University of Cyprus
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RECOMBINANT POLIOVIRUS A THERAPEUTIC AGENT FOR CANCER

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ABSTRACT

Cancer is a leading cause of global mortality, and its burden is growing despite the availability of standard treatment methods. Genetically and Morphologically tumor cells differ from normal cells. However, cancers arise from genetic modifications of normal cells and these cells may be nearly identical to normal cells, hence, making it difficult to selectively target tumor cells with the current standard cancer therapeutic methods. The ability of oncolytic viruses to specifically replicate within a target host cell and stimulate therapeutic response without disrupting normal cells in the immediate environment confers oncolytic virotherapy with a pharmacokinetic advantage over standard cancer therapies and this creates a pathway to reducing the global morality rate attributable to cancer.

Aim:This review is aimed at illustrating the oncolytic potential of Recombinant Poliovirus, PVSRIPO, for cancer therapy.

PVSRIPO is a live attenuated, nonpathogenic virus containing the oral poliovirus Sabin type 1 in which the internal ribosomal entry site (IRES) is replaced with the IRES from human rhinovirus type 2 (HRV2). PVSRIPO leads to tumor regression by activating the host antitumor immune response and this process follows the phases of cancer immunoediting namely tumor cell elimination by immune system, balancing and regulating genetically unstable tumor cells and immune cells while preventing the escape of variant tumor cells.

PVS-RIPO a derivate of Picornavirus, has a small virion size (~30 nm in diameter) which confers it with an oncolytic advantage. Furthermore, Picornaviruses have an attribute of rapid replication cycle, and this attribute enables PVSRIPO to effectively replicate and propagate in tumor cells to reverse the immunosuppressed state thereby resulting in tumor regression. The primary studies in this review have illustrated the oncolytic effect of the rapid propagation of PVSRIPO in tumor cells. Furthermore, these studies have indicated that this cytotoxicity of PVSRIPO, occurs in a time and dose dependent manner. For example, most of the studies reported that a single dose of intratumorally administered PVSRIPO was potent to delay tumor growth with a decline in tumor weight within few days.

PVSRIPO is stable and safe for use in oncolytic virotherapy due to the substitution of the poliovirus' original IRES for the HRV2 IRES. The primary studies in this review have illustrated

that upon successful administration of PVSRIPO, there was no evidence of viral neuropathogenicity or virus shedding. In addition, PVSRIPO can be safely used in combination with other systemic therapies.

Preventing side effect while targeting cancer has been a primary source of concern in cancer therapy and PVSRIPO fills this gap by its ability to selectively target the tumor cells without affecting non-tumor cells in the immediate environment due to its natural tropism for CD155, the poliovirus receptor (PVR).CD155 is present in a lot of cancers thereby making PVSRIPO an appropriate agent for eliminating these cancer cells.

This review has highlighted the oncolytic potentials of PVSRIPO which was evident in the resulting increased survival rates in both the animal models and clinical trials. Given these illustrations in this review, PVSRIPO is an appropriate agent for cancer therapy.

DEDICATION

I dedicate this thesis work to my amazing aunty, Mrs Eno-Abasi Henry, for encouraging me to apply to the University of Cyprus and for her support.

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COMPOSITION OF THE EXAMINATION COMMITTEE

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SEMINAR ANNOUNCEMENT

University of Cyprus
Department of Biological Sciences

BIO 680 Scientific Methodology in Molecular Biology

Student Presentation

Friday, 20 May 2022 at 09:00
Building ΘΕΕ02 (undergraduate teaching lab), Room B172, Panepistimioupoli Campus

This seminar is open to the public

MBONU HELEN SOCHIMA

Thesis Supervisor: Special Teaching Staff, Dr. Annita Charalambous

"Recombinant Poliovirus a therapeutic agent for Cancer"

Cancer is a leading cause of global mortality, and its burden is growing despite the availability of standard treatment methods. Genetically and Morphologically tumor cells differ from normal cells. However, cancers arise from genetic modifications of normal cells and these cells may be nearly identical to normal cells, hence, making it difficult to selectively target tumor cells with the current standard cancer therapeutic methods. The ability of oncolytic viruses to specifically replicate within a target host cell and stimulate therapeutic response without disrupting normal cells in the immediate environment confers oncolytic virotherapy with a pharmacokinetic advantage over standard cancer therapies and this creates a pathway to reducing the global morality rate attributable to cancer.

Recombinant Poliovirus, PVSRIPO, an oncolytic RNA virus, has shown promise in the treatment of a plethora of cancer types. PVSRIPO is a live attenuated, nonpathogenic virus containing the oral poliovirus Sabin type 1 in which the internal ribosomal entry site (IRES) is replaced with the IRES from human rhinovirus type 2 (HRV2). PVSRIPO leads to tumor regression by activating the host antitumor immune response and this process follows the phases of cancer immunoediting namely tumor cell elimination by immune system, balancing and regulating genetically unstable tumor cells and immune cells while preventing the escape of variant tumor cells.

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INTRODUCTION

Globally, cancer is the leading cause of mortality, and it accounts for about 10million deaths that occurred in the year 2020 (World Health Organization 2022a). Cancer is a large group of genetic diseases that emanates from changes in the cell genome leading to rapid and uncontrollable cell growth and spread into adjoining parts of the body and organs (National Human Genome Research Institute 2018, World Health Organization 2022). These changes in the cell genome emanate from the modification of normal or wild type cells into tumor cells in a multistage process that progresses from a precancerous lesion state to a malignant tumor (National Human Genome Research Institute 2018). Cellular modifications leading to precancerous state can take many forms namely, DNA mutations, rearrangements, deletions, amplifications and addition or removal of chemical marks. These cellular modifications cause cells to produce an unusually large amount of some essential proteins or produce misshapen proteins that do not function as they should. A combination of these cellular genomic modifications functions together to promote cancer. The cellular modifications that promote tumorigenesis and malignancy can either be inherited from parents, caused by the influence of environmental factor, or emanate from errors that occur during cell division(National Cancer Institute 2017). Cellular genomic modifications that promote cancer affects three main types of genes in the target host: proto-oncogenes, tumor suppressor genes and DNA repair genes. Proto-oncogenes are involved in normal cell growth and division and upon modification they become more active than usual and transform into oncogenes, thereby allowing the modified cells to grow and survive in abnormal conditions. Tumor suppressor genes function in controlling cell growth and division. Upon alteration of the tumor suppressor gene, the genes divide in an uncontrolled manner. Modifications in DNA repair genes results in the development of mutations of other genes and changes in their chromosomes leading to a cancerous state(National Cancer Institute 2021). Previous research has implicated the involvement of the immune system in the inhibition and promotion of tumorigenesis(O'Donnell, Teng et al. 2019). This process involving the immune system has been termed ‘immunoediting’ and it occurs progressively in three phases namely, elimination, equilibrium, and escape(O'Donnell, Teng et al. 2019). In the elimination phase, the innate and adaptive immune system attacks tumor cells and

eliminates them. However, some tumor cells survive this elimination process and enter the next phase where it experiences growth dormancy. During this equilibrium phase of dormancy, some tumor cells with modified ability to evade immune surveillance are selected into the escape phase(O'Donnell, Teng et al. 2019). In the escape phase, tumor cell proliferates, growth is unlimited, and tumors become clinically visible (Shimizu, Iyoda et al. 2018). The acquired ability of tumor cells to evade immune surveillance is facilitated by a combination of intrinsic and extrinsic factors. An increase in extrinsic factors; Tcells $_{(Treg)}$ and myeloid-derived cells facilitates an immunosuppressive state. Regulatory Tcells $_{(Treg)}$ interact with effector Tcells $_{(Teff)}$ to inhibit its anti-tumor activity while the myeloid cells reinforce an immunosuppressive state. Furthermore, the intrinsic factors select the tumor cells with antigen-loss ability(Shimizu, Iyoda et al. 2018). Elimination phase consists of four subphases, namely, phase 1, where the antitumor immune response is initiated, the activated innate immune surveillance system detects the growing tumor cells. The activation of the immune system induces inflammatory signals such as macrophages, dendritic cells, and infiltrating lymphocytes namely NK cells and NK Tcells that release Interferon gamma (IFN-γ). In the second phase, the released IFN-γ induces Immunogenic Tumor Cell Death (ITCD) and the release of chemokines namely CXCL9, CXCL10 and CXCL11, which inhibit angiogenesis thereby inducing immunogenic necrotic tumor cell death. The tumor apoptotic bodies are phagocytosed by DCs in the draining lymph nodes, as a Bystander Killing Effect (BKE). The subsequent inflammation that occurs releases cytokines and chemokines which attract additional immune cells. In the third phase of elimination, the reciprocal release of cytokines IL-12 and IFN-γ transactivates macrophages and NK cells, expanding tumor cell death by apoptosis or PCD type 1 and releasing reactive oxygen and nitrogen intermediates. Tumor-specific DCs in the draining lymph nodes induces the differentiation of T helper type 1 (Th1) cells, which mediate the production of killer T cells or CD8+ T cells (Giannios 2015) . The fourth phase of elimination entails the infiltration of tumor site by tumor-specific CTL CD8+ and CD4+ T cells after recognition of tumor-associated antigens, such as MHC class I and class II molecules, which function in synergy with B lymphocytes that produce antibodies, such as IgG, IgA, IgM, IgD, and IgE, to facilitate innate and adaptive immune mechanisms, which in turn mediates the release of cytokines thereby resulting to immunogenic tumor cell death. Tumor cells that are not killed by the elimination phases of the immune surveillance enter the equilibrium phase, where IFN-gamma and lymphocytes inhibit expansion of tumor cells that are genetically unstable and mutate rapidly.

All tumor cell variants that have escaped immune pressure due to resistance to elimination, in which the balance between the immune response and tumor cells leads to uncontrollable tumor cell proliferation with non-immunogenic transformed cells, can lead to malignant neoplasms by direct entry into the escape phase (Giannios 2015) . Tumors escape immune surveillance eradication utilizing immunosuppressive molecular and cellular mechanisms. Immunosuppressive regulators are supported by the heterogenous TME which contain tumor cells, extracellular matrix (ECM) cells, local bone marrow-derived stromal progenitor cells, pericytes, endothelial cells, proteins, matrix degrading enzymes, chemokines, cellular factors, immune cells, tumor-associated fibroblasts and angiogenic cells which may cause desmoplasia after stromal cell infiltration and ECM deposition (Giannios 2015). The first category of tumor escape from the immune system involves the development of tumor immuno-resistance namely, the promotion of oncogenic tumor stem cells that are dormant and able to self-renew, proliferate, completely differentiate, and produce immunosuppressive factors to inhibit apoptosis or type 1 PCD. The loss of abnormal surface antigens on tumoral plasma membrane due to mutations and immuno-escape of epitope loss tumor variants after sensing the presence of Cytotoxic T lymphocytes (CTL) in the TME. Hence, only tumor cells expressing the epitope are eliminated while the epitope-negative tumor cells escape elimination by CTL. Another immuno-resistant mechanism exerted by tumor cell is the lack of susceptibility to immune effector cells namely NK, CTL, macrophages and DCs, which cause antibody induced cytotoxicity, phagocytosis, or vaccine effects in cancer immunotherapy (Giannios 2015). The second category of tumor immune surveillance escape mechanisms consists of the interference with the antitumor-induced immune responses, such as reduced expression of costimulatory molecules on tumor cells or antigen presenting cells (APCs). This downregulation of costimulatory molecules on tumor cells or professional APC may inactivate or eliminate TAA-specific CTLs, put in an immature state the dendritic cells conditioned by the tumor cells, and inactivate T cells leading to tumor tolerance by bypassing productive immune responses against the tumor cells. Also, the tumor for escaping the immune system of the host alters the T-cell receptor (TCR) on the tumor infiltrating lymphocytes (TIL), especially in cases with advanced cancer, leading to reduced mediation of tumor cytotoxicity and decreased production of Th1-type cytokines. In addition, death receptor/ligand signaling and tumor-induced counterattack on immune cells that induce apoptosis or type I PCD in the tumor cells by upregulating the expression of Fas (CD95) receptor on the plasma membrane of activated T cells. The tumor cells release

