NATURAL KILLER T CELL IMMUNOTHERAPY IN COMBINATION WITH RECOMBINANT ONCOLYTIC VESICULAR STOMATITIS VIRUS AND IMMUNE CHECKPOINT THERAPY REDUCES TUMOR BURDEN IN MODELS OF PANCREATIC AND BREAST CANCER

by

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Submitted in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

at

Dalhousie University
Halifax, Nova Scotia
March 2022

Dalhousie University is located in Mi'kma'ki, the
ancestral and unceded territory of the Mi'kmaq.
We are all Treaty people.

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ABSTRACT

Cancer is the leading cause of death in Canada. Current treatments, such as chemotherapy and radiation, have high rates of adverse effects leading to dose-limiting toxicities and reduced therapeutic efficacy. Therefore, new treatments that are safe and effective are needed. I examined natural killer T (NKT) cell therapy in combination with recombinant oncolytic vesicular stomatitis virus (VSV-ΔM51) expressing the cytokine IL-15 (VSV-IL-15) or reovirus fusion associated small transmembrane (FAST) proteins in models of pancreatic and metastatic breast cancer, respectively. In the pancreatic model, VSV-IL-15 in combination with NKT cell immunotherapy increased tumor regression and overall survival. The addition of anti-PD-1 to the combination treatment further enhanced tumor immune infiltration, cytotoxicity, and cytokine production, resulting in 20% of mice experiencing complete tumor regression. Combination of VSV-ΔM51 expressing the FAST proteins p14 (VSV-p14) or p15 (VSV-p15) with NKT cell therapy led to 100% survival in the metastatic breast cancer model. In both models, combined therapy enhanced tumor regression and increased survival time over individual treatments and was superior to NKT cell therapy combined with VSV-ΔM51 expressing green fluorescent protein (VSV-GFP). Combination treatments increased tumor immune infiltration, cytotoxic activity, and proinflammatory cytokine production, to mediate increased tumor control. In both models, mice who survived initial tumor challenged had slower tumor growth compared to naïve mice upon rechallenge, demonstrating formation of immune memory. Therefore, combined NKT cell immunotherapy, with VSV-IL-15, VSV-p14, or VSV-p15, presents a promising treatment strategy for pancreatic and metastatic breast cancer, respectively.

LIST OF ABBREVIATIONS

α-C-GalCer	α-C-galactosylceramide
α-GalCer	α-galactosylceramide
γc	gamma chain
ADCC	antibody dependent cell cytotoxicity
APC	antigen presenting cell
ATP	adenosine triphosphate
CALR	calreticulin
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DAMPs	damage associated molecular patterns
DC	dendritic cell
DMEM	Dulbecco's modified eagle medium
EDTA	ethylenediamine-tetraacetic acid
ER	estrogen receptor
FAST	fusion associated small transmembrane protein
FBS	fetal bovine serum
FOXP3	forkhead box protein P3
G protein	glycoprotein
GFP	green fluorescent protein
GM-CSF	granulocyte-macrophage colony-stimulating factor
HER2	human epidermal growth factor receptor 2
HMGB1	high mobility group box 1

ICD	immunogenic cell death
ICI	immune checkpoint inhibitor
IDO	indoleamine-2,3-dioxygenase
IFN	interferon
IFNγ	interferon γ
IL	interleukin
IL-2Rβ	interleukin-2 receptor β subunit
IL-15	interleukin-15
IL-15Rα	interleukin-15 receptor α subunit
ip	intraperitoneal
iv	intravenous
JAK/STAT	janus kinase-signal transducer and activator of transcription
L protein	large protein
LDL	low density lipoprotein
M protein	matrix protein
MDSCs	myeloid derived suppressor cells
MHC	major histocompatibility complex
MMP	matrix metalloproteinases
MOI	multiplicity of infection
N protein	nucelocapsid protein
NK cell	natural killer cell
NKT cell	natural killer T cell

NSCLC	non-small cell lung cancer
OVs	oncolytic viruses
P protein	phosphoprotein
PanINs	pancreatic intraepithelial neoplasms
PD-1	programmed cell death protein 1
PDAC	pancreatic ductal adenocarcinoma
PD-L1	programmed death ligand 1
PFU	plaque forming units
PNETs	pancreatic neuroendocrine tumors
PR	progesterone receptor
PRR	pattern recognition receptor
RAG	recombination activating gene
RAGE	receptor for advanced glycation end-products
Reovirus	respiratory enteric orphan virus
SHP-1	src homology 2 domain-containing PTPs-1
SHP-2	src homology 2 domain-containing PTPs-2
STAT1	signal transducer and activator of transcription 1
STING	stimulator of interferon genes
TAAs	tumor associated antigens
TAMs	tumor associated macrophages
TGFβ	tumor growth factor beta
Th	T helper
TIGIT	T cell immunoreceptor with Ig and ITIM domains

TIM-3	T cell immunoglobulin and mucin-domain containing-3
TLR	toll-like receptor
TNBC	triple negative breast cancer
TNF	tumor necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
Treg	regulatory T cell
TSA	tumor specific antigens
TVEC	Talimogene laherparepvec
VSV	vesicular stomatitis virus
VSV-ΔM51	vesicular stomatitis virus with matrix protein mutation

ACKNOWLEDGEMENTS

I would like to start by acknowledging my supervisor, Dr. Brent Johnston for all of the support and guidance over my graduate degree. I would also like to thank my graduate committee, Dr. Jeanette Boudreau, Dr. Roy Duncan, and Dr. Paola Marcato for their support and expertise during my graduate degree. I would like to thank all the members of the Carleton Animal Care Facility for their assistance with animal studies and the members of the Flow Cytometry Core: Derek Rowter, Renee Raudonis, and Tatjana Brauer-Chapin for all the help with flow cytometry and cell sorting.

I would like to thank both the current members of the Johnston Lab: Laura Korycinska, Rushit Madeka, Jordan Lukacs, Rhea Nickerson, Natasha Osborne, and Emily Pritchard, as well as the past members: Kaitlyn Tanner, Dr. Drew Slauenwhite, Dr. Simon Gebremeskel, Dr. Alison Gareau, and Dr. Dihia Meghnem for making the Johnston lab a great place to work. I would like to extend a special thank you to Terry LeVatte for all the help with injections, harvests, and surgeries over the course of my degree. I would like to thank Dr. Roy Duncan's lab for being great collaborators on the VSV-FAST project and Entos for giving me the opportunity to work for them on the SARS-COV-2 vaccine project. I would like to extend a special thank you to Nichole McMullen for all the help on the VSV-FAST and vaccine projects. Thank you to all the members of the 15th floor and Micro Deptartment. for all the support and great discussions over the years.

