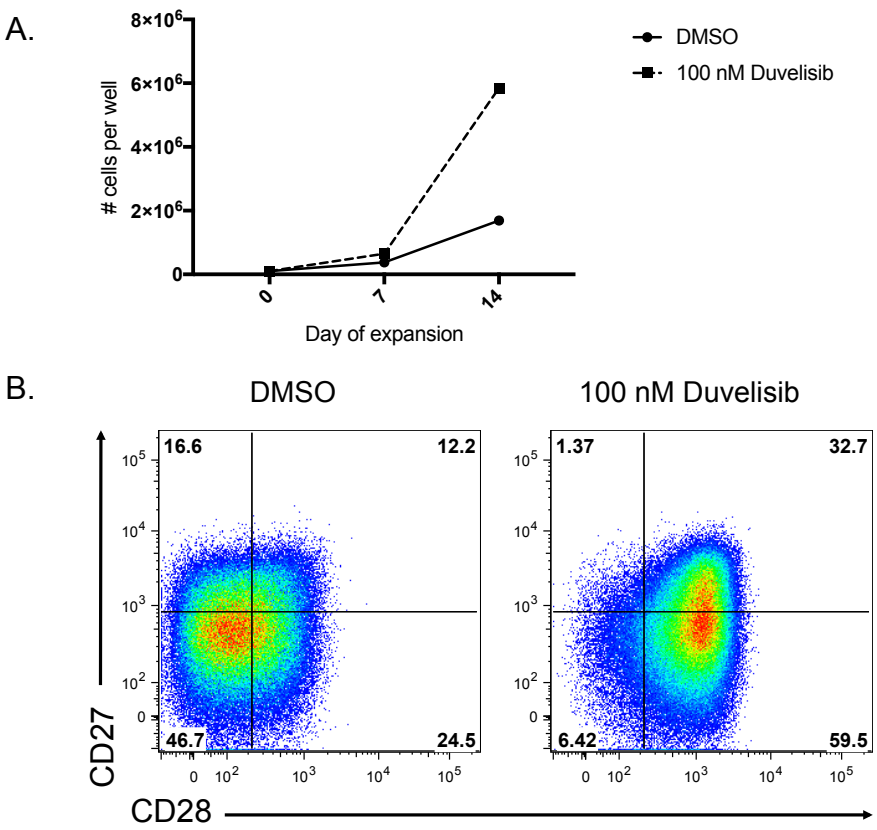

A.
B.
DMSO
100 nM Idelalisib
3 μM VIPhyb
Idelalisib + VIPhyb
CD62L
CD44
C.
% of max
CD62L
D.
D18 post-tumor
Tumor volume (mm³)
No T cells
B6 T cells
Control
100 nM Idelalisib
3 uM VIPhyb
100 nM Idelalisib + VIPhyb
Days post-tumor injection
E.
No T cells
B6 T cells
DMSO
100 nM Idelalisib
3 μM VIPhyb
100 nM Idelalisib + 3 μM VIPhyb
F.
Percent survival
No T cells
Control
B6 only
100 nM Idelalisib
3 μM VIPhyb
Idelalisib + VIPhyb
Days post-tumor injection