PGE2, an immune suppressive factor which functions to downregulate Jak3, thereby inhibiting the IL-2R downstream signaling pathway that is responsible for inhibiting apoptosis via the bcl-2 family, resulting in a deficient signaling which inactivates T cells with subsequent bypassing of tumor cells (Giannios 2015). Also, another immunosuppressive mechanism of this second category entails DC malfunction in tumor-associated antigen (TAA) cross presentation to T cells which results in a deficient immune response against tumor cells, which may deplete dendritic cells (DC) by blocking the induction of TAA-specific immunity comprising of cytokines and chemokines, namely interleukins (IL-1, IL-12, IL-15, IL-18, and IL-23), interferons, and costimulatory molecules, which function as growth factors, and signals for T-cell proliferation, differentiation, and memory development. Maturation of Dendritic cells may be inhibiting by Tumor cells which utilizes Vascular endothelial growth factor (VEGF) and exosomes to block their differentiation. Also, tumor-associated gangliosides (TAG) may downregulate proteasomal constituents of antigen processing machinery (APM) of dendritic cells (DCs). Furthermore, apoptosis or type 1 PCD of DCs can be induced in the TME by the downregulation of antiapoptotic oncogene bcl-2, the production of nitric-oxide (NO), which downregulates cellular inhibitors of apoptotic proteins (cIAPs or cFLIP), the release of ceramide, which blocks PI3K-mediated survival signals, and alterations in intrinsic apoptotic pathways (Giannios 2015). The third tumor-induced immunosuppressive category entails the dysfunction of effector cells in the tumor microenvironment (TEM). Its first mechanism consists of suppression of T-cell immune responses by regulatory T cells (Treg), such as CD4+CD25 highFOXP3+, which accumulate in tumors, and in the peripheral circulation. They downregulate the immune response of the effector T cells by releasing TGF-b1 and IL-10 and involve the Fas/FasL and pathways linked to granzyme/perforin, and enzymatic ATP degradation to adenosine exerting immunosuppressive effects, which create tumor resistance. The second mechanism of this immunosuppressive category consists of suppression of immune cells by bone marrow myeloid-derived immature suppressor cells (MDSC), such as CD13+, CD33+, and CD34+, which are located in the peripheral circulation of cancer patients, and they are recruited to the tumors after they release soluble immunosuppressive factors, such as PGE2, IL-6, GM-CSF, IL-10, VEGF, and TGF-b1, which produce the arginase-1 enzyme that metabolizes L-arginine, activate iNOS, and control the tumor release of indoleamine-2,2-dioxygenase (IDO), which catabolizes the essential for the differentiation of T-cell amino acid tryptophan, leading to the immunosuppression of T-cell responses that promotes the survival of

tumor cells (Giannios 2015) The third immunosuppressive mechanism of this third category consists of tumor-derived micro vesicles (MV) or exosomes, which express TAA, human leukocyte antigen (HLA) class I molecules, and death ligands, which exert their immunosuppressive action by the induction of apoptosis or type I PCD in activated CD8+ effector T cells, eradicating their antitumor action. Also, these tumor-derived exosomes exert an additional immunosuppressive action by blocking the differentiation of monocytes to dendritic cells. Subsequently, the monocytes are transformed by the tumor-induced exosomes (MV) into CD14-negative HLA-DR low TGF-b+ myeloid suppressor cells (MSC), blocking the differentiation of immune cells, which inactivates their antitumor properties by releasing TGF-b, downregulating HLA class II molecules, and inhibiting the proliferation of lymphocytes (Giannios 2015) The fourth mechanism of this immunosuppressive mechanism consists of induction of apoptosis or type I PCD in effector T cells in the tumor and its periphery (Giannios 2015). Tumor cells may cause apoptotic DNA fragmentation in a proportion of activated CD8+ T lymphocytes and their effector subpopulations, such as CD8+CD28– and CD8+CD45RO+CD27–, in the tumor site, and the peripheral circulation may lead to tumor progression due to apoptotic death of effector T-cell functions, which significantly compromises antitumor immune response (Giannios 2015) The last tumor-induced immunosuppressive category comprises of insufficiency in tumor recognition signals consisting of four mechanisms. The first sub mechanism illustrates the downregulation of expression of HLA molecules on the surface of tumor cells. As the tumor progresses, it downregulates all HLA class I allospecificities, HLA-A, HLA-B, and HLA-C loci. Tumor cells disrupt the expression of antigen components and cause defects in the b2-microglobulin, and HLA class I heavy chain synthesis hence enabling the tumor cells to bypass immune surveillance resulting in tumor progression. Second sub-mechanism of the category involves the downregulation of antigen processing machinery (APM) components in tumor cells or antigen presenting cells (APCs) that affect all the peptides, which are presented by HLA class I molecules to T cells enhancing tumor resistance to CTL lysis. The downregulation of total loss of expression of the HLA class I or peptide complexes enables the bypass of immune surveillance and successive tumor cell destruction by CTL (Giannios 2015) . The third sub mechanism consists of the suppression of natural killer cells (NK) in the tumor microenvironment (TME). The downregulation of the cytolytic activity against tumor cells is mediated by the action of inhibitory receptors, such as ILT2/LIRI, CD94/NKG2A, and KIR, which blocks lysis of cells expressing

normal HLA class I. The NK cells respond spontaneously to cytokines by expressing IL2Rβγ, such as IFN-a, IFN-γ, IL-2, and IL-15. Upon activation, NK cells release TNF-a and IFN-γ for eradicating tumor cells. They also interact with dendritic cells (DCs) for exerting synergistic apoptotic cell death in tumor cells. However, tumor cells release TGF-b1, which downregulates the expression of NKG2D on NK cells impairing their antitumor activity, especially in advanced stages. Thus, tumors may escape the cytolytic activity of NK cells by the inhibition of interactions between receptors and ligands, the downregulation of tumoral ligands MICA or MICB, the eradication of activated NK cells mediated by overexpression of tumoral death-ligands, and the suppression of interactions between NKs and DCs in the tumor microenvironment (TME) promoting tumor growth and subsequent metastasis, which may kill the cancer patient (Giannios 2015) . The final mechanism of the last immunosuppressive category consists of loss or downregulation of surface antigens TAA by tumor cells, which evade the host's immune system by circumventing the cytolytic action of effector T cells (CTLs) due to genetic or epigenetic alterations, which may alter the tumoral protein expression, misleading recognition by the immune system, which promotes uncontrollable tumor growth. Thus, the loss or downregulation of epitopes, such as TAA, and differentiation antigens, such as TRP-1, tyrosinase, MART-1, gp100, and MUC-1, may promote tumoral growth due to escape from the host immune system. Furthermore, mutations caused by the tumor in the TAA may circumvent the generation of epitopes, which are recognized immunogenically by cognate CTL regardless of the expression of TAA. These genetic alterations of tumor cells at the coding RNA level may affect posttranslational mechanisms at the protein level, including glycosylation, ubiquitination, and proteolytic enzymes, such as endopeptidases and metalloproteinases (MMPs), which degrade extracellular matrix (ECM), leading to the downregulation or even total loss of TAA, which mediates tumor escape from the immune system of the host promoting tumor growth (Giannios 2015). Based on the evident role of the immune system in tumorigenesis, there are two major approaches to treating cancer namely, reversal of tumor immune suppression and immune based therapies targeting specific immune cell types(Shimizu, Iyoda et al. 2018). Many therapeutic methods have been applied to target immune suppressive cells and reverse their effects but the primarily used methods are surgery, chemotherapy, and radiation therapy. These named treatments though they have benefits, they also consist of side effects which are deleterious to the patient (Denniston, Crewdson et al. 2016). For example, chemotherapy targets immunosuppressive cells in two ways; either by

reducing immunosuppressive myeloid cells which is an advantage or by entirely depleting immune cells which is deleterious(Shimizu, Iyoda et al. 2018). The chemotherapeutic agents inhibit tumor proliferation and metastasis by exerting cytotoxic effect to tumor cells and myeloid cells(Shimizu, Iyoda et al. 2018). While targeting mitotic cancer cells, they also target non-cancerous mitotic cells as collateral damage. This may lead to reduced bone density, cognitive deficits, cardiotoxicity, sexual disfunction and other reported side effects (Denniston, Crewdson et al. 2016). Radiation therapy utilizes high energy radiation to kill the target cancerous cells by initiating DNA damage(O'Donnell, Teng et al. 2019). This resulting DNA damage prevents cell replication thereby inducing apoptosis of the cancerous cells. However, during the process, like in the case of chemotherapy, non-cancerous cells surrounding the target cancerous cells are killed. Although surgery, is the most confined of the three primary therapeutic methods, it is the most invasive and it also has complications(Denniston, Crewdson et al. 2016). These illustrations can show that despite the benefits these afore mentioned primary methods may offer for cancer therapy, their deleterious side effects remain a cause for concern. Hence, this has triggered the need for an alternative and less invasive method for cancer therapy(Denniston, Crewdson et al. 2016) . The recent breakthrough with the human genome sequencing and technological advances has paved the pathway for developing the model method for cancer therapy. Current clinical investigations are using viruses to target tumor cells and this field is called Virotherapy (National Cancer Institute 2017, Denniston, Crewdson et al. 2016). The viruses, tagged "oncolytic viruses", are a novel type of diversely programmed cancer therapeutic agents that possess the ability to perform both targeted therapy and immunotherapy without affecting the normal cells of the body and they are less invasive (National Cancer Institute 2017, Denniston, Crewdson et al. 2016, Yang, Gu et al. 2021) . **Evolution of Virotherapy** The evolution of Virotherapy dates to 1904 when Dr. George Dock hypothesized that viruses could have therapeutic use, and this served as a Landmark for Virotherapy. His hypothesis was based on a case report of a 42-year-old female leukemia patient who experienced remission after a presumed infection with influenza. Following this landmark case, in 1912 complete remission of tumor was observed in a cervical cancer patient following administration of life attenuated rabies virus after a dog bite. In addition, tumor remissions were also observed in other cases like Hodgkin Lymphoma and Burkitt's Lymphoma after a measles virus infection(Larson, Oronsky et al. 2015) . These observed remissions gave rise to many clinical trials aimed at cancer treatment using the "Good Viruses". Most clinical trials were done before

the genomic and biotechnological era. Based on this fact, most of these clinical trials were not up to the modern-day clinical trial standards and as such the experimental data is difficult to understand. Over the last decade, Virotherapy has evolved due to advances in biotechnology and gene therapy(Larson, Oronsky et al. 2015) . Gene therapy is a procedure for the treatment of genetic diseases which involves the introduction of a foreign or treated gene sequence into a target cell to restore it into a normal condition. The introduction of the genetic material into the target cells for therapy is carried out using gene delivery vehicles called Vectors(Denniston, Crewdson et al. 2016). There are two types of vectors are used namely, viral vectors and non-viral vectors. For this review, only viral vectors will be discussed. The use of these viral vectors for gene delivery in combating diseases has positively impacted the outcome of Virotherapy(Denniston, Crewdson et al. 2016) . Viruses are the most frequently used gene delivery vehicles in gene therapy because they are highly efficient. They are highly efficient because of the characteristic of the virus structure to prevent degradation and they function to replicate their own DNA or RNA into the patients target cell (Bulcha, Wang et al. 2021, Sung, Kim 2019) . Viral vectors may be categorized into two namely, DNA-viral based vectors and RNA- viral based vectors. Both categories are frequently used in modern day therapy, and each has their merits and demerits which largely depend on the expected therapeutic outcome. For this review, discussion will be centered only on RNA -viral based vectors(McFarland, Stout 2010). **RNA-viral based Vectors** These RNA-based viral vectors are commonly derived from retroviruses namely poliovirus, Rhinovirus, monloney murine leukemia virus, rous sarcoma virus and many others(ZLATEVA, DE VRIES, Jutte J. C et al. 2014, McFarland, Stout 2010, Denniston, Crewdson et al. 2016). These retroviral vectors containing the therapeutic gene are delivered into the hosts target cells using gene delivery systems. Gene delivery systems are of two categories namely, the germline gene delivery system and the somatic gene delivery systems (Sung, Kim 2019). Germline gene delivery involves the introduction of "normal" human genes into the eggs or sperm of parents or into a fertilized egg of an offspring(Greely 2001). Although the germline gene delivery system has promising potentials, it has great ethical limitations and is not currently being used (Sung, Kim 2019). Based on this limitation of the germline gene delivery system, only the somatic gene delivery system is being used in practice for gene therapy involving humans (Sung, Kim 2019) . The somatic gene delivery system involves the introduction of a normal or therapeutic gene into the patients target somatic cells. Somatic cells are the cell lines that cannot be passed unto progeny or inherited by

offspring(Greely 2001). Many different organs and cell types have been used in clinical studies when considering somatic gene therapy, including bone marrow, liver, muscle, skin, thyroid gland, and many others(O'Malley, Ledley 1993). This review will be illustrating the introduction of a named retroviral vector into the somatic cell of cancer patients. Delivery of retroviral vectors into cancer patients can be carried out using three systemic methods namely, in vivo delivery, ex vivo delivery and in situ delivery(Sung, Kim 2019, Bulcha, Wang et al. 2021). In vivo delivery involves the direct transfer of the RNA-based vector carrying the therapeutic gene into the target cell or tissue of a patient. In the ex vivo delivery system, faulty genes are extracted from a patients target tissue or bone marrow, it is manipulated in vitro by genetic modification in cell culture and insertion into RNA-based viral vector. The modified or treated gene is further expanded in cell culture and then infused back into the patient (Figure 1.). In situ delivery systems involves the administration of therapeutic gene product directly to the target tissue or cell(Sung, Kim 2019) .