Lastly, I would like to thank my mom, Shelley, my brother, Eric, and my grandparents, for all their support over the course of my degree. To all my family and friends who supported me over the years, thank you.

CHAPTER 1: INTRODUCTION

1.1 Cancer

Cancer is the leading cause of death in Canada, responsible for 30% of all fatalities in 2019.[1] It is estimated that 50% of Canadians will be diagnosed with cancer in their lifetime, with 25% of cases being fatal.[1] Those who survive will live with physical challenges after treatment.[2] Despite recent research advances in mechanistic understanding, and treatment strategies, cancer remains a significant burden on society. Therefore, safer, more effective therapeutic strategies are needed.

Cancer can be broadly classified into five groups based on the tissue of origin. These categories are carcinoma, sarcoma, myeloma, leukemia, and lymphoma.[3] Carcinomas are neoplasms of epithelial origin and account for 80-90% percent of all cancer cases. Carcinomas can be broken down into two subcategories: adenocarcinomas, which develop in an organ or gland; and squamous cell carcinoma, which develops from squamous epithelia cells.[4] Sarcomas are neoplasms that originate from supportive and connective tissue including bones, cartilage, muscle, and fat.[5] Myelomas originate from plasma cells in bone marrow and may produce different immunoglobulin isotypes.[6] Leukemias derive from the bone marrow and overproduce immune cells leading to patients being prone to infection.[7] Lymphomas develop from the lymphatic system and can be sub-divided into Hodgkin's lymphoma and non-Hodgkin's lymphomas.[8,9] The subcategories are differentiated by the presence of Reed-Sternberg cells, large cells derived from B lymphocytes.[9]

Cancer is a disease driven by the accumulation of genetic mutations over time, converting normal cells into transformed cells. Genetic mutations can be hereditary or

environmental, giving rise to cancer by promoting the hallmarks of cancer. The hallmarks of cancer describe various mechanisms that underly tumor development and progression.[10] The immune system and its interaction with cancer cells plays an important and complex role in many of the hallmarks of cancer. Chronic inflammation caused by immune cells increases genetic mutations leading to faster cancer progression.[11] Tumors often have their own microbiome, which can induce Toll like receptor (TLR) signalling increasing inflammation and tumor growth.[12] Under normal conditions, immune cells survey tissues, looking for transformed cells and removing them.[13] Transformed cells can downregulate receptors or release cytokines to dampen the function of these immune cells, allowing them to evade immune recognition and supress immune cell activity.[14,15] The immune system can both promote and inhibit tumor associated angiogenesis, promoting or inhibiting tumor growth, respectively.[16] Many immune cells release enzymes that degrade the extra cellular matrix, leading to increased metastasis.[17]

1.2 Pancreatic cancer

In 2019, pancreatic cancer became the third leading cause of cancer death in Canada, overtaking breast cancer.[1] This is largely due to its low 5-year survival rate of <10%, resulting in approximately 5,300 deaths a year in Canada.[1,18,19] This low survival rate is due to the lack of early symptoms, difficulty of diagnosing pancreatic cancer early, quick metastatic rate, and treatment resistance.[20–22] The incidence of pancreatic cancer is also on the rise, with approximately 6000 Canadians being diagnosed each year, making it the 12th most prevalent cancer in Canada.[1]

Pancreatic cancer develops from precursor lesions, mainly pancreatic intraepithelial neoplasms (PanINs). PanINs are small, microscopic lesions composed of neoplastic epithelium.[23] Pancreatic cells that develop activating KRAS mutations turn into stage one PanIN lesions.[24,25] Stage one PanINs further develop into stage two and three PanINs by additional inactivating mutations resulting in the loss of cyclin dependent kinase inhibitor 2A and p53 function.[26,27] Stage two and three PanINs are characterized by a loss of polarity and nuclear atypia.[26] All PanINs are non-invasive and are considered pancreatic cancer once they invade the basement membrane.[26]

While there are many types of pancreatic cancer, they can be subdivided into two main categories: pancreatic neuroendocrine tumors (PNETs) and pancreatic exocrine tumors. PNETs, arising from hormone-producing islet cells, make up about 7% of pancreatic cancer cases and are slower-growing than exocrine tumors.[28] While some PNETs are functional, producing large amounts of hormones, most are non-functional and produce no hormones.[29] Due to this, they are often not diagnosed until they are large enough to cause pain and jaundice.[29]

Non-functional PNETs make up 60-90% of PNET diagnoses and have a 5-year survival of 30-50%.[30] Due to the lack of hormone secretion, non-functional PNETs are diagnosed later, when 60-77% of patients have formed liver metastases.[30] Treatment of non-functional PNETs often involves chemotherapy and surgical resection of the liver and/or pancreas.[30,31]

Functional PNETs make up 10-30% of PNET diagnoses and are classified by the hormone they secrete (i.e. insulinoma, glucagonoma).[29] Functional PNETs secrete large amounts of their associated hormone, leading to earlier symptoms and diagnosis.[29] This

earlier diagnosis combined with targeted therapeutics gives functional PNETS a higher survival rate (59-80%) compared to other forms of pancreatic cancer.[32–34]

Exocrine tumors comprise the other 93% of pancreatic cancer diagnoses and arise from the exocrine cells that comprise the exocrine gland and ducts of the pancreas, which produce and release enzymes into the duodenum. The most common exocrine tumor is pancreatic ductal adenocarcinoma (PDAC). PDAC accounts for 90% of all pancreatic cancer diagnoses and has a 5-year survival rate of <10%, largely due to its lack of early symptoms, quick rate of metastasis, and chemotherapy resistance.[20–22] Surgical resection with subsequent chemotherapy remains the best treatment option, increasing the 5-year survival rate to ~20%; however, by the time the cancer is diagnosed, 80% of patients are not eligible for surgery.[35,36] Therefore, many patients receive only chemotherapy, either gemcitabine, FOLFIRFINOX, or a combination of gemcitabine and nab-paclitaxel depending on the patient's performance score.[37] However, the majority of patients do not respond to chemotherapy.[38] Patients who maintain a good performance score after gemcitabine treatment may receive second-line treatment with nanoliposomal irinotecan plus 5-FU and folinic acid.[39] Recent work has tried to detail molecular subtypes of PDAC and potential therapies that may be more effective for patients with certain subtypes. While there are many systems to classify molecular subsets of PDAC, there is no current universal, clinically applied classification.[40,41]

1.3 Breast cancer

Breast cancer is the most common female cancer and second-leading cause of cancer death in Canadian women.[1] In 2020, approximately 27,000 Canadian women were diagnosed with breast cancer and 5,100 women succumbed to the disease, accounting for

25% of all cancer cases and 13% of cancer deaths in Canadian women.[1] Since 1991, the breast cancer death rate has been declining, largely due to increased screening and new treatment options.[1] However, many of these treatments are associated with severe adverse events, highlighting the need for safe and effective treatments.