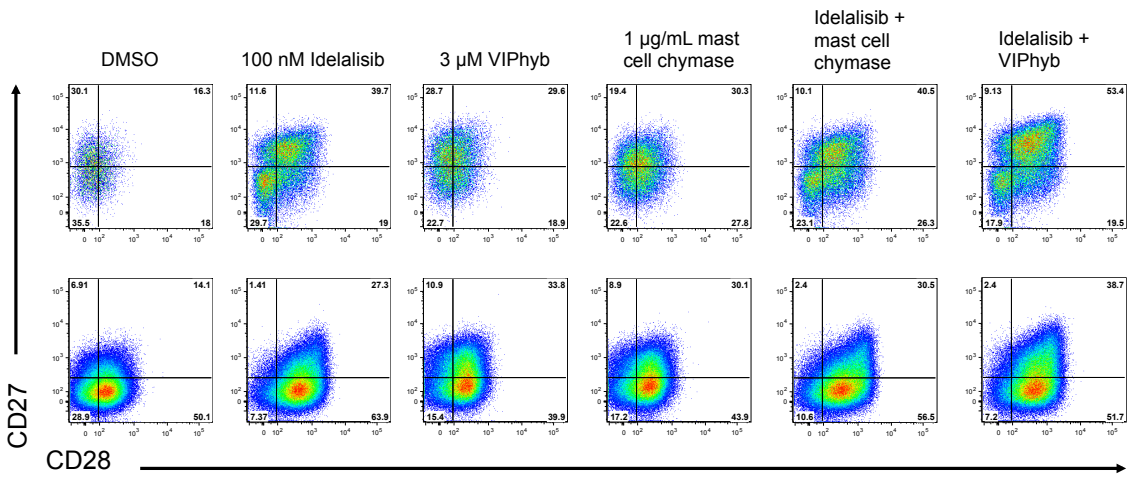
Figure 3-7-6



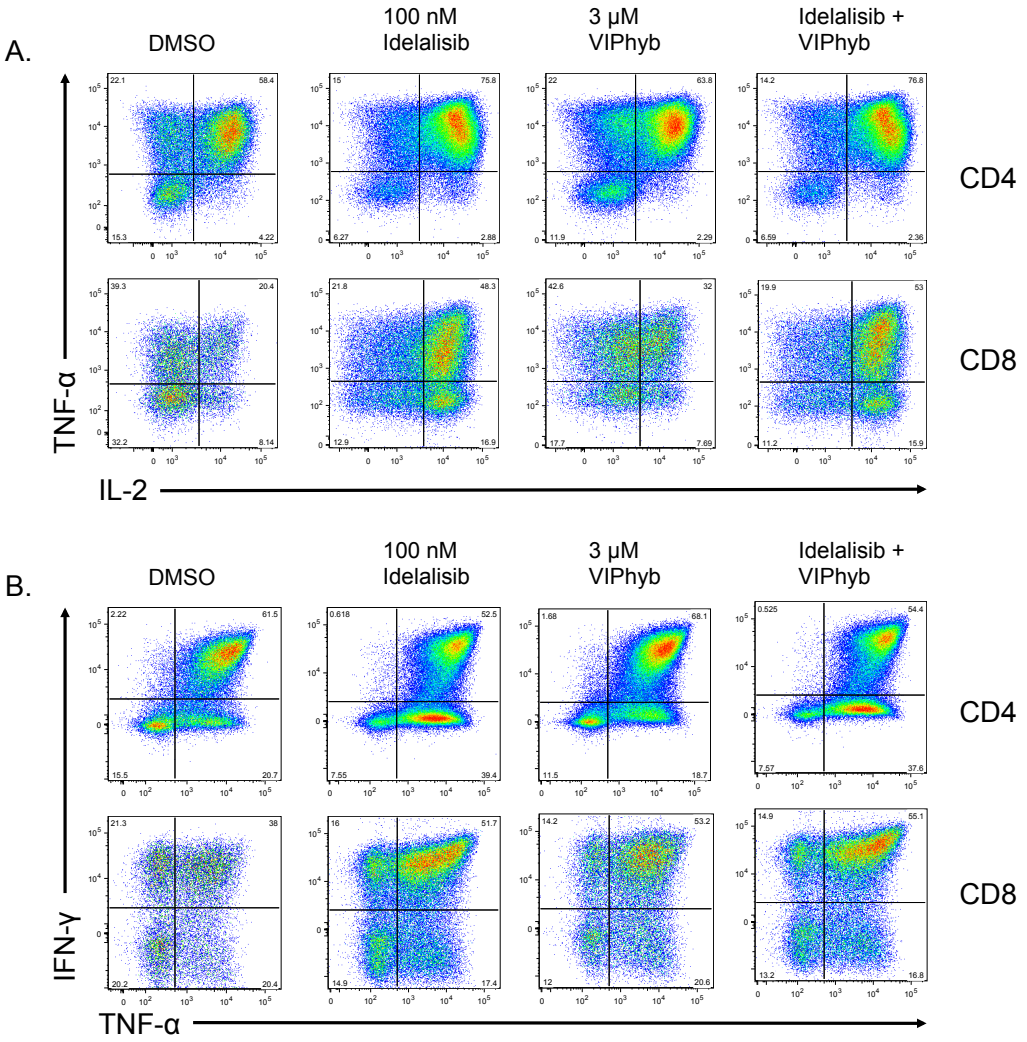
Supplementary Figure 3-7-1

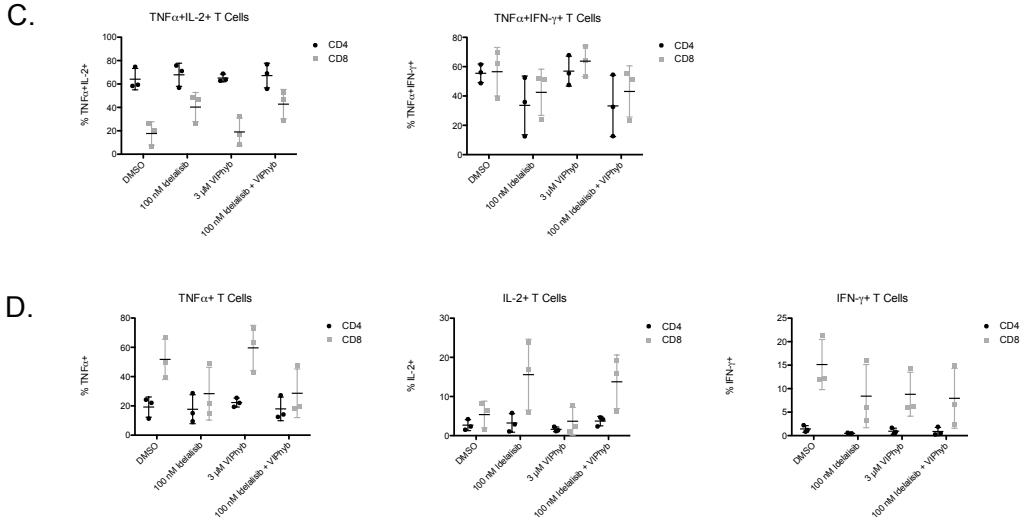


Supplementary Figure 3-7-2



Supplementary Figure 3-7-3





Supplementary Figure 3-7-4

Chapter 4:

Discussion

4-1-0 Summary

Immunotherapeutic approaches to leukemia and lymphoma treatment have made tremendous progress since the introduction of HSCT six decades ago. Although HSCT remains a go-to treatment for many different hematological malignancies, therapies designed to enhance autologous anti-cancer T cell responses have moved to the forefront and received much attention in recent years. Enhancing T cell responses through immunomodulation is an attractive approach as transplant-associated morbidity and mortality would be avoided. The complexities of T cell-tumor cell interactions and the variety of immune evasion tactics employed by tumor cells to subvert the natural T cell response have opened up several possibilities for therapeutic intervention. Immune modulatory agents have shown promising results in solid tumors, but these treatments have seen more limited success as monotherapies in leukemia and lymphoma. This is likely due to the non-inflammatory nature of these cancers and the failure of T cells to initiate a response. Genetically modified T cells that bypass the requirement for antigen processing and presentation have largely resolved this issue, but problems inherent with manufacturing processes have prevented them from reaching their full potential. As such, there is an urgent need to discover new pathways and develop interventions that work around the barriers that currently hold back T cell therapies for leukemia and lymphoma.