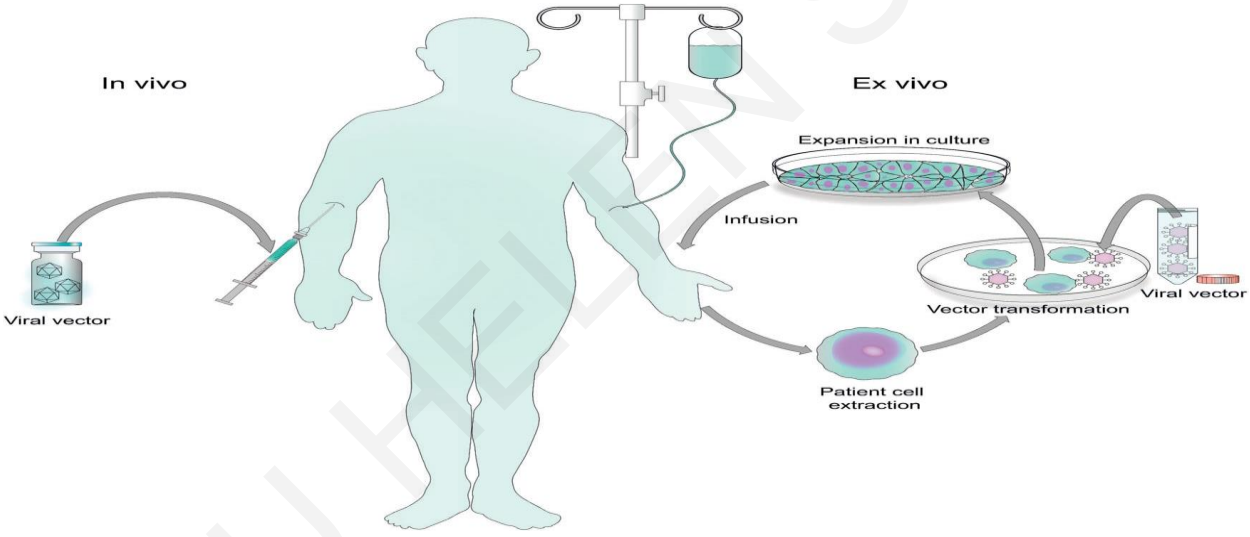


Figure 1: Illustration of RNA-viral based delivery Systems. In vivo gene delivery entails the direct administration of RNA-based viral vector carrying a therapeutic transgene into the patient. Ex vivo gene delivery involves the extraction of a patient's cells or tissue, genetic modification by a vector carrying a therapeutic transgene, selection and expansion in culture, and infusion to re-introduce the therapeutic cells back into the patient(Bulcha, Wang et al. 2021) . Retroviral vectors can transcribe directly for infectious RNA transcripts. The delivery of RNA -viral based vector is not permanent and transient. They are enveloped with a diameter of 80-130nm and a genome size of 8 -11 kilobases (Kb). Its viral genome is located with the capsid along with integrase and reverse transcriptase(Sung, Kim 2019). In the RNA-based viral vector gene delivery systems, the

retroviruses used as delivery vehicles are categorized in to three namely, human foamy virus, oncoretroviral vectors, and lentiviral RNA-based vectors (Sung, Kim 2019). **Oncolytic Retroviral Vectors** Oncolytic retroviruses are a novel type of diversely programmed cancer therapeutic agents that have a combined ability of performing targeted therapy and immunotherapy. Reprogramming the genome of oncolytic viruses improves its tumor targeting and immunogenic potential (Yang, Gu et al. 2021). **Mechanism of Oncolytic Retrovirus** Selectivity: Genetically and morphologically, tumor cells differ from normal cells, and this enables oncolytic viruses effectively target them for therapeutic purposes possible. Secondly, surface receptor proteins and apoptosis-related genes are differentially expressed between the tumor cells and normal cells which enhances selective targeting of tumor cells by oncolytic retroviruses without destroying normal cells. Thirdly, the dysregulated signaling pathways of tumor cells comprise of mutations of key protein-coding genes, oncolytic retroviruses capitalize on these loopholes to promote viral replication, infection and spread. In addition, some cancerous non-immune signaling pathways, for example Ras pathway, favors the antitumor activities of oncolytic retroviruses. Fourthly, tissue-specific miRNA sequences can be inserted into the oncolytic virus genome to prevent its replication in non-tumor cells. Fifthly, key genes unrelated to viral replication in tumor cells can be targeted to enhance viral selectivity. For example, targeting p53 gene to inhibit host cell apoptosis promotes oncolytic viral replication and enhances therapy(Yang, Gu et al. 2021) Tumor Lysis: Oncolytic viruses can lyse tumor cells in two ways namely, direct effect on cell and targeting tumor vasculature to inhibit angiogenesis (Yang, Gu et al. 2021)Tumor cells depend on angiogenesis for growth, supply of nutrients and oxygen, and metastasis (Yadav, Puri et al. 2015). Therefore, oncogenic virus functions to inhibit angiogenesis thereby leading to ischemic death of tumor cells. Also, oncolytic viruses utilize tumor cells as a processing factory and replicate in large quantities. During proliferation, the oncolytic virus functions to inhibit the synthesis of cellular nucleic acids and proteins, which causes cell metabolism dysfunction and ultimately lyses tumor cells. In addition, the nucleus, lysosomes, endoplasmic reticulum, and mitochondria of the infected cells can all be damaged because of the high viral replication volumes. Furthermore, in addition to the effects of massive proliferation in tumor cells, the structures of oncolytic viruses, namely the capsid protein, can induce direct oncolysis (Yang, Gu et al. 2021) Antitumor Immunity: Innate and Adaptive immunity of host can be activated by oncolytic viruses to attack and kill tumor cells. Upon viral entry and replication in

tumor cells, Endoplasmic reticulum and genotoxic stress is induced in tumor cells which lead to the release of key viral elements, Tumor-Associated Antigens (TAAs), Pathogen-Associated Molecular Patterns (PAMPs) and cell-derived Damage-associated molecular patterns (DAMPs). The release of these key elements promotes the production of type 1 interferons and other cytokines. These cytokines recruit and activates the innate immune system namely dendritic cells and natural killer cells to turn the "cold" tumor in "hot tumor', thereby reversing the immunosuppressed state (Yang, Gu et al. 2021). Major Histocompatibility Complex (MHC) molecules function to bind peptide fragments from pathogens and make them visible for recognition by immune system Tcells (Charles A Janeway, Travers et al. 2001)Tumor cells do not express MHC molecules, and this enables them to evade immune surveillance. Upon invasion of tumor cells by oncogenic retroviruses, DC maturation is enhanced, thereby increasing antigen visibility of tumor cells for destruction by immune Tcells. In addition, Oncolytic Viruses function to inhibit tumor recurrence by enacting immune memory in the host (Yang, Gu et al. 2021) Transgene Expression: Oncogenic retroviruses can serve as vectors to express therapeutic genes to kill tumor cells in various ways. The therapeutic genes used are suicide genes, anti-angiogenesis genes, immune related genes, and tumor microenvironment related genes (Yang, Gu et al. 2021). Suicide genes which encode a target sensitivity factor as introduced into the tumor cells and this makes the tumor cells sensitive to the target gene and furthermore induces cell death. Oncolytic viruses can be engineered to continually express anti-angiogenesis genes in the tumor microenvironment to inhibit angiogenesis factors, significantly reduce activation and spread of endothelial cells. Also, inserting cytokine related genes into the oncolytic viral genome can effectively increase local immune inflammatory mediators in the tumor microenvironment, thereby activating the hosts antitumor immune surveillance system and enhances anti-tumor effects of the oncolytic virus. In addition, oncolytic viruses can be engineered with therapeutic genes to eliminate specific factors in the tumor microenvironment while exerting their effect on the tumor cells (Yang, Gu et al. 2021). Based on the illustration of the antitumor potency of oncolytic RNA-based viruses, a variety of these viruses have been used clinically in the treatment of cancers namely, Newcastle disease virus, coxsackievirus, Vesicular Stomatitis Virus, Poliovirus, and Rhino Virus(Yang, Gu et al. 2021). In this review, Poliovirus and its therapeutic efficacy in cancer treatment will be illustrated. **Oncolytic Poliovirus** Polio virus is a positive sense single stranded RNA virus that causes poliomyelitis disease in both animals and humans

(Nomoto 2007, Bowers, Readler et al. 2017, Denniston, Crewdson et al. 2016)Poliovirus is transmitted through the fecal-oral route, nasal and pharyngeal passageways, and it rapidly proliferates within the epithelial cells in this route (Bowers, Readler et al. 2017, Denniston, Crewdson et al. 2016). The virus is a human enterovirus belonging to the Picornaviridae family and it is classified into three serotypes namely, type 1, type 2 and type 3(Nomoto 2007) The polio virion consists of non-enveloped capsid comprised of 60copies each of four viral polypeptides namely, VP1, VP2, VP3 and VP4. The arrangement of the proteins in the capsid creates icosahedral symmetry (Shaffer Catherine 2018) The virion surface consists of canyons which serve as attachment sites for the poliovirus receptors (Denniston, Crewdson et al. 2016) The poliovirus genome contains complex RNA secondary and tertiary structures which are crucial for translation and replication of the viral host genome (Burrill, Westesson et al. 2013). These structures also play a role in packaging the genome and recruiting the hosts' antiviral immune surveillance response for example Poliovirus's natural pathogenicity can be converted into a therapeutic response by replacing its Internal Ribosomal Entry Site (IRES) with that of a less pathogenic virus to inhibit its ability to cause polio after inoculation. A commonly used IRES is that of the human Rhino Virus type 2 which inhibits polios neurovirulence and enables its propagation in tumor cells (Burrill, Westesson et al. 2013, Jammal, Michelin et al. 2018) Receptor Binding and translation of viral genome defines the anti-tumor potential of poliovirus (Jammal, Michelin et al. 2018). Poliovirus has tropism for CD155, the poliovirus receptor in the host. The CD155 belongs to the immunoglobin-like superfamily, and it is commonly expressed in solid tumor cells, myeloid cells, and endothelial cells. Upon expression of CD155 in solid tumors, the tumors become susceptible to poliovirus infection. Upon binding with the CD155 receptors, the innate signaling pathway is activated. CD155 serves as a ligand for activating the DNAM-1 receptor "CD226" expressed in natural killer (NK) cells, in CD8+ T cells and other immune cells, thereby leading to an abundant immune cell invasion (Jammal, Michelin et al. 2018) Antigen Presenting Cells (APCs) such as macrophages and dendritic cells express CD155 and as such, upon PVR trigger, proinflammatory epithelial effects are provoked namely, Tumor Associated Macrophages M1 (TAM) which leads to the production of cytokine interactions between the cells and the extracellular matrix. This interaction results in an immunogenic tumor microenvironment (Jammal, Michelin et al. 2018) In all, PVSRIPO, a recombinant, live attenuated, nonpathogenic oncolytic virus containing the oral poliovirus Sabin type 1 in which the internal ribosomal entry site (IRES) is replaced with the IRES

from human rhinovirus type 2 (HRV2), with potential antineoplastic activity. PVSRIPO leads to tumor regression by recruiting the hosts antitumor immune response and this process follows the phases of cancer immunoediting namely tumor cell elimination by immune system, balancing and regulating genetically unstable tumor cells and immune cells while preventing the escape of variant tumor cells (Jammal, Michelin et al. 2018) **Significance of Study** According to the world health Organization, cancer is a leading cause of global mortality, and its burden is growing (World Health Organization 2022b). Global cancer burden crossed a new threshold in 2021- 20 million people were diagnosed and 10 million died. Furthermore, these numbers have been predicted to continually rise in the decades ahead and yet all these cases can be treated (World Health Organization 2022). This new cancer threshold predicted rise in coming years and the fact that all these cases can be treated, reiterates the importance for a safe and effective treatment approach for all cancers. Genetically and Morphologically tumor cells differ from normal cells. However, cancers arise from genetic modifications of normal cells and these cells may be nearly identical to normal cells, hence, it is difficult to selectively target tumor cells with the current standard cancer therapeutic methods. Furthermore, difficulty arises from the inability to ascertain the specific changes the precancerous cells undergo to become cancerous. Therefore, the ability of oncolytic virus to specifically replicate within a target host cell and stimulate therapeutic response without disrupting normal cells in the immediate environment confers oncolytic virotherapy with a pharmacokinetic advantage over standard cancer therapies and this creates a pathway to evading the predicted continual rise in cancer burden (Denniston, Crewdson et al. 2016). As previously illustrated, oncolytic viruses can recruit the hosts immune surveillance system while replicating within the tumor cells. This immune activation helps to override immune tolerance to current standard therapeutic methods that may have developed. In summary, oncolytic viral immunotherapy helps to limit drug resistance and tolerance (Denniston, Crewdson et al. 2016). Most importantly, Viral immunotherapy is emerging as a promising treatment approach for cancer with recombinant poliovirus at the forefront of this innovative method as highlighted by Food and Drug Administration granting breakthrough therapy Designation. Recombinant Poliovirus has shown promise in clinical trial treatment of a plethora of cancer types of namely human breast cancer, prostate cancer, glioblastoma multiform (GBM) (Carpenter 2019) The next chapter will review clinical studies to illustrate how recombinant poliovirus functions to abrogate immune

suppression, induce immunogenic cancer cell death, enhance antigen presentation, and stimulate activation and survival of immune effector cells.

OVERVIEW

PVSRIPO functions to lyse tumor cells in a time and dose-dependent manner

The induced cell death that occurs following PV Lysis occurs in a time- and dose-dependent manner (Zhand, Hosseini et al. 2018a). Poliovirus infection confers a cytopathic effect which results in change of the morphology and physiology of host cells visible by light and electron microscopy. The virus induces the accumulation of membrane vesicles in large numbers within 3 to 4 hours (Yang, Gu et al. 2021). Poliovirus-induced vesicles predominantly are double-membraned structures with cytoplasmic contents. By 5 or 6 hours, several hours before the lysis of most cells, infected cells begin to lose attachment to the substratum, and the cellular microtubule and intermediate filament networks collapse (TAYLOR, BURGON et al. 2009).

PVSRIPO must be inoculated intratumorally to unfold its range of activities leading up to antitumor immunity. Systemic administration of any tumor-targeting viral agent faces high hurdles, e.g., the blood brain barrier, circulating serum neutralizing antibodies, complement, a failure to reach the intended target and/or reach critical concentration in the intended target. Since all 'oncolytic' viruses are naturally devoid of pathogenicity or attenuated versions of pathogenic precursors, their ability to harm the intended target is reduced (Gromeier, Nair 2018)Several studies have illustrated the time- and dose-dependent manner of tumor cell lysis following an intratumoral administration of PVSRIPO:

A recent study assessed cytotoxic effects and viral propagation of PVSRIPO in prostate and breast cancer xenograft models ((Holl, Brown et al. 2016). They administered PVS-RIPO intratumorally to lyse Prostate (DU145) and breast cancer (SUM149) cell lines. They measured tumor cell lysis after PVSRIPO infection using crystal violet to stain remaining live cells post infection. For this purpose, virus was added to tissue culture wells and infected cultures were stained at the indicated intervals with crystal violet. The Observations indicated that the cancer cell lines were completely lysed within 72 hrs. (SUM149, Figure 2a) or 48 hrs. (DU145, Figure 2b) at all multiplicities of infection (MOI) tested. After cell lysis, the tumor cells were assessed for viral propagation (Figure 2c) over a 72-hour time utilizing a one-step growth curve assay. The findings indicated that PVSRIPO propagates and has potent cytotoxic effects in SUM149/DU145 cells in vitro within hours. Also, the researchers reported a dose dependent manner of tumor cell lysis following an

intratumoral administration of PVSRIPO. They utilized two different rodent tumor xenotransplantation models: orthotopic SUM149 breast cancer and subcutaneous DU145 prostate cancer for the PVSRIPO dose dependence experiments. Once tumors reached a volume of 150-200 mm^3, a single dose of PVSRIPO (10^8 pfu) was injected intratumorally. Tumor growth was monitored, and mice were sacrificed when tumors reached 2000 mm^3 or mice became moribund. A single dose of PVSRIPO was sufficient to delay tumor growth by day-7 post injection, as measured by tumor weight, with ~7-fold (SUM149) and ~3-fold (DU145) decline in weight as compared to mock-treated tumors (Figure 3A, 3B). This finding indicated that low dose level PVSRIPO was sufficient to exert an anti-tumor cytotoxic effect and thus highlighting its potential as a therapeutic agent for treatment of patients with breast and prostate cancer.