Breast cancer carcinoma can be separated into four main groups, based on the expression of the estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2).[42] These groups can be further separated into molecular subtypes based on cellular origin (basal or luminal), Ki67 proliferation index, and receptor expression (Table 1).[43]

Intrinsic Breast cancer subtype	Molecular subtype	Associated Markers
Luminal A	N/A	Estrogen Receptor (+), Progesterone Receptor (+/-), Low Ki67, HER2 (-)
Luminal B	N/A	Estrogen Receptor (+), Progesterone Receptor (+/-), High Ki67, HER2 (+/-)
HER2 Positive	N/A	Estrogen Receptor (-), Progesterone Receptor (-), HER2 (+)
TNBC (basal-like) **Estrogen Receptor (-), Progesterone Receptor (-), HER2 (-)** **FOXC1 (+)** **High expression of laminins**	Basal-like 1	High expression of cell-cycle related genes (e.g. CDKN2A). High expression of Ki67.
	Basal-like 2	High levels of growth factor receptors (e.g. EGFR). High levels of glycolysis and gluconeogenesis. High expression of Ki67.
TNBC (Normal-like) **Estrogen Receptor (-), Progesterone Receptor (-), HER2 (-)**	Luminal Androgen receptor	Androgen receptor (+), high level of steroid biosynthesis
	Claudin low	Low expression of claudin. Increased expression of genes associated with epithelial-mesenchymal transition (e.g. Twist1)
	Mesenchymal-like	High expression of TGF-beta, mTOR, and VEGFR signaling pathways.
	Immunomodulatory	High immune gene expression (e.g. NF-κB, TNF signalling). Increased immune infiltration.

Table 1: Molecular classification of breast cancer. Table describing the molecular classification of breast cancer by receptor and gene expression. *CDKN2A=cyclin dependent kinase inhibitor 2A, EGFR=epidermal growth factor receptor, FOXC1= forkhead box C1, HER2=human epidermal growth factor receptor 2, NF-κB=nuclear factor-kappa B, TNBC=triple negative breast cancer, TNF=tumor necrosis factor, TGF-β=tumor growth factor-beta, TWIST1=twist-related protein-1, VEGFR=vascular endothelial growth factor receptor, Positive= (+), negative= (-).* Adapted from [43]

Treatment of breast cancer depends on the type and stage of cancer. Most patients will have surgery to remove the tumor mass and draining lymph nodes.[44] Patients with

HER2, ER, or PR expression can be treated with anti-HER2 and hormone therapy, respectively.[45–47] Patients that do not express HER2, ER, or PR (triple negative breast cancer) (TNBC) lack any targeted therapy and are treated using chemotherapy.[48] This TNBC subset also is highly metastatic and has the lowest survival rate, demonstrating the need for new, more effective therapies.[49]

While TNBC patients are conventionally treated using chemotherapy, newer work has identified that patients with certain molecular subtypes of TNBC may benefit from treatment with different therapeutics. The luminal androgen receptor subtype has an upregulated estrogen signalling pathway, indicating it may respond to anti-estrogen or anti-androgen therapy.[50,51] And in fact, androgen receptor inhibitors used in prostate cancer have shown efficacy in treating the luminal androgen receptor subtype of TNBC.[52,53] Due to its high level of tumor infiltrating lymphocytes, the immunomodulatory TNBC subtype has the best prognosis out of the any subtype.[54,55] Due to this higher immune activity, patients may respond better to immunotherapy. Indeed, patients with TNBC that expressed high levels of programmed death ligand-1 (PD-L1) responded better to checkpoint blockade.[56,57] And, while the other subtypes of TNBC listed above have not shown susceptibility to any targeted or immune based therapy, they do show differing responses to different classes of chemotherapeutic drugs.[58] As a further area for development, most subtypes are associated with high expression of certain pathways, therefore therapies that target those pathways may be more effective against specific subtypes.[43]

1.4 Cancer and the immune system

One of the hallmarks of cancer is immune evasion, highlighting the importance of the immune system's interaction with cancer cells.[10,59] Cancer immunoediting is the process by which the immune system can both promote and constrain cancer growth.[60] Cancer immunoediting has three phases: elimination, equilibrium, and escape.[60,61] The elimination phase begins before tumors are detectable and involves the recognition and removal of transformed cells. Normal cells express intracellular antigens loaded on major histocompatibility (MHC) I on their cell surface.[14] Lymphocytes monitor the presented antigens and the level of MHC I expression on the cell. When a transformed cell expresses a neo-antigen or downregulates the expression of MHC I, lymphocytes remove the transformed cell, a process termed immunosurveillance.[14] If the immune system is unable to fully eliminate the cancer, immunoediting moves on to the second phase, equilibrium, where the tumor neither grows nor shrinks. If the immune system is unable to clear the tumor, the escape phase starts, leading to unrestrained growth and clinically detectable tumors.[61,62] The escape phase is largely attributed to the activation of immunosuppressive and immunoevasion pathways. Tumor cells release chemokines and immunosuppressive cytokines, including CCL2 and tumor growth factor beta (TGFβ), recruiting immunosuppressive cell types and induce alterations in stromal cells, leading to an immunosuppressive microenvironment and therapeutic resistance.[63–67] Furthermore, tumor cells and tumor associated myeloid cells increase inhibitory receptors including immune checkpoint ligands, which bind to receptors on anti-tumor immune cells, further suppressing the immune system.[68,69]

1.4.1 Elimination phase

Both the innate and adaptive immune system have important roles in the elimination of transformed cells. The innate immune system uses germline-encoded receptors to recognize damage associated molecular patterns (DAMPs) and tumor associated ligands to eliminate transformed cells.[70] The lysis of transformed cells by the innate immune system allows for antigen uptake and presentation by dendritic cells (DCs) leading to the activation of T cells and the adaptive immune response. The key immune cells in the elimination phase are $CD4^+$ and $CD8^+$ T cells, natural killer (NK) cells, natural killer T (NKT) cells and DCs. The role of NKT cells in cancer immunity will be discussed in detail in section 1.5.