The works presented here highlight the potential for both the vasoactive intestinal peptide and PI3K δ signaling pathways in T cell modulatory therapies for leukemia and lymphoma. A relatively small body of work regarding the role of VIP in T cell responses exists with even fewer works describing the role of VIP in anti-tumor T cell responses. The data presented here expand on these studies and extend them to settings of hematological malignancy, an area that has yet to be explored in detail. The role of PI3K δ in T cell responses has been described in numerous studies, but these studies largely utilize kinase inactive mouse cells rather than pharmacological inhibition in human T cells. Additionally, much of the focus of the pro-inflammatory effects of PI3K δ antagonism have focused on Treg inhibition whereas the data presented here highlight a crucial role for PI3K δ in CD8 T cell responses *in vitro* and *in vivo.* Further, the works presented here provide a possible synergism between the VIP and PI3K δ pathways as the effects seen during human T cell expansion were enhanced when both pathways were inhibited. The intersection of these two pathways has yet to be described in this context. Combinations of immune modulators to treat cancer have been tested and proposed as superior approaches to immunotherapy. As such, the use of VIP antagonists in combination with PI3K δ antagonists may prove to be a powerful strategy in the treatment of hematological malignancies as well as the manufacture of T cell therapies. Dissection of the molecular mechanisms underlying the beneficial effects of VIP and PI3K δ antagonism and the precise signaling events linking these two pathways will require further study.