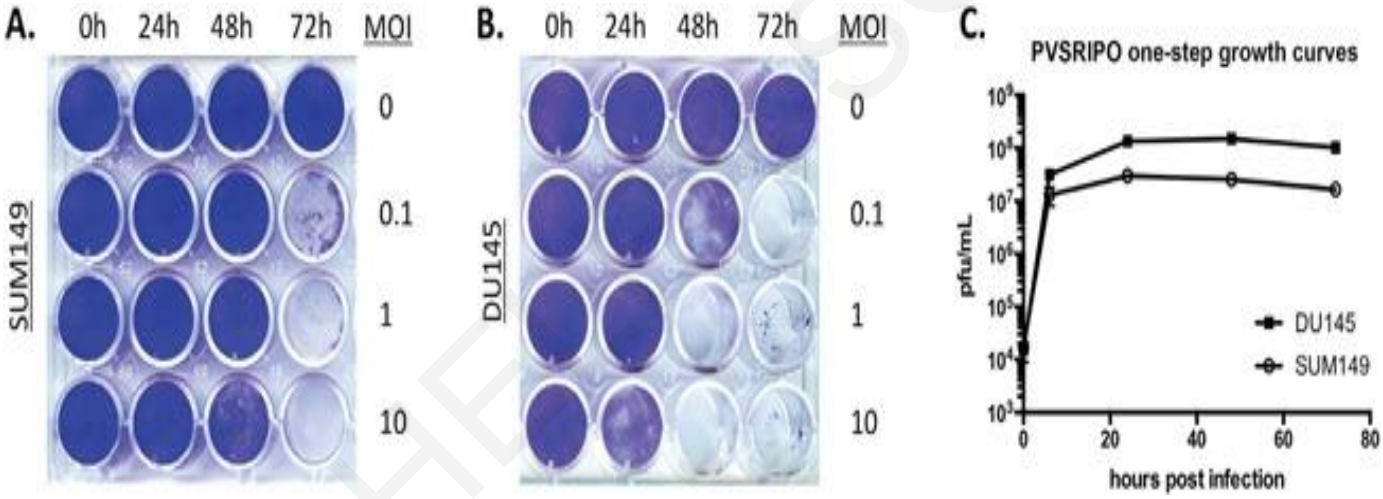


Figure 2: PVSRIPO effectively lyses SUM149 breast cancer cells and DU145 prostate cancer cells

SUM149 (A) and DU145 (B) cells were seeded at a density of 5 × 104 cells per well. Cells were exposed to various concentrations of PVSRIPO (MOI of 0, 0.1, 1 and 10 MOIs) for 0, 24, 48 and 72 hours. (C) PVSRIPO propagation following infection (MOI of 10) of DU145 and SUM149 cells was assessed by plaque assay at the designated time points. Data are representative of at least 3 independent experiments; note that titers in (C) are from an assay distinct from (A) and (B) adapted from figure 1 from (Holl, Brown et al. 2016).

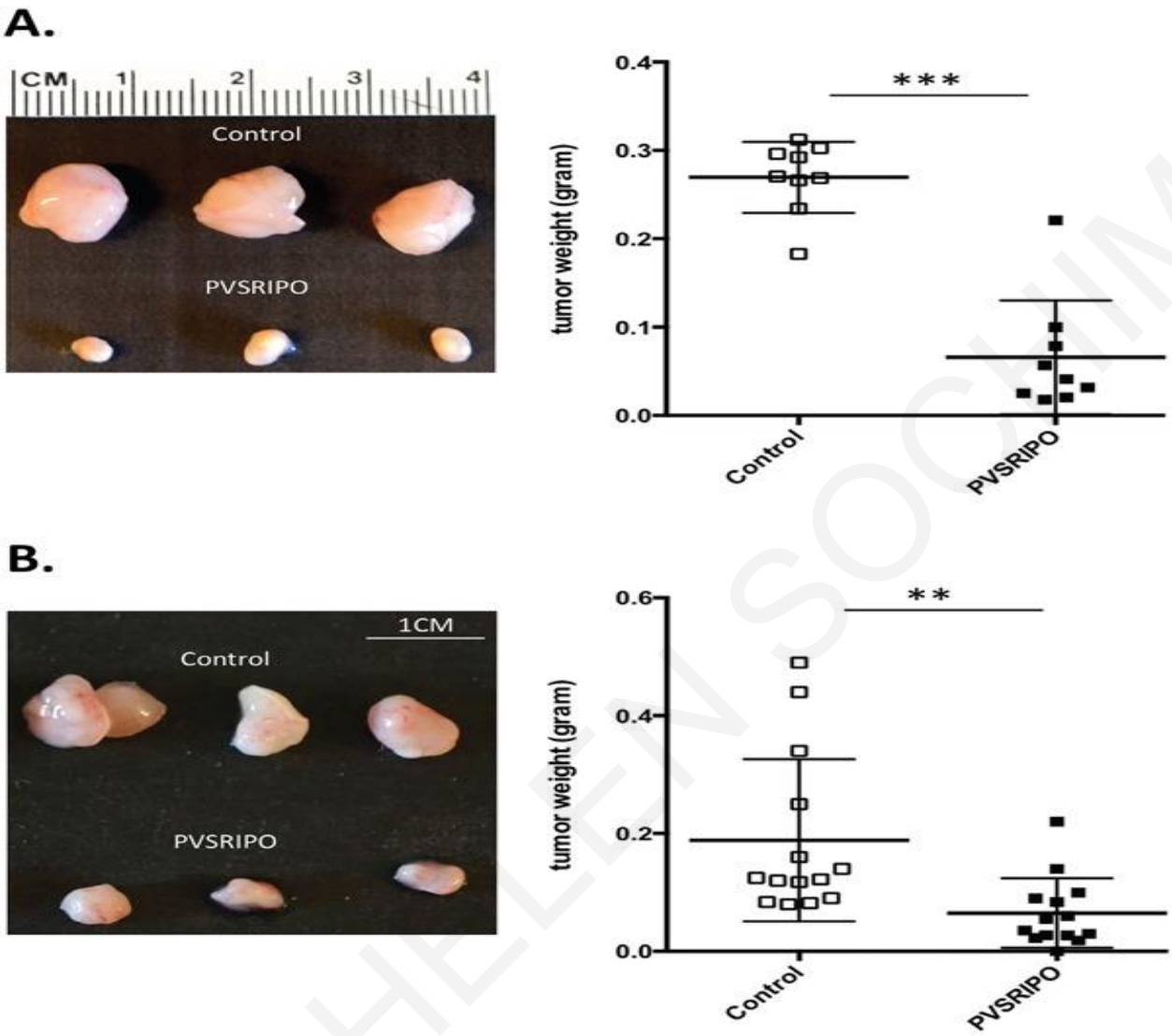


Figure 3: Single intratumoral administration of PVSRIPO results in tumor regression in SUM149 and DU145 xenografts.

SUM149 and DU145 cells were implanted in athymic nu/nu mice. Tumors were injected with 108 pfu of PVSRIPO when they reached 150-200 mm^3. Tumors were collected at 7 days post injection and size and weight were assessed. (A) SUM149 tumor data are representative of five different experiments and a total of 25 mice per group. Graph represents tumor weights (grams) from one representative experiment. (B) DU145 tumor data are representative of five different experiments and a total of 25 mice per group. Graph represents tumor weights (grams) from one representative experiment. ***p<0.00. Adapted from figure 2 from (Holl, Brown et al. 2016).

A similar study was conducted to assess viral propagation and cytotoxicity in melanoma cells (Walton, Brown et al. 2018). PVSRIPO was intratumorally administered to lyse human melanoma cell lines and assays were conducted to assess viral propagation and cytotoxicity over time. The findings indicated 100% tumor cell killing by 36h post infection (hpi) (DM440, DM443) or 48 hpi (DM6) and these results were further supported by similar findings from the immunoblotting assay. The researchers also infected the DM440 cells at three different MOIs (0.1, 0.01 and 0.001) and tracked viral cytopathogenicity and cytotoxicity over time. The findings showed that after PVSRIPO attached to cell receptors at MOIs of ~0.0006 and ~0.0004 for the intended MOIs of 0.1 and 0.001 were observed respectively but could not recover virus from DM440 cells infected at an MOIs of 0.001. However, the infection of these cells with PVSRIPO revealed significant viral translation and host cytotoxicity at all tested MOIs. Also, the findings indicated an independence of input dose, at least at MOIs of ≥0.001.

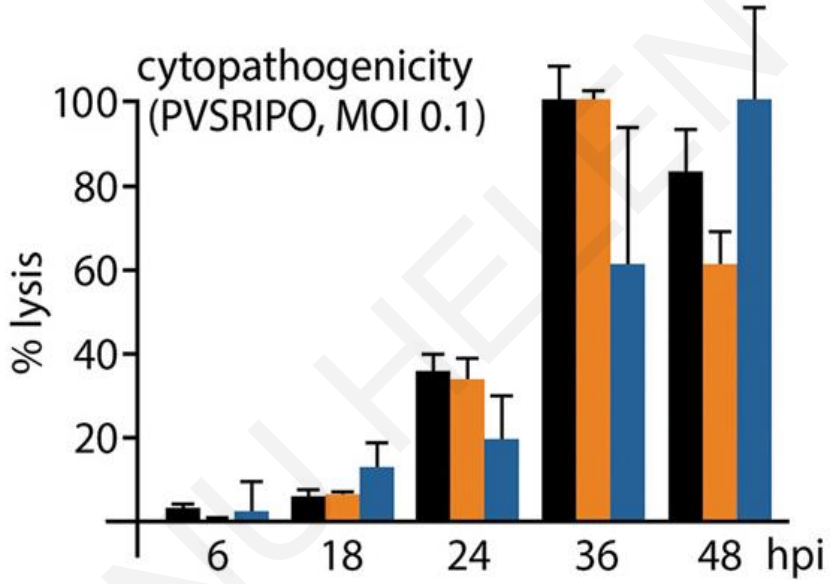


Figure 4A: PVSRIPO induces 100% cell death at 36h or 48h post infection in (DM440, DM443) and (DM6) melanoma cells respectively. Adapted from figure 1E from (Walton, Brown et al. 2018).

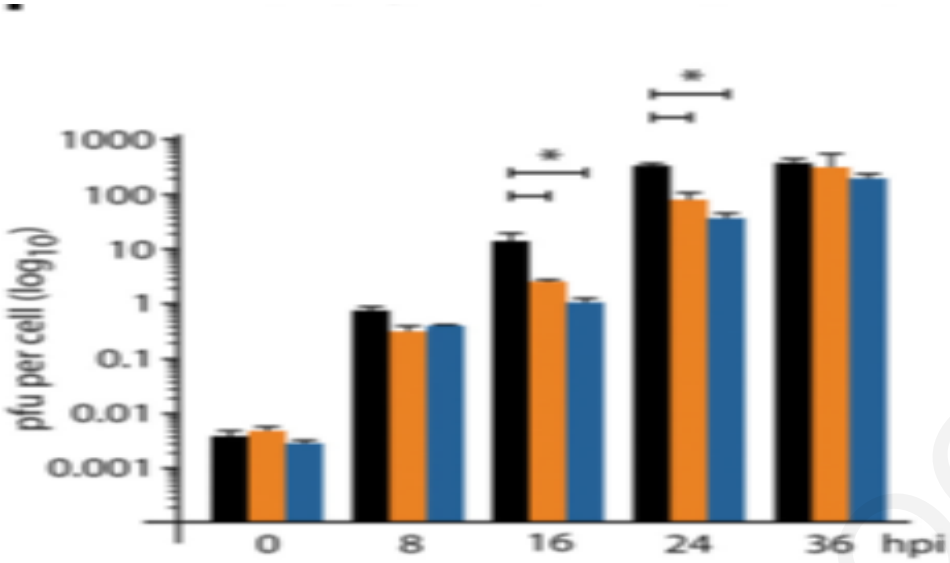


Figure 4B: Multistep growth curves of PVSRIPO in melanoma cells (n=3; p <0.0P, for results with DM440 versus those with DM443 and DM6 cells at 16 and 24 hpi). Adapted from figure 1F from (Walton, Brown et al. 2018).

A recent study evaluated the therapeutic potential of PVSRIPO on human CRC cells and elucidated its mechanism of inducing apoptosis (Zhand, Hosseini et al. 2018b). Normal and CRC cell lines were suspended in a 96-well micro titer plate and incubated at 37°C in a humidified atmosphere containing 5% CO2 for 24 and 48 hours, during which the activity of caspase-8 and caspase-9 was assayed by FLICE/caspase-8 colorimetric assay kit. CRC cells were exposed to PVSRIPO for 24 hours (at the same titer used for apoptosis assay), and the quantity of viral yields (cells associated and extracellular) was determined using TaqMan PCR. The results indicated that PVSRIPO induced cell death in a time- and dose-dependent manner in human CRC cells. Also, a monolayer of ~106 cells were infected with PVSRIPO with appropriate MOIs. Results showed that PV induced the activation of caspase-8 and caspase-9 in a time- and dose-dependent manner (Table 1). In the studied MOIs, cancer cell lines rather than normal FHC cell lines show the highest caspase activity in both extrinsic and intrinsic pathways.