1.4.1.1 NK cells

NK cells express many receptors, including NKG2D, CD94/NKG2C/E, and natural cytotoxicity receptors (NKp30, NKp44, and NKp46) that recognize surface markers on transformed cells leading to elimination of transformed cells.[71–73] Furthermore, inhibitory members of the Ly49 (mouse) and killer-cell immunogloblin-like (humans) families bind MHC I to prevent cytotoxicity against healthy cells.[74,75] Transformed cells often downregulate MHC I, activating NK cells via missing-self recognition and overcoming an important immunoevasion mechanism.[76] NK cells also express CD16, which binds the FC portion of IgG1, mediating antibody dependent cell cytotoxicity (ADCC) of transformed cells.[77] Following activation, NK cells release interferon γ (IFNγ) and exocytose cytotoxic granules containing perforin and granzyme B, lysing transformed cells.[78,79] Furthermore, NK cells can kill transformed cells by engagement of tumor necrosis factor receptor superfamily ligands, including tumor necrosis factor (TNF)-

related apoptosis-inducing ligand (TRAIL) and Fas ligand (FasL) with their corresponding receptors on transformed cells.[80]

Mice depleted of NK cells or with deficient NK cell function often have more aggressive tumor growth and increased metastasis, demonstrating the role of NK cells in tumor immunosurveillance.[81] Mice genetically deficient in important NK cell effector molecules, including perforin-1 and signal transducer and activator of transcription 1 (STAT1), have an increased frequency of spontaneous tumors and tumors induced by the carcinogen methylcholanthrene.[82,83] Furthermore, mice deficient in recombinase activating genes (RAG) and STAT1 develop spontaneous tumors at a higher rate then RAG mice, demonstrating the loss of STAT1 in NK cells contributes to increased tumor development.[84]

Transformed and stressed cells upregulate expression of NKG2D ligands, which bind to NKG2D expressed on NK cells, NKT cells, $CD8^+$ T cells, and γδ T cells, leading activation of cytotoxic effector function.[85] In humans, there are two families of NKG2D ligands, the MHC class I chain-related molecules A and B and the UL16-binding proteins,[86,87] whereas mice have three families (Rae1, MULT1, and H60) with many isoforms.[88] NKG2D ligands have low expression on healthy tissue and are induced by stressors such as DNA damage and hyperproliferation, rendering transformed cells susceptible to killing by cytolytic effectors.[85,89–91] However, NKG2D ligands can be cleaved and shed from the surface of transformed cells, preventing killing of these cells and contributing to immune escape.[85] The shedding of NKG2D ligands leads to their increased serum levels, which can be used as an early detection method for various cancers.[92,93]

1.4.1.2 $CD8^+$ T cells

$CD8^+$ T cells are an important mediator of the adaptive immune system that recognize peptides derived from intracellular antigens to eliminate infected and transformed cells directly through cytolytic activity and indirectly through cytokine expression. Generation of cytotoxic $CD8^+$ T cells is initiated by DCs in the regional lymph node. DCs uptake antigen in the periphery and cross-present the antigen to $CD8^+$ T cells via MHC I.[94] Furthermore, DCs provide many co-stimulatory signals, resulting in optimal priming of the $CD8^+$ T cell.[94] $CD8^+$ T cells can eliminate transformed cells through various mechanisms. $CD8^+$ T cells which recognize neo-antigens presented by MHC class I on transformed cells can release pro-inflammatory cytokines (IFNγ and TNF) and cytolytic enzymes (perforin and granzyme B), resulting in killing of transformed cells. Furthermore, $CD8^+$ T cells express NKG2D and can bind to NKG2D ligands on transformed cells, leading to further release of pro-inflammatory cytokine and cytolytic enzymes.[95] Lastly, $CD8^+$ T cells can also kill transformed cells via TRAIL and FasL-mediated apoptosis via binding to their receptors on the tumor surface, resulting in caspase activation and subsequent cytochrome c release.[96] After initial activation, $CD8^+$ T cells can become tissue resident memory $CD8^+$ T cells ($CD69^+$ $CD103^+$) that also contribute to cancer immunosurveillance. Tissue resident memory $CD8^+$ T cells infiltrate into a wide range of cancers, are associated with improved patient outcomes, and may limit cancer recurrence.[97]

1.4.1.3 $CD4^+$ T cells

$CD4^+$ T cells are important regulators of immune function and can be separated into different lineages with distinct immune functions. $CD4^+$ T helper (Th) cells recognize

peptides presented on MHC II by antigen presenting cells (APCs). Following activation, $CD4^+$ T cells differentiate into different lineages based on their cytokine profile and master transcription factor. $CD4^+$ T cells can be polarized into three major subsets: Th1 cells, which express T-bet and produce IFNγ; Th2 cells, which express GATA3 and produce interleukin (IL)-4, IL-5, and IL-13; and Th17 cells, which express RORγT and produce IL-17A/F. Forkhead box protein P3 ($FOXP3^+$) $CD4^+$ T cells, also known as regulatory T cells (Tregs), will be discussed in section 1.4.2.

$CD4^+$ T cells play variable roles in cancer immunity based on their Th lineage. Th1 $CD4^+$ T cells serve an important role in priming the anti-tumor immune response. Th1 $CD4^+$ T cells in the draining lymph node can stimulate tumor-specific $CD8^+$ T cells through CD40-CD40L interactions and IL-2 production.[98,99] Furthermore, $CD4^+$ T cells can license DCs, allowing them to activate tumor-specific $CD8^+$ T cells via cross-presentation.[100] Both processes are essential to enhance $CD8^+$ T cell cytolytic function against cancer cells. Th1 $CD4^+$ T cells also have an important anti-tumor role at the tumor site. Tumor-specific $CD4^+$ T cells can promote expansion, differentiation, and trafficking of tumor-specific $CD8^+$ T cells, enhancing anti-tumor immunity.[101] Reduced numbers of $CD4^+$ T cells at the tumor site decreases $CD8^+$ T cell recruitment and cytolytic function, resulting in increased tumor progression.[101]

Th2 $CD4^+$ T cells play a more controversial role in cancer progression. In murine lung metastasis models, Th2 $CD4^+$ T cell secretion of IL-4, IL-5, and IL-13 increased eosinophil tumor infiltration and release of eosinophil cytotoxic proteins, resulting in increased cytotoxicity and decreased metastatic burden.[102] However, many studies assocaite Th2 $CD4^+$ T cells are associated with increased tumor progression and immune

evasion.[103–106] More research is needed to better understand the role of Th2 $CD4^+$ T cells and how they can be used to target tumors.