4-2-0 Conclusions and Perspectives

The data presented provide significant insight into the role of the VIP and PI3K δ signaling pathways in T cells. More specifically, the data describe the functions of these pathways in CD8 T cells and the consequences of antagonizing them in contexts of T cell expansion, leukemia, and lymphoma. Among the observed effects of this antagonism on T cell function, one of the most striking observations was the significant enhancement of anti-leukemia T cell responses in acute myeloid leukemia-bearing mice treated with VIPhyb. The enhanced cytokine secretion and reduction in PD-1 highlight the relevance of the VIP pathway in CD4 and CD8 T cell function in settings of acute leukemia. Due to the reduced expression of VIP receptors on the surface of CD8 T cells, antagonism of VIP may be particularly effective in this subset as the receptors are easier to saturate with peptide antagonists. As such, the use of VIPhyb may be most appropriate in settings of cancer and viral infection in which CD8 T cells are the main effectors. Extension of these findings to humans may prove more difficult due to the inherent difficulties associated with peptide therapeutics, the most notable of which being poor stability *in vivo.*[225] Several strategies may be employed to work around this issue including modification of potential enzymatic cleavage sites or introduction of a zinc finger to produce a more stable peptide. Reaching a sufficient concentration of VIPhyb is easy to achieve in mice due to their small size and plasma volume, but this may prove to be an additional challenge in humans. Increased peptide stability may help overcome this issue,

as smaller amounts of peptide would be needed if the persistence were increased. Prolonging the plasma half of VIPhyb will thus be crucial if it is to be used as an immunotherapeutic agent in leukemia and lymphoma patients.

Current methods to generate sufficient numbers of T cells for adoptive cellular therapies typically employ strong stimulatory agents such as anti-CD3/CD28 beads. Although these methods often give an adequate yield, the quality of the T cells may be reduced due to persistent stimulation leading to exhaustion and senescence. This is particularly relevant in CAR T manufacturing which requires expansion periods of up to 14 days or more. Terminal differentiation and senescence led to severely impaired *in vivo* persistence of expanded lymphoma patient T cells in NSG mice and a failure of the anti-lymphoma T cell response in B6 mice receiving tumor antigen-specific T cells. Similar effects may be observed in humans receiving CAR T cells which can help account for the failure of the therapy in some patients. This is particularly relevant in lymphoma patients as evidenced by the high frequency of CD27-CD28- T cells in patients who had received multiple cycles of chemotherapy. Interventions with VIPhyb and the PI3K δ antagonist Idelalisib prevented the negative functional consequences associated with anti-CD3/CD28 bead-mediated expansion. These results support a role for both pathways in terminal differentiation and senescence. The likely mechanism underlying the effects of VIPhyb and Idelalisib is a prevention of replicative senescence. Replicative senescence occurs in CD4 and CD8 T cells expanded for extended periods *in vitro* or in older CMV seropositive individuals

and leads to functional impairments in cytokine secretion and proliferative capacity.[226-228] Phenotypically, cells that have reached replicative senescence show significant loss of CD28 due to changes in transcription factor binding to the two CD28 regulatory elements.[227, 229] Data from the current studies supporting reduced replicative senescence in T cells expanded in VIPhyb and Idelalisib is the sustained expression of CD28. While control cells lost a large portion of CD28+ cells, T cells expanded in Idelalisib or a combination of Idelalisib and VIPhyb had significantly higher frequencies of CD28+ cells. This difference was most pronounced in CD8 T cells. T cells expanded in the presence of VIPhyb alone had frequencies of CD28+ cells similar to control, but these cells showed reduced loss of the co-stimulatory molecule CD27. Further supporting a delay in replicative senescence as the mechanism behind VIPhyb and Idelalisib were observations of increased frequencies of TNF-α+IL-2+ CD8 T cells following re-stimulation with PMA and ionomycin. No differences in the secretion of IFN-γ, IL-2, or TNF-α were observed in CD4 T cells suggesting a more prominent role for PI3K δ and VIP signaling in CD8 T cell differentiation. These results show that T cells expanded under conditions of reduced VIP and PI3K δ signaling have greater functional capacities, as they have not reached a terminal or exhausted state. In further support of this notion were observations that T cells expanded in the presence of Idelalisib and VIPhyb had greater frequencies of naïve and central memory T cells. This is likely due to reduced induction of Tbet via the mTOR pathway and increased expression of eomesodermin in CD8 T cells.[230] Taken together, these results are particularly

relevant in the setting of CAR T therapy as CAR T cells expanded in these conditions would have greater proliferative and cytokine-secreting capacities following antigen encounter *in vivo*.