Furthermore, they assessed the viral load of virus after virotherapy to be confident that the observed apoptosis is directly for virus replication and they confirmed that the viral load was directly correlated with the infected cell caspase activity level, supporting the fact that productive viral infection can be associated with the cell death. The polio viral load was significantly

associated with apoptosis via extrinsic (R=0.945, P=0.0001) and intrinsic (R=0.756, P=0.001) pathways, hence supporting existing literature that tumor cell death via apoptosis depends on the PVSRIPO replication.

Table 1: Viral load of PV in all normal and cancerous cell lines after 24 hours exposure

Adapted from table 3 from (Zhand, Hosseini et al. 2018b).

Cell line	Viral load at MOI=1	Viral load at MOI=0.5	Viral load at MOI=0.25
HCT116	4.00E+05	3.07E+05	2.00E+05
SW480	3.80E+05	2.98E+05	1.46E+05
HT-29	2.08E+05	5.97E+04	4.70E+04
CaCo-2	5.30E+04	3.76E+04	3.66E+04
FHC	5.27E+04	4.84E+04	3.076E+03

Abbreviations: FHC, fetal human colon; MOI, multiplicity of infection; PV, poliovirus.

Another recent study profiled the dose dependence and toxicity level of PVSRIPO in 61 adult patients with WHO grade IV malignant glioma ((Desjardins, Gromeier et al. 2018a). PVSRIPO was intratumorally administered by means of convection-enhanced delivery over a period of 6.5 hours at a rate of 500µl per hour. A total of seven doses were evaluated ranging between 107 and 1010 50% tissue-culture infectious doses (TCID50), first in a dose-escalation phase and then in a dose-expansion phase. The patients were continuously monitored for toxic effects during the study. The findings indicated that of all the patients who received PVSRIPO, 69% had a grade 1 or 2 event that was attributed to PVSRIPO as their most severe adverse event. In the dose-expansion phase, only 19% of the patients had a PVSRIPO-related adverse event of grade 3 or higher. PVSRIPO infusion was not associated with evidence of encephalomyelitis, poliomyelitis, meningitis, or systemic autoimmune reactions in any patient at any dose level. However, two deaths occurred during the trial, one patient (who received dose level −2) had a seizure related to cerebral edema that was related to autopsy-confirmed tumor progression at 4.8 months after the PVSRIPO infusion and the second patient (who received dose level −1) died 10.5 months after the PVSRIPO infusion from complications of an intracranial hemorrhage while receiving anticoagulation and bevacizumab; this event was attributed to bevacizumab.

Recently, an open-label phase 1 trial of intratumorally administered PVSRIPO in unresectable stage IIIB, IIIC, or IV melanoma patients was conducted to assess the dose-limiting toxicities (DLTs) of PVSRIPO ((Beasley, Nair et al. 2021). Twelve patients in four cohorts received a total of 1, 2, 3 injections of PVSRIPO, 21 days between injections. The findings indicated all adverse events (AEs) were either at grade (G) 1 or G2 (G1 pruritus most common at 58%); all but two PVSRIPO-treatment related AEs were localized to the injected or adjacent lesions. In summary, no serious adverse events or dose-limiting toxicities were reported and most of the patients achieved an optimum immune-related response thereby reaffirming the previous reports of low toxicity levels and therapeutic efficacy of PVSRIPO.

PVSRIPO's proinflammatory effect stimulates a T-cell mediated antitumor response

The multitude of different interactions that arise between tumor cells and the tumor microenvironment influences the development of cancer. As earlier illustrated, the immune system is implicated in these interactions that occur between the tumor cells and TME. Tumors exploit the immune system to recruit tumor-associated myeloid cells and immunoregulatory leukocytes which in turn induces immunosuppressed and inflamed TME. To reverse this immunosuppressed state that promotes tumor progression, T cells need to be activated and upon their activation, the innate and adaptive immune signals are recruited to the tumor site (Chang, Beatty 2020).

As earlier illustrated, the intratumoral administration of PVSRIPO induces a cytotoxic effect in vitro and in vivo. Viral propagation and cell lysis stimulate a T-cell mediated antitumor response which involves the recruitment of the innate and adaptive immune system. The tandem events that lead to the recruitment of the innate and adaptive immune system is termed "cancer-immunity cycle" and it consists of six basic steps namely, (i) uptake of tumor antigens, (ii) cross-presentation of tumor antigens to T cells by antigen presenting cells (APCs), (iii) priming and activation of naïve T cells (Chang, Beatty 2020), (iv) trafficking of activated T cells to the tumor microenvironment (TME), (v) T cell infiltration into tumors and (vi) elimination of malignant cells by activated cytotoxic T cells (Chang, Beatty 2020).

In summary, these steps can be divided into two major phases: T-cell priming phase (comprising of step i – iv) and the effector phases (step v – vi) figure 5. These two phases of the "cancer-immunity cycle" are regulated by innate immunity in such a way that the generation of tumor antigen-specific T cells reflects a coordinated interaction between the innate and adaptive immune system. Adaptive immunity is also defined by the cancer-immunity cycle (Chang, Beatty 2020).

To achieve effective elimination of tumor cells, it is important that the stimulated tumor antigen-specific T cells go across the TME into the tumor bed, thereby infiltrating it. Hence, sufficient intratumoral T cell infiltration is a prerequisite for T cell effector activity.

The TME constituent can exhibit an incredible degree of spatial heterogeneity, and the composition of the immune infiltrate may differ markedly both within and across tumors. T cell-infiltrated tumors are characterized by a robust infiltration of $CD8^+$ T cells which function as strong indicators of improved prognostic and survival outcomes across several cancer types. On the molecular level, the transcriptomic profiling of T cell-rich tumors indicates that immunoreactive tumor beds upregulate the expression of IFN signaling genes. On the contrary, T cell 'cold' tumors, which correlate with poor patient survival, are marked by an abundance of Tregs and immunosuppressive myeloid cells. These observations imply that the innate immune system regulates the phenotypic plasticity of the TME and may dictate the formation of T cell 'hot' and 'cold' tumors (Chang, Beatty 2020).

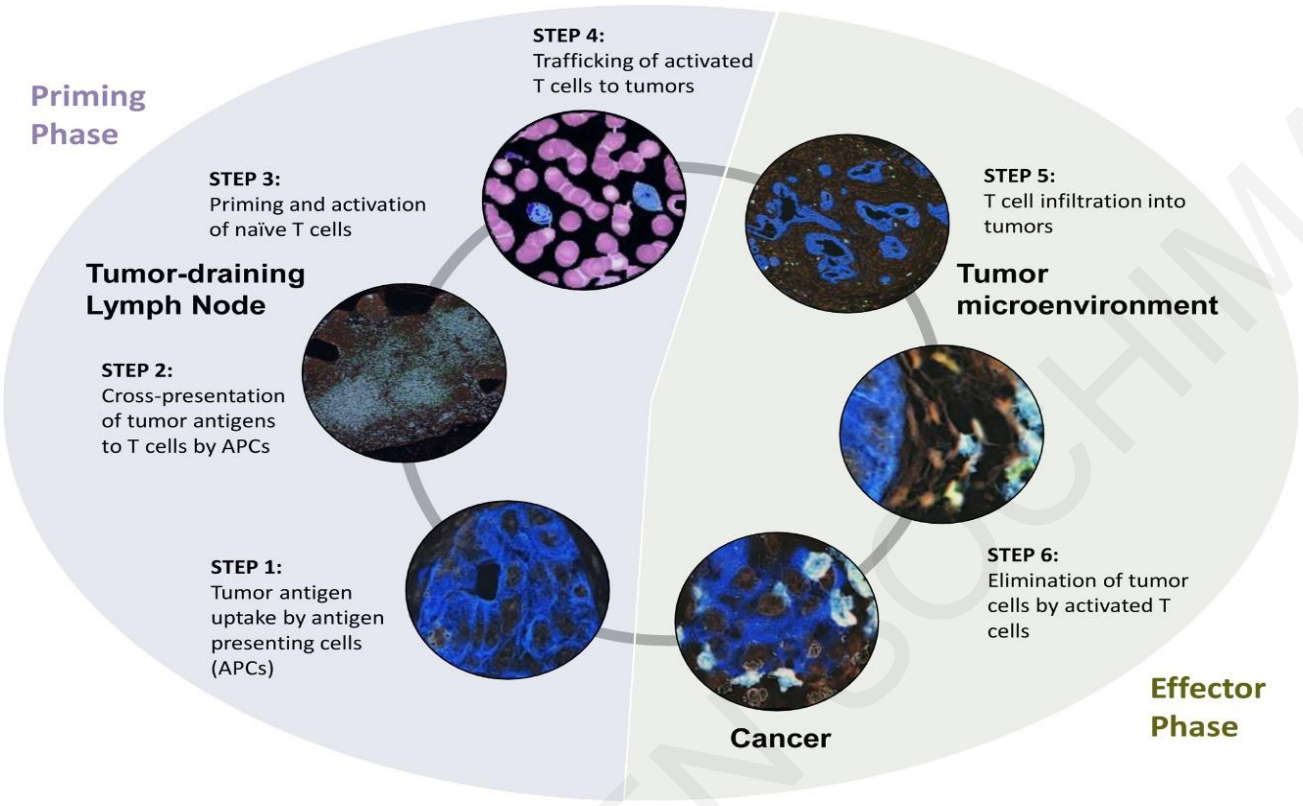


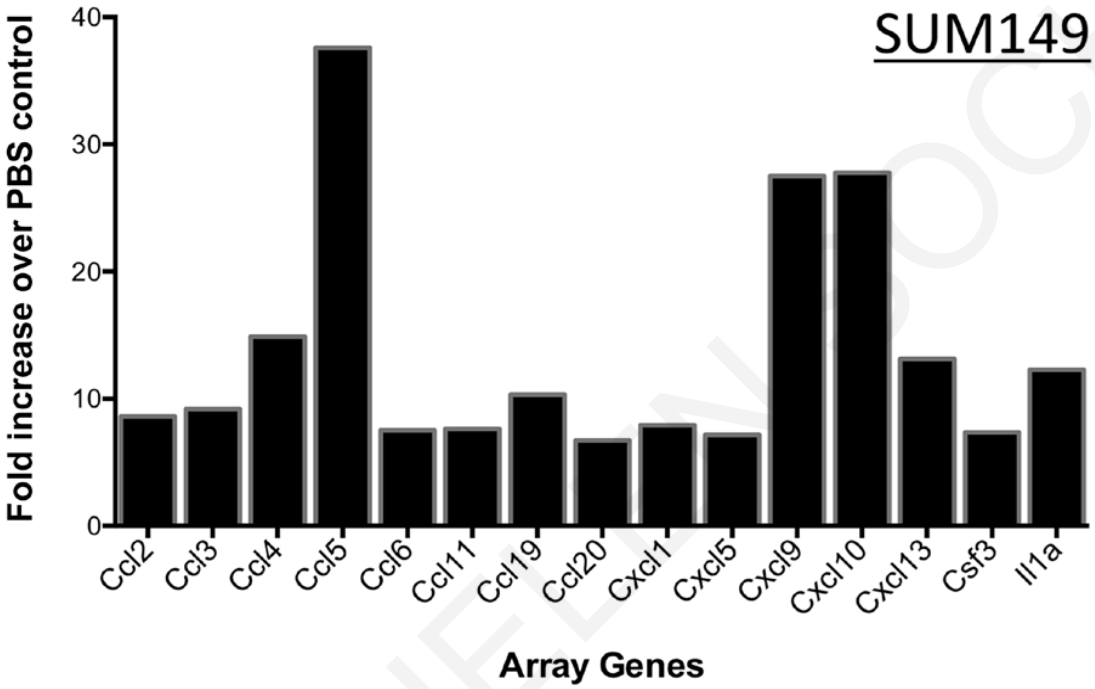
Figure 5: **Innate immunity in cancer is defined by T-cell priming (comprising of step 1- 4) and effector phases (step 5 - 6).**

As already elucidated, the T-cell Priming Phase encompasses (1) the release of tumor antigens which are captured by antigen presenting cells (APCs), (2) cross-presentation of tumor antigens to T cells by APCs in tumor-draining lymph nodes, (3) priming and activation of naïve T cells by antigen-loaded APCs, and (4) trafficking of T cells from lymph node into the blood stream and to tumors. While the Effector Phase encompasses T cell infiltration into tumors and T cell recognition and elimination of tumor cells, thus accounting for step 5 and step 6 respectively. Adapted from figure 2 from (Chang, Beatty 2020).

Immune response stimulated by PVS(RIPO) was investigated using human breast and prostate cancer xenograft models in mice (Holl, Brown et al. 2016). The researchers harvested mRNA 24 hours post- PVSRIPO or PBS administration and analyzed for presence of murine pro-inflammatory cytokines using gene arrays. They observed a significant upregulation or expression of pro-inflammatory chemokines and cytokines in PVSRIPO-treated tumors as compared to the PBS-treated controls. This powerful upregulation of proinflammatory chemokines coincided with

myeloid cell infiltration in tumors for example basophil, eosinophil, and neutrophil cells, were reportedly induced in both tumor cell lines (CDll1+Ly6C+Ly6G+ neutrophils). Intriguingly, CCL5 and CXCL10, two chemokines that were significantly upregulated, are noted to be involved in the recruitment of T-cells which in turn stimulates an antitumor response.

A.