Similar to Th2 cells, Th17 $CD4^+$ T cells play a controversial role in tumor immunity. Th17 $CD4^+$ T cells are highly pro-inflammatory, and their production of IL-17 increases cancer progression in many gastric cancers.[107] Furthermore, cytokines associated with Th17 $CD4^+$ T cells drive angiogenesis and accumulation of immunosuppressive immune cells, driving tumor progression. [108,109] However, Th17 $CD4^+$ T cells also have roles in promoting anti-tumor immunity. Th17 $CD4^+$ T cells secrete IL-2 and promote CCL20 production from tissues, leading to the expansion and trafficking of cytotoxic $CD8^+$ T cell to the tumor.[110,111] While some cytokines produced by Th17 $CD4^+$ T cells increase angiogenesis, others decrease angiogenesis, overall making the role of Th17 $CD4^+$ T cells in angiogenesis cancer-dependent.[112,113] Further studies are needed to understand the role of Th17 $CD4^+$ T cells in tumor immunity.

1.4.1.4 Dendritic cells

DCs are potent APCs and are essential in shaping the adaptive immune response. DCs take up tumor associated antigens (TAAs) and tumor specific antigens (TSAs) and present them to T cells in draining lymph nodes, initiating and sustaining an anti-tumor immune response.[94] Furthermore, DCs can perform a unique type of antigen presentation called cross-presentation. Extracellular antigens are generally degraded and presented through MHC II. However, through cross-presentation, TAAs and TSAs released by tumor cells can be presented through MHC I, stimulating a cytotoxic $CD8^+$ T cell response.[114] Mice with impaired cross-presentation are unable to mount effective anti-

tumor immune responses, demonstrating that cross-presentation is essential for creating the effective anti-tumor immune response.[115]

Mature DC tumor infiltration is associated with positive prognosis in cancer patients.[116–118] Tumor-infiltrating DCs produce many important chemokines (eg. CXCL9, CXCL10), increasing NK, NKT, and T cell infiltration into the tumor microenvironment (TME).[119] Furthermore, DCs secrete many proinflammatory cytokines, such as IL-12, which polarize T cells towards a Th1 phenotype and activate NK cells, inducing secretion of IFNγ and enhancing tumor control.[120] Overall, DCs play an important role in orchestrating and sustaining the anti-tumor immune response.

1.4.2 Immune escape

Over time, tumors can acquire genetic mutations that allow the tumor to escape the immune system and grow to become clinically detectable. Tumors employ many immunosuppressive and evasive tactics to escape killing by the immune system. Tumors can downregulate surface receptors (eg. MHC I) to lower their antigenicity and evade immune detection by $CD8^+$ T cells.[14,121] Tumors can decrease their immunogenicity by increasing expression of immunoinhibitory surface receptors, including immune checkpoint receptors.[122] Lastly, tumors can create immunosuppressive microenvironments by modulating the function of stromal cells and recruiting suppressive immune cells that inhibit elimination of cancer cells.[63–66,123] Two key immune cells that work to establish an immunosuppressive microenvironment in the escape phase are Tregs and myeloid derived suppressor cells (MDSCs).

1.4.2.1 Regulatory T cells

Tregs are a subset of $CD4^+$ T cells that suppress immune activity and maintain immune homeostasis. Tregs express the master transcription factor FOXP3, which is critical for Treg development and their suppression of anti-tumor immune cells.[124] Tumor cells secrete chemokines such as CCL5 that recruit Tregs to the tumor site,[125] where they are activated by immature DCs,[126] creating an immunosuppressive environment. Cancer patients often have increased Treg function and accumulation compared to healthy individuals.[125,127] Furthermore, a high Treg/T cell ratio is associated with a poor prognosis in various cancer types.[128,129] Within tumors, Tregs produce large amounts of immunosuppressive cytokines, including TGFβ and IL-10, inhibiting the function of anti-tumor immune cells in the tumor microenvironment.[130] Furthermore, Tregs can release perforin and granzyme B, killing APCs, NK cells, and T cells, depleting anti-tumor immune cells at tumor sites resulting in reduced anti-tumor immunity.[131,132] Tregs also express high amounts of CD25, which binds IL-2 at a high affinity, depleting free IL-2 and inhibiting T cell proliferation.[133] Tregs constitutively express immune checkpoint receptors, such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4).[134] CTLA-4 binds CD80 on APCs, causing them to release immunosuppressive indoleamine-2,3-dioxygenase (IDO) which limits the proliferation and function of anti-tumor lymphocytes.[135] PD-1/PD-L1 signalling is essential for Treg development and function,[136,137] and the accumulation of tumor-infiltrating $PD\text{-}L1^+$ Tregs increases the immune exhaustion of tumor-infiltrating $CD8^+$ T cells, leading to a poorer prognosis.[138,139]

Due to their strong immunosuppressive abilities, depleting Treg numbers or reducing their immunosuppressive function is an important immunotherapy strategy. Depleting and/or inhibiting Tregs effectively reduces tumor growth in various cancer models and many therapeutics that interfere or deplete Tregs are currently in clinical trials.[140–143]