One of the most intriguing observations in experiments testing combinations of VIPhyb and Idelalisib was the additive effects on the preservation of CD28+ cells as well as naïve and central memory T cells. The possible crosstalk between these two pathways in T cells has not been fully explored and may be partially explained by the ability of VIP to activate the PI3K pathway and induce differentiation in prostate cancer cells.[231] One possible mechanism for this is the ability of $G_{\beta\gamma}$ subunits to activate PI3K.[144] Since VIP mainly signals through VPAC1/2 via $G_{\alpha s}$ and $G_{\beta\gamma}$ subunits it is possible that the addition of VIPhyb further suppresses PI3K signaling when combined with Idelalisib. PI3K γ is predominantly activated via association with GPCR $G_{\beta\gamma}$ subunits, but blockade of this pathway through VIP antagonism cannot account for the observed effects since the dual PI3K δ/γ antagonist duvelisib did not give results different from PI3K δ antagonism alone. As such, other PI3K isoforms may also associate with VPAC1/2 $G_{\beta\gamma}$ accounting for the additive effects observed with VIPhyb and Idelalisib.

In summation, the work presented here identifies two additional pathways that may be suitable targets for T cell modulation in leukemia and lymphoma patients: the vasoactive intestinal peptide pathway and the PI3K δ pathway. The results

obtained also show intersection between these two seemingly unrelated pathways and their profound effects on T cell function and differentiation. Further development of treatment approaches that take advantage of these pathways may result in another addition to the arsenal of immunotherapeutics currently available to treat hematological malignancies.

4-3-0 Future Directions

The data presented show enhancement of T cell responses through antagonism of the vasoactive intestinal peptide and PI3K δ signaling pathways, but the precise molecular mechanisms underlying this enhancement remain to be elucidated. In order to determine the specific downstream pathways that are affected during the blockade of VIP and PI3K δ signaling, phosphorylation status of key signaling proteins will need to be examined at various time points during T cell stimulation. The Akt/mTOR pathway is of particular interest due to its large role in T cell activation and differentiation. The retained expression of CD28 on T cells expanded in the presence of VIPhyb and Idelalisib supports a role for enhanced CD28 signaling in the observed effects. Whether additional signals emanating from CD28 play a role could be examined in murine CD28 knockout T cells. In addition, differential gene expression in T cells stimulated with VIP could help shed light into other possible pathways that may be modulated by VIPhyb. In particular, repeated observations of decreased PD-1 expression on T cells could be explained by analysis of PD-1 transcript in VIP-stimulated T cells in the

presence or absence of anti-CD3 stimulation. Epigenetic modification of the PD-1 locus has been described as the mechanism behind differential PD-1 expression, but methylation status was not different between VIPhyb-treated mice and PBS controls when tested using a viral infection model. As such, another unknown mechanism is responsible for the decreased PD-1 expression in VIPhyb-treated AML-bearing mice. The ability of VPAC1/2 to activate the PI3K pathway must also be examined and could be performed by measuring PIP3 production in response to incubation with VPAC1/2 $G_{\beta\gamma}$ subunits.

Expression of VPAC1 and VPAC2 is dynamic during T cell activation. Despite many similarities between the two receptors and their downstream signals, they are not fully redundant. It is thus imperative to determine which of the two receptors is primarily responsible for the effects mediated by VIPhyb. In order to accomplish this, VPAC1 and VPAC2 knockout mice could be inoculated with C1498 and evaluated for survival and immune activation. Additionally, knockout of VPAC1, VPAC2, or both receptors in human T cells may be performed using a CRISPR/Cas9 system. VPAC1/2 single and double knockouts could be expanded *in vitro* and evaluated using the same parameters as described. The results of such an experiment could also provide further insight into the roles of each receptor during T cell activation and proliferation.