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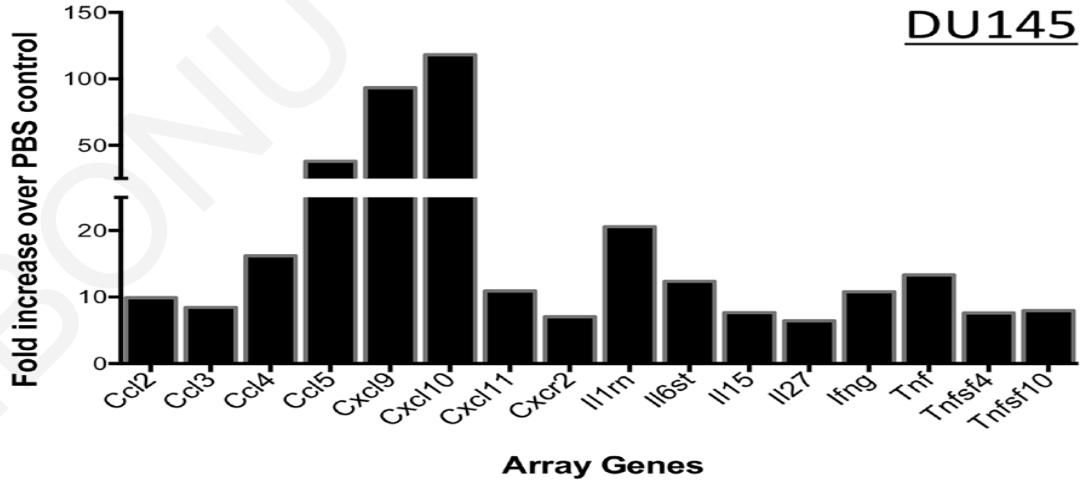

Figure 6: PVSRIPO upregulates transcripts encoding for innate immune-related gene products in SUM149 and DU145 xenografts. SUM149 and DU145 cells were implanted in athymic nu/nu mice. Tumors were injected with 108 pfu of PVSRIPO when they reached 150-200 mm3. Tumors were collected at 24 hours post injection and innate immune-related transcript abundance was assessed. Data are representative of two different experiments and a total of 6 mice per group. Only changes of ≥8-fold are reported. Adapted from figure 3 from ((Holl, Brown et al. 2016).

The observed rapid upregulation of chemokines and cytokines in PVSRIPO treated cells hinted that innate immune cell infiltration will ensure. Based on this, they conducted immunoblot and ELISA assays on the tumor lysate extract from both SUM149 and DU145 cells to assess for pro-inflammatory innate response at 24 and 48 hrs post PVSRIPO treatment.

The findings from the immunoblot assay indicated a significant increased intratumoral expression of iNOS, an enzyme responsible for producing NO; myeloperoxidase, an enzyme predominately expressed by neutrophils that catalyzes the production of cytotoxic free radicals; p-Stat1 (Y701), phosphorylated downstream of both type-I and -II IFN signaling; Stat1, which is induced by its own phosphorylation; and higher H2O2 concentration (Figure 7b). Also, the ELISA assay indicated presence of TNF-α and IFN-β in both tumor cell lines and TNF-α and IFN-β, are intricately involved in the direct or indirect elimination of tumor cells (Figure 8). In summary, these findings indicate that PVSRIPO intratumoral administration induces a cytopathic effect and recruits' innate immune system to eliminate tumor cells.

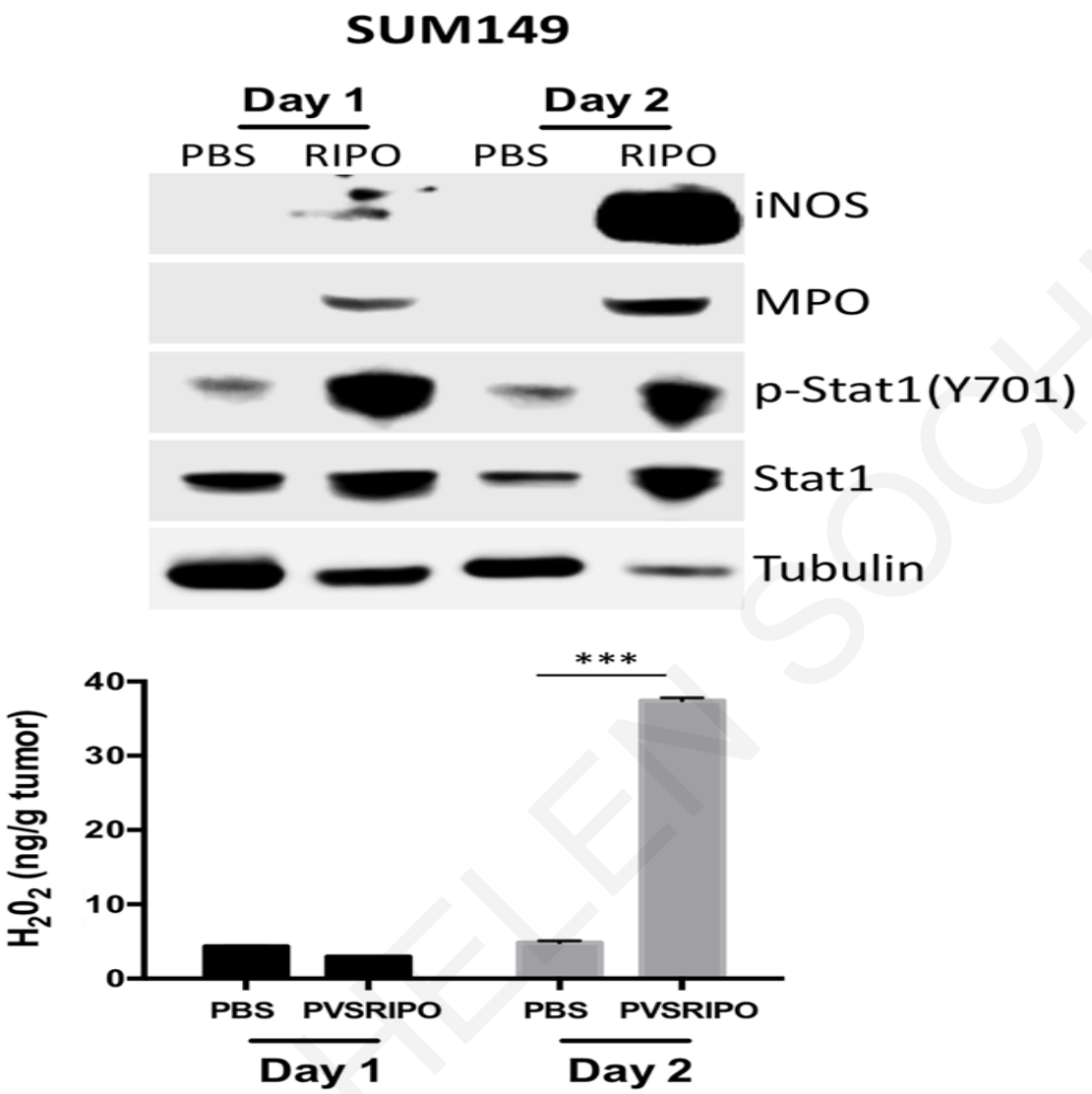

Figure 9: Immunoblot of tumor lysate extract

SUM149 tumor homogenates from mock (PBS) or PVSRIPO-treated mice were tested (7A, top) for markers of neutrophil and innate immune cell inflammation by immunoblot; (7B, bottom) for the presence of H2O, adapted figure 5C from (Holl, Brown et al. 2016).

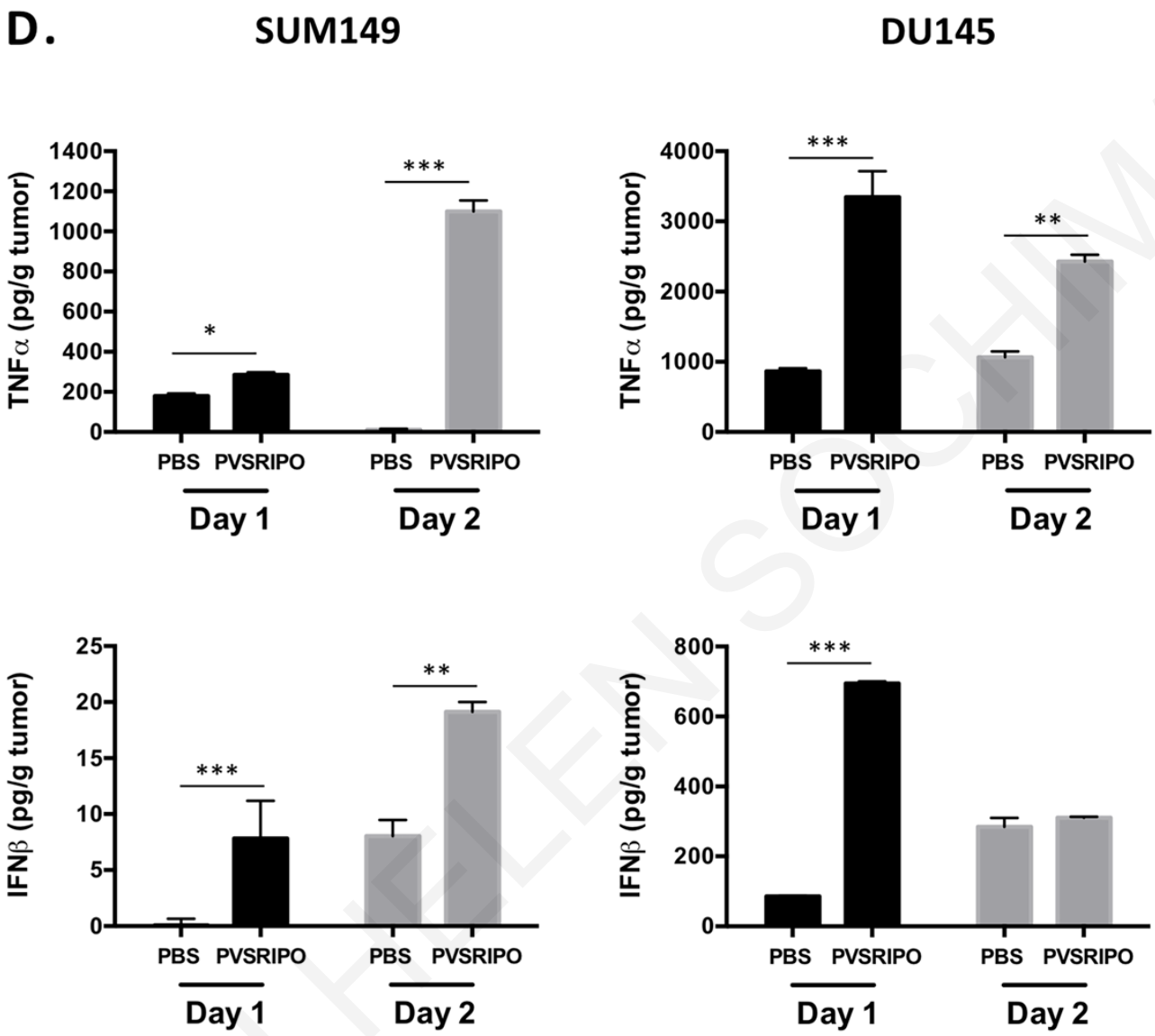

Figure 8: ELISA assay of tumor lysate extract indicating presence of TNF-α and IFN-β and its association with tumor elimination, adapted from figure 5D from ((Holl, Brown et al. 2016)

A similar study revealed active PVS-RIPO replication in vivo and a vigorous host response which induced complete tumor elimination resulting in scar formation (Dobrikova, Broadt et al. 2008). Bilateral HTB-15 xenografts were implanted in the flanks of 12 athymic Balb/c mice. Ten of these animals were inoculated bilaterally with PVSRIPO at a tissue culture infectious dose ($TCID_{50}$) of 1 x 10^8 administered intratumorally while 2 mice received bilateral inoculations of vehicle. Ten days post PVS-RIPO administration, the median xenograft size had shrunk by 45%. Six of these PVS-RIPO treated animals were euthanized and utilized for virus recovery while four were retained and observed for tumor regression. In the two vehicle treated animals, the xenografts continued to progress throughout the observation interval. In the first six animals, a histopathology analysis of the xenografts 10days post PVS-RIPO inoculation revealed advanced tumor lysis and although a small middle area of the xenograft retained the dense hypercellular architecture with the vehicle treated animals, the bulk of the tumor no longer had the appearance of proliferation. The other four PVSRIPO intratumorally administered animals showed tumor regression and by the end of the study HTB-15 xenograft had almost obliterated in all animals. Most of the tumor mass had significantly reduced cell content and was diffusely invaded by infiltrates. After 28 days (about 4 weeks) post PVS-RIPO intratumoral administration., xenograft was replaced by scar and the base of the scar composed of collapsed tissue containing profuse infiltrates.

PVSRIPO increases survival rate and functions to induce antitumoral memory in host cell

As earlier illustrated, PVSRIPO propagation recruits the innate and adaptive immune system to eliminate tumor cells in two phases namely, the T cell priming phase and the effector phase. In the effector phase, neutrophils and macrophages recruit antigen presenting cells and adaptive immune cells to generate anti-tumor memory, eliminate tumors and increase survival rate (Gromeier, Nair 2018). PVSRIPO, as a positive stranded RNA virus is unable to chronically persist, except in rare instances of persistence in the enteric tract of patients with severe inherited/acquired immune deficiencies, and its presence and spread in tumors is limited and diminished upon elimination of tumor cells hence conferring it with an anti-tumoral memory and increasing survival (Holl, Brown et al. 2016).

An evaluation of the anti-tumor effects of intratumorally administered PVSRIPO in SUM149 and DU145 xenografts indicated a suppressed tumor growth and an overall improved survival rate of the PVSRIPO treated mice when compared with PB-treated mice (Holl, Brown et al. 2016). The researchers measured tumor volume post PVSRIPO intratumoral administration in mice bearing SUM149 and DU145 tumor. They observed that the cohort of the PVSRIPO-treated mice in both cancer models achieved complete tumor regression and extended overall survival. Furthermore, they assessed for PVSRIPO persistence in SUM149 and DU145 cells, 1-week post PVSRIPO treatment. The findings indicated the presence of PVSRIPO in both SUM149 and DU145, however tumor-associated antigens did not increase overtime in SUM149, and they totally declined in DU145 tumors.