1.4.2.2 Myeloid derived suppressor cells

Chronic tumor inflammation stimulates the generation, trafficking, and activation of MDSCs, a heterogenous immature myeloid cell population with immunosuppressive functions. Increased numbers of MDSC in cancer patients are associated with a worse prognosis and decreased overall survival.[144] Chronic inflammatory signals lead to the generation of MDSCs and their recruitment to the tumor site.[145] Once there, MDSCs suppress the function of anti-tumor immune cells and remodel the TME, increasing tumor progression and promoting metastasis, respectively.[63,146,147] MDSCs can limit the function of anti-tumor immune cells in multiple ways (Figure 1). MDSCs release IDO to metabolise amino acids essential for lymphocyte proliferation and function, limiting lymphocyte reactivity towards tumor cells.[148,149] MDSCs release many immunosuppressive mediators, including nitric oxide, TGFβ, and IL-10, that inhibit NKT, NK, DCs, and T cell functions.[145,150,151] Furthermore, the release of these suppressive cytokines increases the function of other immunosuppressive immune cells such as Tregs and M2 macrophages, further supporting a pro-tumor TME.[152] Importantly, MDSCs also express high levels of PD-L1, allowing them to bind programmed cell death protein 1 (PD-1)-positive NKT, NK, and T cells in the tumor microenvironment, inhibiting their function.[153,154] MDSCs can also promote tumor metastasis via increased

production of multiple matrix metalloproteinases (MMPs). MMPs produced by MDSCs degrade extracellular matrix proteins and cell-cell attachments, allowing cancer cells to break away from the tumor and metastasize to other locations.[17,155]

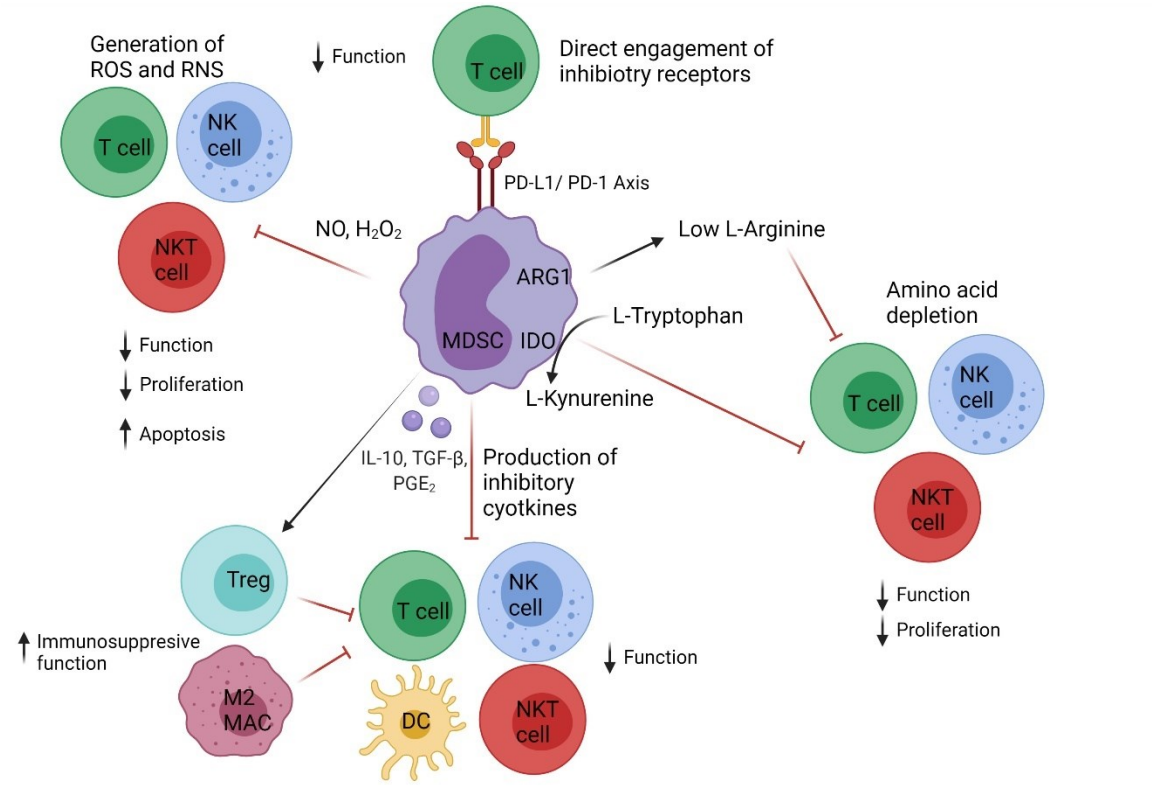


Figure 1: Mechanisms of MDSC suppression of anti-tumor immunity. MDSCs inhibit the antitumor immune response through multiple mechanisms. MDSCs can deplete amino acids from the environment, limiting NK, NKT, and T cell proliferation and function. MDSCs produce high amounts of arginase- 1(ARG1) increasing L-arginine uptake. MDSCs also produce high levels of idolamine-2,3-dioxygenase (IDO), which converts L-tryptophan into L-kynurenine. The low levels of L-arginine and L-tryptophan, combined with the presence of L- kynurenine, limit NK, NKT, and T cell proliferation and function. MDSCs produce reactive nitrogen species (RNS) and reactive oxygen species (ROS) that can promote TCR downregulation and reduced IL-2 signalling, limiting function and proliferation, respectively. Furthermore, ROS and RNS can lead to T cell and NK cell apoptosis. MDSCs produce a wide range of immunosuppressive cytokines including IL-10, TGF-β, and prostaglandin E2 (PGE-2). The production of these immunosuppressive cytokines limits the function of anti-tumor immune cells. Furthermore, these immunosuppressive cytokines increase Treg functionality and polarize macrophages to an M2 phenotype, further increasing immunosuppression. MDSCs also express high levels of immunoregulatory receptors, such as PD-L1. Binding of MDSC PD-L1 to PD-1 on NK, NKT, and T cells limits their anti-tumor functions.