Recent publications have described the potential use of Idelalisib as an immune modulatory agent in settings of cancer.[162, 175] Both of these studies largely focus

on the effects of PI3K δ signaling blockade on the generation and function of Tregs. The effects of PI3K δ signaling blockade in the presented work were tested in models of T cell expansion and adoptive cellular therapy, but not in endogenous T cells. Based on the numerous observed effects of Idelalisib on CD8 T cell function, administration of Idelalisib alone or in combination with VIPhyb to leukemia and lymphoma-bearing mice is of particular interest. The inflammatory responses observed in patients taking Idelalisib supports a potential for the compound to enhance anti-tumor responses *in vivo.*[174]

In addition to T cells, NK cells, dendritic cells, and myeloid cells express VIP receptors and PI3K δ. While the presented work has focused exclusively on T cells, the contribution of other immune cell subsets cannot be ignored. The effects of VIP and PI3K δ signaling blockade on NK and myeloid cell function is of particular interest due to their prominent role in anti-tumor responses. Secretion of VIP by myeloid-derived suppressor cells has recently been described as a mechanism to suppress T cell proliferation as shown in the figure below:

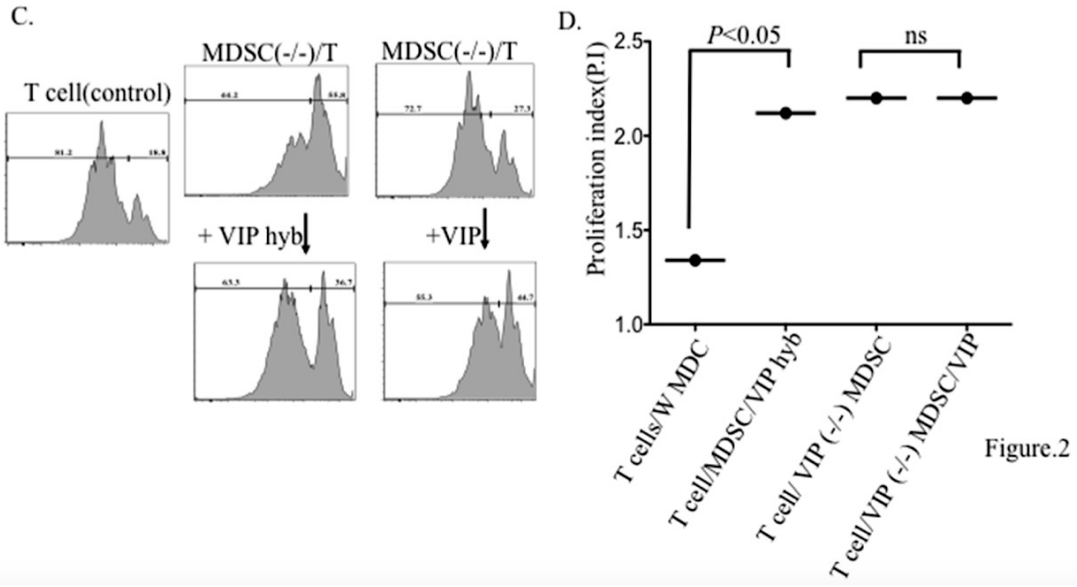
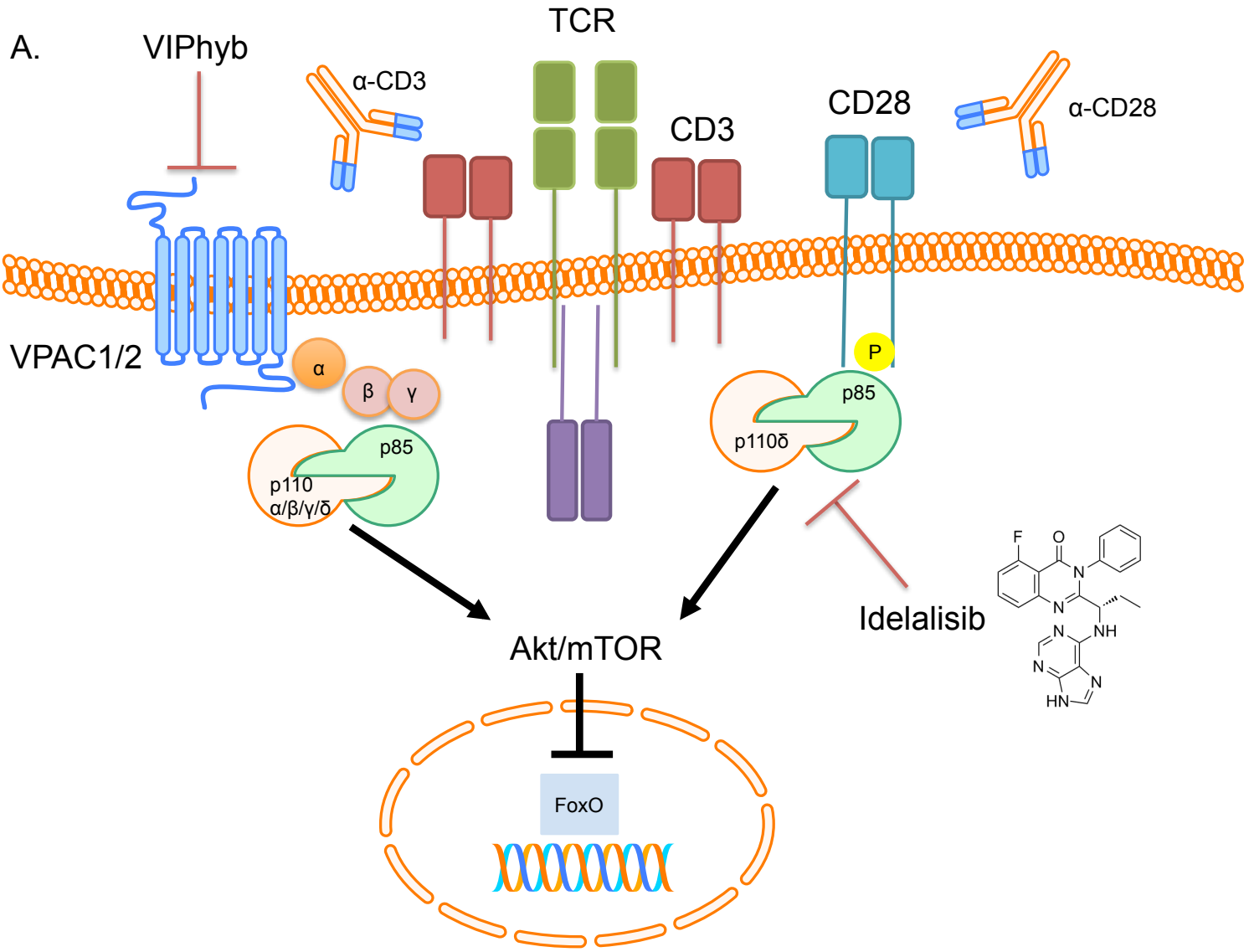