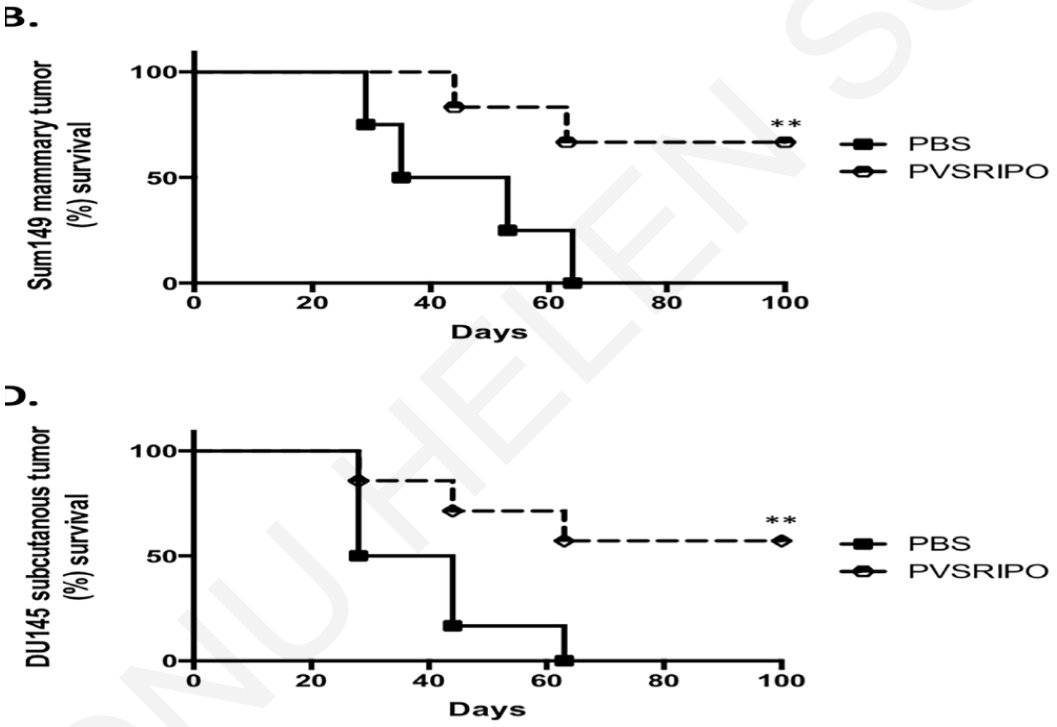

Figure 9: Single intratumoral administration of PVSRIPO in SUM149 and DU145 xenografts results in tumor regression and survival benefit. SUM149 and DU145 cells were implanted in athymic nu/nu mice. Tumors were injected with 108 pfu of PVSRIPO when they reached 150-200 mm^3. Tumor growth was monitored daily, and mice were sacrificed when tumors reached 2000 mm^3. Data are representative of 3 different experiments (n=30). SUM149 and DU145 tumor growth 7 days post PVRIPO injection and overall survival up to 100 days. Adapted figure 6C and 6D from (Holl, Brown et al. 2016)

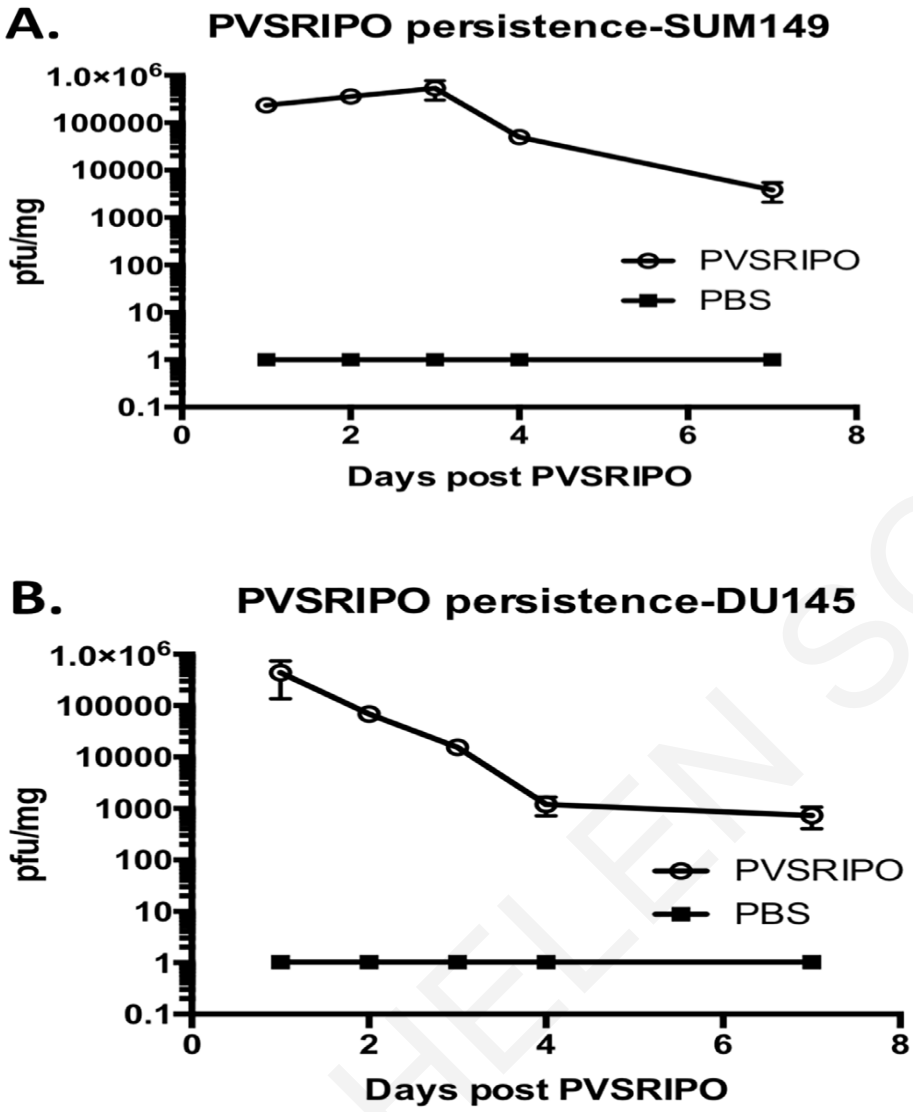

Figure 10: PVSRIPO persistence in SUM149 and DU145 xenografts following single intratumoral injection. SUM149 **(A)** and DU145 **(B)** cells were implanted in athymic nu/nu mice. Tumors were injected with 108 pfu of PVSRIPO when they reached 150-200 mm3. Tumors were isolated at 1, 2, 3, 4- and 7-days post virus inoculation. Pfu per mg of tumor were determined by plaque assay and plotted; data are representative of two independent experiments (n=30). Adapted figure 7A and 7B from (Holl, Brown et al. 2016).

The clinical trial conducted by (Desjardins, Gromeier et al. 2018), indicated an overall increased survival rate of 61 adult patients with WHO grade IV malignant glioma compared with a historical control group following an intratumoral infusion of PVSRIPO. All but 1 patient in the historical control group are known to have died (the remaining patient was lost to follow-up). The median overall survival among all 61 patients who received PVSRIPO was 12.5 months (95% CI, 9.9 to 15.2), which was longer than the 11.3 months (95% CI, 9.8 to 12.5) in the historical control group

and the 6.6 months in the Novo-TTF-100A treatment group. However, overall survival among the patients who received PVSRIPO reached a plateau beginning at 24 months, with the overall survival rate being 21% (95% CI, 11 to 33) at 24 months and 36 months, whereas overall survival in the historical control group continued to decline, with overall survival rates of 14% (95% CI, 8 to 21) at 24 months and 4% (95% CI, 1 to 9) at 36 months.

Advantage of PVSRIPO over other Oncolytic Viruses

A study was conducted to compare PVSRIPO to a distant relative, EMCV, with similar IFN sensitivity in human host cells (Dobrikova, Broadt et al. 2008). The researchers generated stable MDA5-depleted DM440 and DM6 cells with lentiviral short hairpin RNA (shRNA) transduction. The findings indicated that MDA5 depletion had no significant effect on PVSRIPO translation at an MOI of 0.1. Also, there was an increase in viral propagation in MDA5-depleted cells, with viral titers elevated 6-fold at 18 hpi. Similarly, MDA5 depletion abolished IFN-release from DM6 cells but had no significant effect on PVSRIPO translation and propagation. In sharp contrast, MDA5 depletion in DM440 cells had a profound effect on EMCV. MDA5-depleted cells permitted earlier expression of viral 3Dpol than the parental line, as well as accelerated lytic cell death as measured by global loss of cellular proteins. EMCV 3Dpol levels were higher in wild-type than in MDA5-depleted cells at 24 hpi because the infected MDA5-depleted cells were already destroyed at this interval. MDA5 depletion elevated EMCV propagation 240-fold at 18 hpi. These results give evidence to the potency of the rapid viral propagation of PVSRIPO. In addition, the lack of an effect of MDA5 depletion on PVSRIPO translation and growth resonates with an earlier illustration of an unimpeded PVSRIPO spread/propagation at extremely low MOIs in type I IFN-competent cultures.

DISCUSSION

PVSRIPO has been utilized successfully in a plethora of cancer therapeutic research namely GBM, breast and prostate cancer; and melanoma (Holl, Brown et al. 2016, Beasley, Nair et al. 2021, Desjardins, Gromeier et al. 2018, Zhand, Hosseini et al. 2018). This review has highlighted and illustrated the core mechanisms by which PVSRIPO functions in these named cancers. According to the world health organization, the most common causes of cancer death in 2020 were: lung (1.80 million deaths); colon and rectum (916 000 deaths); liver (830 000 deaths); stomach (769 000 deaths); and breast (685 000 deaths) (World Health Organization 2022). The primary studies discussed in this review have provided a rationale and served as baseline data for further research utilizing PVSRIPO in the treatment of these cancers that already have an existing high mortality and morbidity rate. Also, the WHO report stated that some of these most common cancer types, namely, breast cancer, cervical cancer, oral cancer, and colorectal cancer, have high cure probabilities even when cancerous cells have metastasized to other areas of the body provided an appropriate treatment is available (World Health Organization 2022). The primary objective of this review is to illustrate the potential of oncolytic poliovirus (PVSRIPO) as a therapeutic agent for these cancers. PVS-RIPO a derivate of Picornavirus, has a small virion size (~30 nm in diameter) which confers it with an oncolytic advantage. Furthermore, Picornaviruses have an attribute of rapid replication cycle from 5 to 10 hours and this attribute can enable PVSRIPO to reduce tumor size more effectively than a slower replicating virus (Zhand, Hosseini et al. 2018). The primary studies in this review have illustrated PVSRIPO's rapid replicative attribute and how this attribute confers cytotoxic therapeutic potency to the host cell. The findings from these studies have indicated that PVSRIPO stimulates cytotoxicity upon intratumoral administration at extremely low MOIs (multiplicities of infection) (Holl, Brown et al. 2016, Walton, Brown et al. 2018, Brown, Dobrikova et al. 2014) A single dose of intratumorally administered PVSRIPO (10^8 pfu) is potent to delay tumor growth with a decline in tumor weight (Walton, Brown et al. 2018, Holl, Brown et al. 2016). Cancer cell lines were completely lysed within 48 hours or 72 hours. However, despite this illustrated cytotoxic mechanistic attribute of PVS-RIPO, there is an impending drawback because tumors have diverse compartments which are infected with vastly different outcomes. PVS-RIPO infection is associated with profuse release of inflammatory responses towards each compartment is not well elucidated (Walton, Brown et al. 2018). This review earlier mentioned the mechanisms by which

oncolytic viruses induce apoptosis. The findings from the study conducted by (Zhand, Hosseini et al. 2018) indicated that OPV possesses an inherent capacity to induce apoptosis in CRC cell lines in vitro and has good potential for use in in vivo studies. Furthermore, the researchers also reported that PV infection can trigger apoptosis in neuroblastoma and bone and soft tissue sarcoma cells expressing CD155, as shown by DNA fragmentation, activation of effector caspase, mitochondrial dysfunction, and TUNNEL assay. Similarly, as reported by other researchers in this review, PV infection leads to programmed cell death in CRC cell lines in a time- and dose-dependent manner (Zhand, Hosseini et al. 2018) Preventing side effect while targeting cancer has been a primary source of concern in cancer research and therapy. As earlier illustrated, PVSRIPO binds to nectin-like molecule 5 (Necl-5) or CD155, a poliovirus receptor (PVR), found on distinct types of cancers and this attribute allows the virus to have a high binding affinity to tumor cells (Jindal, Lee et al. 2018). PVSRIPO targets tumor cells without affecting non-tumor or surrounding cells. The first step in initiating host immune response is the ability for PVSRIPO to target the lone PVR, Necl-5 or CD155 receptor (Jindal, Lee et al. 2018). Upon viral entry and binding to CD155, the PVSRIPO capsid undergoes a conformational expansion, extruding the myristoylated capsid protein VP4 and externalizing the N-terminus of VP1. This leads to the disintegration of the viral capsid and mediated transmembrane cytosolic transfer of the viral RNA genome. Upon entry into the cytoplasm, viral RNA genomes are immediately translated, and then they initiate the process of oncolytic efficacy which involves the recruitment of the immune system to induce tumor cell death. PVSRIPO recruits immune surveillance responses through a two-pronged mechanism: infecting and directly lysing cancer cells while simultaneously activating inflammatory anti-viral pathways (Brown, Gromeier 2015)