Due to their strong immunosuppressive abilities, reducing MDSC number and function in the TME is an effective immunotherapy strategy. Decreasing the number of MDSCs as well as limiting their function has been shown to decrease tumor growth and increase overall survival in both animal models and cancer patients. Inhibition of cyclooxygenase-2 and phosphodiesterase-5 production from MDSCs resulted in increased anti-tumor immune function, leading to tumor reduction in mice and humans, respectively.[156,157] Furthermore, some chemotherapeutics such as gemcitabine, can kill MDSCs directly, resulting in increased anti-tumor immune activity.[158,159]

1.5 NKT cells

NKT cells are a specialized subset of glycolipid-reactive T lymphocytes that express NK cell markers (NKp46, NK1.1) and play an important role in tumor immunosurveillance.[160,161] NKT cells can be broken down into two major subsets: type I NKT cells and type II NKT cells. Type I NKT cells express an invariant TCRα chain rearrangement composed of Vα14-Jα18 paired with Vβ8.2/7/2 in mice and Vα24-Jα18 paired with Vβ11 in humans.[162] Unlike traditional T cells, which recognize peptide antigens in the context of MHC I or II, type I NKT recognize endogenous and exogenous glycolipids presented by the MHC I-like molecule CD1d.[163] NKT cells can be detected using CD1d tetramers loaded with α-galactosylceramide (α-GalCer) analogs such as PBS-57. Type II NKT cells have a more diverse repertoire of Vα rearrangements (Vα1/3/8) and recognize sulfatides instead of α-GalCer.[164,165] Multiple studies have shown that activation of type II NKT cells via administration of sulfatides increases tumor growth and metastasis, demonstrating a pro-tumor role for these cells, in contrast to the robust anti-tumor activity exhibited by type I NKT cells.[166,167] As type II NKT cells

are not a focus on this dissertation, NKT cells will be used to refer to type I NKT cells only.

NKT cells can be further broken down into subsets based on their expression of CD4 and CD8. Double-negative ($CD4^-$ $CD8^-$) and CD4-positive ($CD4^+$ $CD8^-$) are found in both mice and humans, whereas CD8-positive ($CD4^-$ $CD8^+$) NKT cells are only found in humans.[168–171] Furthermore, NKT cells can be broken down into functionally distinct subsets based on their transcription factor and cytokine profiles. These subsets are NKT-1, NKT-2, NKT-10, and NKT-17, which are analogous to Th1, Th2, Th10, and Th17 subsets in conventional T cells.[170,172]

1.5.1 NKT cells and cancer

It is well-established that NKT cells have a direct role in antitumor immunity and immunosurveillance. Treatment with the carcinogen methylcholanthrene led to rapid growth of spontaneous tumors in NKT cell deficient $J\alpha18^{-/-}$ mice.[173] In the transgenic adenocarcinoma of the mouse prostate (TRAMP) model, mice who were also deficient in NKT cells exhibited increased tumor development and higher rates of metastasis, leading to reduced survival compared to WT TRAMP mice.[174] Consistent with this, mice lacking NKT cells exhibit enhanced pancreatic intraepithelial neoplasia lesions when crossed with mice harboring an activating Kirsten Rat Sarcoma viral oncogene (KRAS) mutation.[175] Furthermore, $J\alpha18^{-/-}$ $p53^{-/-}$ double-knockout mice exhibit accelerated tumor onset compared to $p53^{-/-}$ single-knockout mice, further demonstrating the importance of NKT cells in cancer immunosurveillance.[176] In humans, cancer patients often have reduced numbers or impaired NKT cell function,[177] while an increase in NKT cell tumor infiltration is associated with a good prognosis in neuroblastomas, colorectal carcinoma,

and pancreatic adenocarcinoma.[178–181] NKT cell immunosurveillance is mediated by their production of inflammatory cytokines as well as direct recognition of tumor-associated or stress-induced glycolipid antigens presented by CD1d in contrast to traditional T cells that recognize peptides presented by MHC I or II.

Therapeutic activation of NKT cells via exogenous glycolipids, such as α-GalCer, increases anti-tumor immunity and provides protection from tumor progression. Activated NKT cells can mediate anti-tumor immunity by three mechanisms: direct tumor lysis, recruitment of innate and adaptive immune cells, and inhibition of suppressive cells in the TME.

NKT cells recognize and are activated by the presentation of glycolipid antigens by CD1d-positive tumor cells or APCs.[182,183] CD1d-positive tumors are more susceptible to NKT cell-mediated lysis compared to CD1d-negative tumors, and cancer cells will often downregulate CD1d to avoid detection by NKT cells, demonstrating an important role for CD1d-TCR interactions in immunosurveillance.[184–186] Furthermore, CD1d-antibody fusion proteins that bind to TAAs on cancer cells increase NKT cell targeting of the tumor, resulting in increased NKT cell activation and NKT cell-mediated tumor cell lysis.[187] NKT cells can directly lyse CD1d-expressing tumor cells using perforin, granzyme B, FasL, and TRAIL.[188] Surface expression of CD1d on tumor cells is directly correlated with NKT cell-mediated cytotoxicity, with higher levels of CD1d expression leading to increased tumor cell lysis and lower metastatic rate, while decreased CD1d expression can lead to tumor escape, as previously discussed.[184–186]

NKT cells can also target non-CD1d-expressing tumor cells by increasing recruitment of innate and adaptive immune cells. Following activation, NKT cells release

large quantities of pro-inflammatory cytokines, including IL-2, IFNγ and TNF, which activate NK cells and $CD8^+$ T cells.[172,189] Their ability to activate both NK and $CD8^+$ T cells is important as it allows for the targeting of both MHC-positive and MHC-negative tumor cells.[190] Activated NKT cells can also recruit and induce the maturation DCs. DCs found in the TME are mostly immature and inept at activating T cells.[66,191] The binding of DC-expressed CD40 to NKT cell-expressed CD40L matures the DC, increasing its co-stimulatory receptor expression and IL-12 production.[192] IL-12 can then stimulate NKT, NK, and Th1 $CD4^+$ and $CD8^+$ T cells, leading to a robust anti-tumor immune response.[193,194]

Tumor associated macrophages (TAMs) and MDSCs are immunosuppressive immune cells which are highly prevalent in the TME and contribute to tumor progression and suppression of NK and T cell responses.[63,195] In neuroblastomas, NKT cells can co-localize and lyse TAMs via a CD1d-dependent mechanism.[196,197] Furthermore, release of granulocyte-macrophage colony-stimulating factor (GM-CSF) from NKT cells can reprogram M2 TAMs to inflammatory M1 macrophages, reducing TAM-mediated immunosuppression.[198,199] NKT cells can inhibit the arginase 1-mediated suppressive activity of MDSCs in a CD1d and CD40-dependent manner.[200] Activation of NKT cells by α-GalCer leads to reduced frequency of MDSCs in a 4T1 model.[201] Furthermore, α-GalCer-loaded MDSCs can convert immature MDSCs to mature APC capable of eliciting an NK and T cell immune response.[202] In summary, activation of NKT cells can limit the function and quantity of several immunosuppressive populations in the TME.