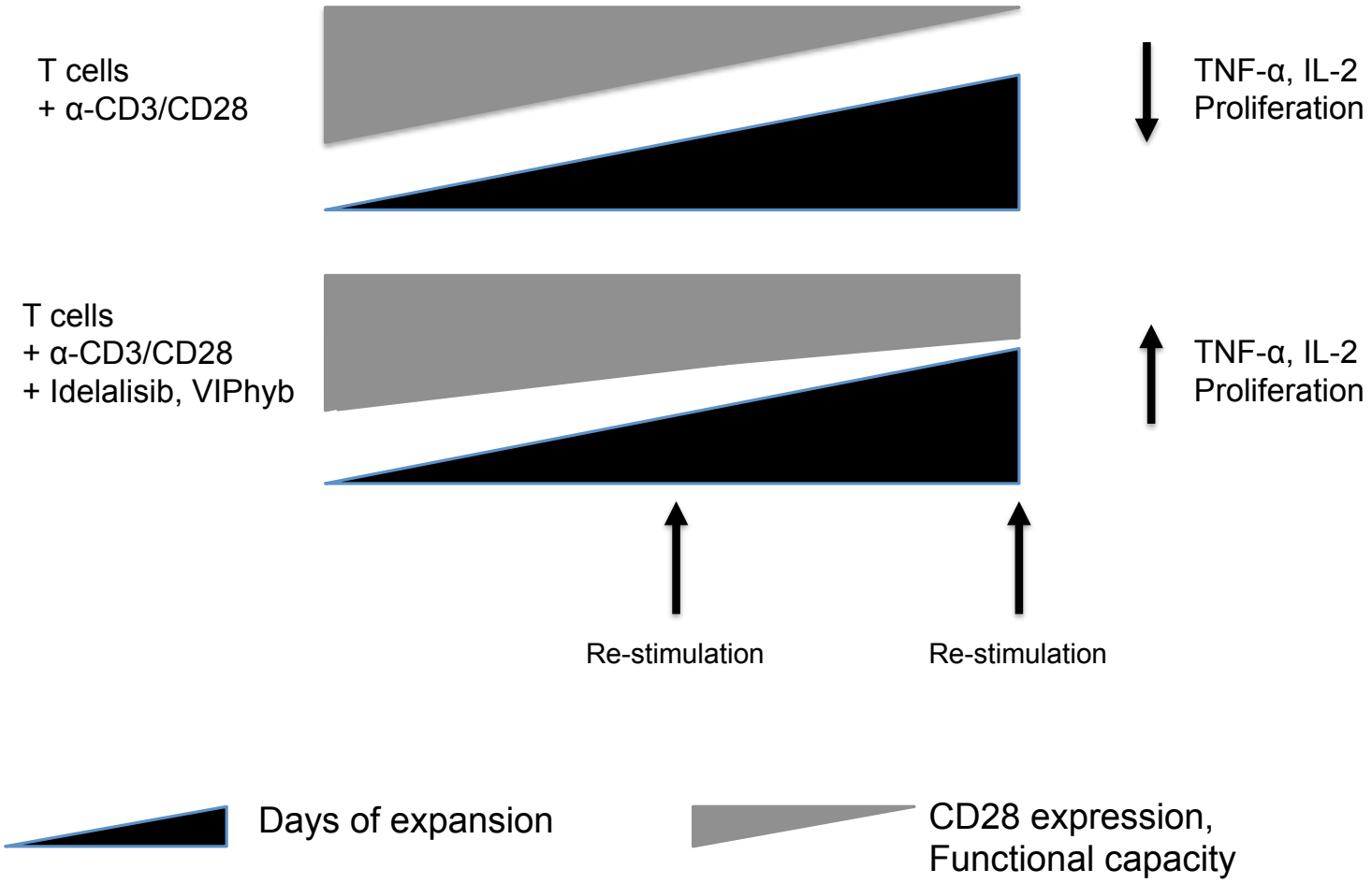
Figure.2

Prevention of this T cell suppression through VIPhyb administration could have beneficial effects on anti-tumor immune responses. The effect of PI3K δ blockade on MDSC generation and function remains to be studied. Additionally, possible additive or synergistic effects of VIP and PI3K δ antagonism on MDSC-mediated T cell suppression should be examined.

4-4-0 Model


A.
VIPhyb
α-CD3
TCR
CD3
CD28
α-CD28
VPAC1/2
α
β
γ
p85
p110
α/β/γ/δ
P
p85
p110δ
Idelalisib
Akt/mTOR
FoxO

B.


T cells
+ α-CD3/CD28
TNF-α, IL-2
Proliferation
T cells
+ α-CD3/CD28
+ Idelalisib, VIPhyb
TNF-α, IL-2
Proliferation
Re-stimulation
Re-stimulation
Days of expansion
CD28 expression,
Functional capacity

Proposed model for the effects of Idelalisib and VIPhyb on CD8 T cells. (A) The PI3K pathway is activated by anti-CD3 stimulation and co-stimulation through CD28 along with VIP binding to VPAC1/2. Production of PIP3, recruitment of Tec kinases, and activation of the Akt/mTOR pathway results in inhibition of FoxO and transcription of genes that leads to differentiation. Idelalisib blocks this signaling sequence at the level of PI3K δ while VIPhyb prevents activation of unknown isoforms PI3K isoforms through VPAC1/2 $G_{\beta\gamma}$ subunits. (B) Persistent stimulation of T cells during *ex vivo* stimulation leads to replicative senescence and reduced proliferative and functional capacities. Idelalisib and VIPhyb delay senescence as evidenced by increased expression of CD28, increased proliferation following re-stimulation, and increased frequencies of TNF-α and IL-2 dual-secreting CD8 T cells.

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