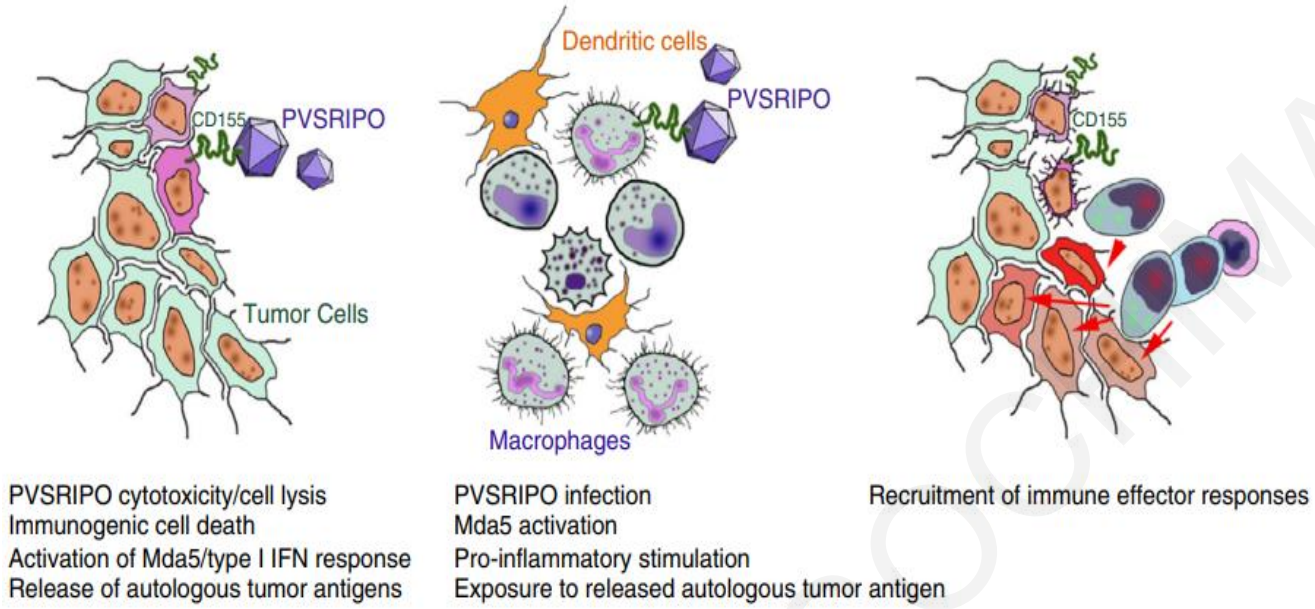

Figure 11: Model of PVSRIPO oncolytic immunotherapy mechanisms. A combination of (left) direct viral tumor cytotoxicity and engagement of Mda5/the anti-viral IFN response; and (middle) PVSRIPO non-lethal infection and pro-inflammatory stimulation of tumor-associated macrophages (TAM) and/or dendritic cells; (right) recruits immune effector responses directed against tumor neo-antigens. A clinical study in this review utilized CRC viral therapy to illustrate how PVS-RIPO specifically targeted only CRC cells. Furthermore, the researchers reported that this effective targeting of only CRC cells was because they expressed more PVR (CD155) than normal colon cells (Zhand, Hosseini et al. 2018) Despite the availability of a variety of anticancer therapeutic methods, only anticancer therapies that engage the innate and adaptive immune system and promote lasting systemic immune surveillance against tumors are the most effective at attaining total elimination of cancer (Holl, Brown et al. 2016). Tumors are characterized by an extensive influx of innate immune cells such as macrophages, mast cells and plasma proteins. These innate immune cells function to promote tumor growth through angiogenesis as previously mentioned. These angiogenic tumor growth is enabled due to the tumors ability to advert the effects of antitumor cytotoxic-T cells (Jindal, Lee et al. 2018). This review has illustrated that upon intratumoral administration of PVSRIPO, the immunosuppressed state is reverted, and the cytotoxic-T cells are recruited to attack and lyse the tumor cells. The study by (Holl, Brown et al. 2016), reported a significant upregulation of pro-inflammatory chemokines and cytokines in PVSRIPO-treated DU145 and SUM149 tumors and this coincided with myeloid cell infiltration in tumors for example basophil, eosinophil, and neutrophil cells, were reportedly induced in both

tumor cell lines (CDll1+Ly6C+Ly6G+ neutrophils). It was also illustrated that the potent induction of an array of chemokines and chemokine receptors within the tumor microenvironment explains the recruitment of innate immune cells, including neutrophils, observed as early as 2 days post-PVSRIPO treatment and persisting to 7 days posttreatment. Notably, CCL5 and CXCL10, chemokines responsible for T cell recruitment were induced. Also, another research work reviewed in this study also reported similar findings of the presence of diffused cytotoxic infiltrates in diminishing tumor cell mass post-PVSRIPO administration (Dobrikova, Broadt et al. 2008) However, the innate immune mechanisms involved remains unexplored (Jindal, Lee et al. 2018) PVSRIPO can be safely used in combination with other systemic therapies. The findings from a reviewed clinical study conducted by (Desjardins, Gromeier et al. 2018), Clinical and radiographic responses were observed after the first cycle of chemotherapy administered, lomustine, for tumor progression that occurred 7 months post-PVSRIPO administration (Fig. 10). Early preliminary tests of immune-cell frequencies in the periphery illustrated a reduction of immunosuppressive regulatory T cells with the recruitment of effector T cells at the nadir (4 weeks after the administration of lomustine). However, potential beneficial effects stemming from such events can be probably lost with standard multicycle chemotherapy, because sustained lymphodepletion dampens immune capacity (Desjardins, Gromeier et al. 2018)

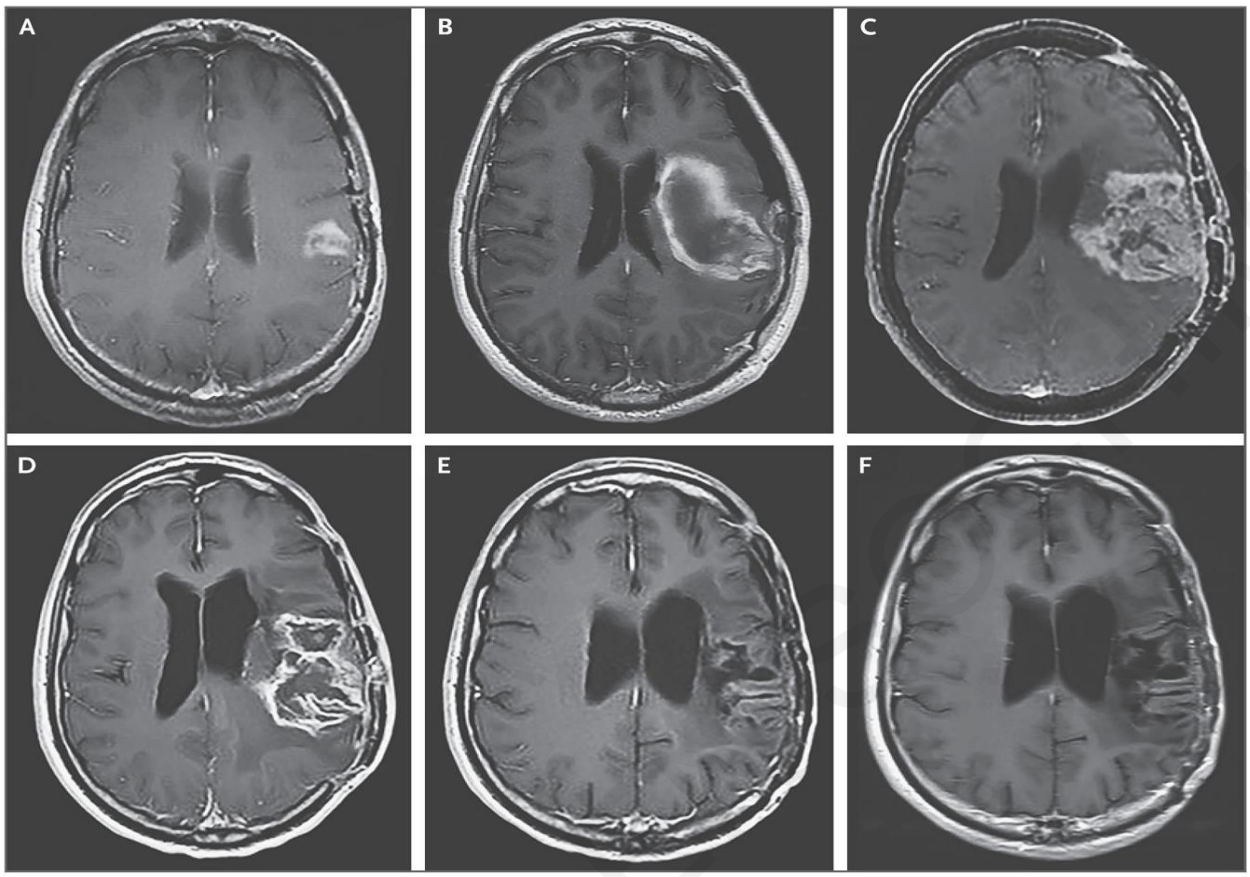

Figure 12: **Axial T1-Weighted MRI of Patient 8 post-PVSRIPO and lomustine treatment, at Dose Level 5** Panel A shows the baseline MRI of this patient, who received dose level 5 (1010 $TCID_{50}$). Panel B shows the image obtained after surgery for evacuation of intracranial hemorrhage after the removal of the infusion catheter. Panel C depicts the image obtained at the time of disease progression, 7 months post-PVSRIPO administration. Panel D illustrates the image obtained after the first cycle of lomustine, and Panel E the image obtained at the completion of nine cycles of lomustine. Panel F shows the results 1 year after the discontinuation of lomustine and 32 months after the PVSRIPO infusion. Adapted from figure 10 from (Desjardins, Gromeier et al. 2018) Furthermore, the researchers reported a safe use of a known antiangiogenic drug that promotes immunotherapy**,** bevacizumab, in compliment with PVSRIPO. The administration of bevacizumab helped control symptoms of locoregional inflammation but did not treat the tumor itself (Desjardins, Gromeier et al. 2018b) PVSRIPO is more stable and has better diminution that its' precursors in oncolytic virotherapy due to the substitution of the poliovirus' original IRES for the HRV2 IRES. For example, clinical research using PVSRIPO on glioblastomas have shown positive results, the genetic composition of the PVSRIPO remained unchanged after eliminating

the tumor in glioma xenografts (Dobrikova, Broadt et al. 2008). In addition, PVSRIPO receded and was eventually eliminated the tumors. Some other studies reviewed in the previous chapter have also reported similar findings upon successful infusion of PVSRIPO and no evidence of viral neuropathogenicity or virus shedding. (Holl, Brown et al. 2016, Walton, Brown et al. 2018, Desjardins, Gromeier et al. 2018) This review has also illustrated how PVSRIPO increases survival rate in cancer patients and induces antitumoral memory. The findings from the clinical trial conducted by (Desjardins, Gromeier et al. 2018) among fifteen PVSRIPO-treated patients with recurrent glioblastomas indicated an increased survival rate after twenty-four months, an increased when compared to the survival rate of the historical control group (Jindal, Lee et al. 2018). Another similar clinical study conducted among 61 patients also reported an increased survival rate at 24 months and 36 months, with patients remaining alive more than 70 months, more than 69 months, and more than 57 months after the PVSRIPO infusion (Desjardins, Gromeier et al. 2018) Furthermore, a study conducted by (Dobrikova, Broadt et al. 2008) illustrated how PVS-RIPO induces antitumor memory. The researchers reported that viral titers declined with the demise of HTB-15 cells in which PVS-RIPO can replicate. These analyses also suggest that PVS-RIPO exclusively resides in the central area morphologically resembling viable tumor in the receding xenograft (Dobrikova, Broadt et al. 2008). Another clinical investigation conducted by (Beasley, Nair et al. 2021) also reported similar PVS-RIPO induced antitumor effects. **CONCLUSION** PVS-RIPO has revealed great potentials as an oncolytic agent for cancer therapy. This review has illustrated that PVS-RIPO functions through direct viral tumor cell toxicity which in turn recruits the innate and adaptive immune system to eliminate tumor and deplete tumor cells in the microenvironment to reverse the immunosuppressed state. However, the mechanisms through which PVSRIPO stimulates immune inflammatory response towards the vast tumor compartments is not well elucidated and this calls for well detailed investigative research aimed at addressing this. PVS-RIPO's small virion size confers it with an oncolytic advantage targeted at efficient viral propagation. Viral propagation is rapid and occurs within few hours in the target tumor cells. Upon sufficient viral propagation, tumor cell lysis begins almost immediately. Small intratumorally administered doses of PVSRIPO is potent enough to delay tumor growth and shrink tumor cells. CD155 or Necl-5 receptors have been reported to be present on the surface of various cancers. PVS-RIPO ability to selectively bind to CD155 or Necl-5 receptors upon entry has further elucidated its oncolytic advantage and efficiency for use as an oncolytic agent for cancer therapy.

The application of PVSRIPO in human clinical trials has shown promising results. Patients introduced to PVSRIPO have shown greater survival rates compared to the historical control group. However only a very few cancers have been targeted in these human clinical trials, for example most of the available data are on glioblastomas and hence there is still a huge unexplored plethora of cancers. Therefore, based on the illustrations from this review, I recommend that successful research findings from the intratumoral administration of PVSRIPO at the preclinical stages should be used as baseline data and escalated into phases of human clinical trials. PVS-RIPO is genetically stable and remains unchanged after tumor elimination and this property makes it completely safe for use in human clinical trials. Furthermore, as already previously discussed, it can be safely used in complement with systemic therapies namely chemotherapeutic drugs or antiangiogenic drugs. In summary, the effectiveness of PVSRIPO as an oncolytic virus makes it the perfect therapeutic agent despite the drawbacks which can always be mitigated upon further research.

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