1.5.2 α-GalCer and NKT cell activation

α-GalCer is a glycolipid NKT cell antigen derived from the marine sponge *Agelas mauritianus*. α-GalCer is comprised of a galactose carbohydrate α-linked to a ceramide backbone comprised of a C26:0 acyl chain and 18-carbon phytosphingosine chain (Figure 2).[203] The acyl chain and phytosphingosine are placed in the A' and F' pockets of the CD1d binding groove, leaving the galactose carbohydrate exposed directly to the NKT cell.[203] Upon recognition of glycolipid antigen, NKT cells secrete large amounts of cytokines, including IFNγ, TNF, and IL-4, which influence downstream immune activity.[172,189] Chemical modifications to α-GalCer can produce derivative compounds that skew NKT cell production towards either a Th1 or Th2 immune response. Three analogs that promote a stronger Th1 response compared to α-GalCer are α-C-galactosylceramide (α-C-GalCer), 7DW8-5, and ABX196, while one analog that promotes a stronger Th2 response is OCH (Figure 2).

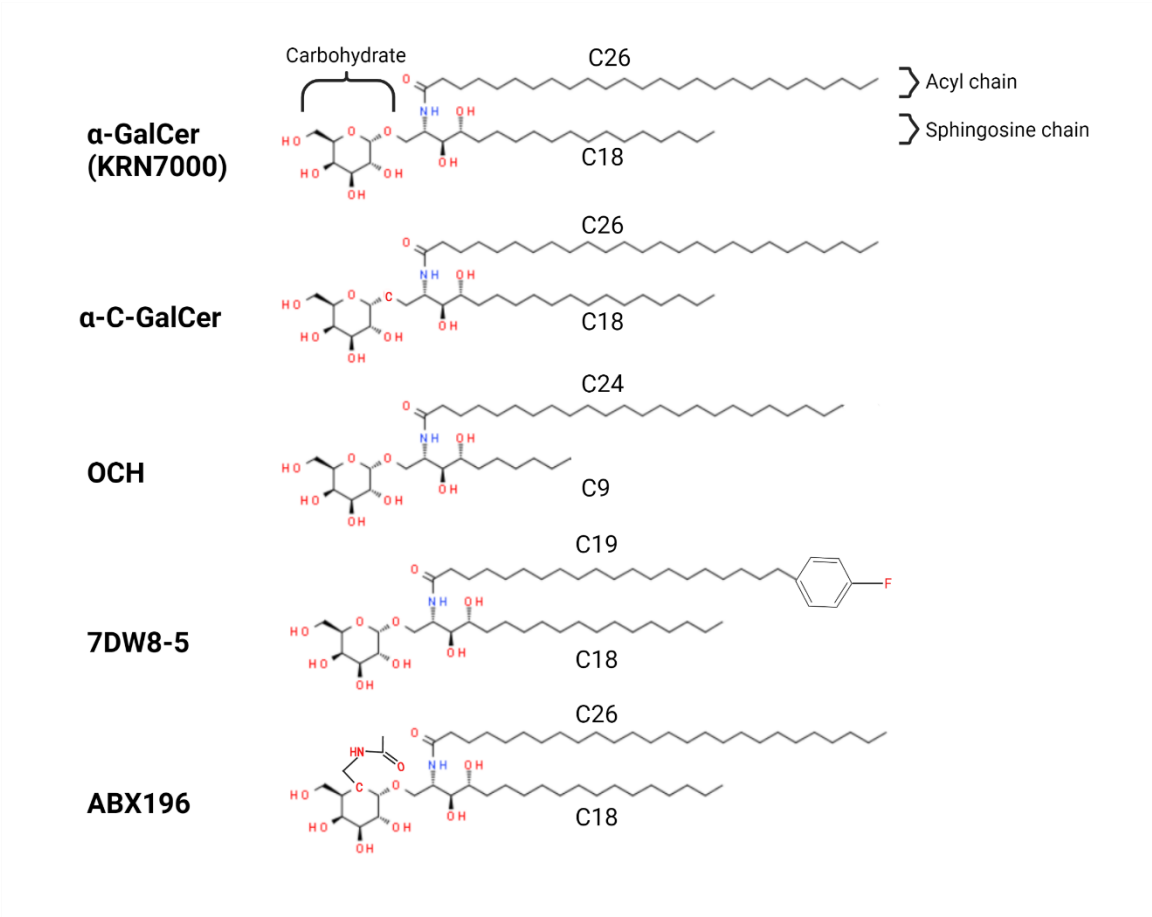


Figure 2: Structure of α-GalCer and its analogs. α-GalCer is comprised of a galactose carbohydrate α-linked to a ceramide backbone comprised of a C26:0 acyl chain and 18-carbon phytosphingosine chain. The acyl chain and phytosphingosine are placed in the CD1d binding groove, leaving the galactose carbohydrate exposed directly to the NKT cell. α-GalCer contains a O-glycoside linkage whereas α-C-GalCer contains a C-glycoside linkage. OCH has a shorter acyl and phytosphingosine chain compared to α-GalCer. 7DW8-5 has a shorter acyl chain which ends in a fluorinated benzene ring. ABX196 has an amide attached to the C6 carbon of the galactose. Adapted from[204]

α-GalCer contains O-glycoside linkage whereas α-C-GalCer contains a C-glycoside linkage, further skewing NKT cells towards Th1 and producing higher levels of IFNγ compared to activation by α-GalCer.[205,206] This is largely due to *C*-Glycoside analogs being more resistant to degradation.[206] 7DW8-5 has a shorter acyl chain which ends in a fluorinated benzene ring.[207] Compared to α-GalCer, 7DW8-5 has a stronger binding affinity to CD1d and the TCR of NKT cells, stimulating greater production of IFNγ and IL-2 by NKT cells.[207] Administration of ABX196 resulted in similar levels of IFNγ production by NKT cells compared to α-GalCer, however NKT cells activated by ABX196 produced significantly less IL-4, demonstrating increased Th1 skewing.[208] In melanoma, colon carcinoma, and bladder cancer models, ABX196 alone and in combination with anti-PD-1 increased tumor regression and overall survival.[209] ABX196 in combination with anti-PD-1 is currently being examined in a phase I/II clinical trial (NCT03897543). Compared to α-GalCer, OCH has a shortened sphingosine chain and induces preferential production of IL-4 by NKT cells, inducing a Th2 phenotype.[210,211] Therefore, OCH is useful in treating autoimmunity, where a Th2 phenotype is more desirable. These analogs of α-GalCer continue to be an active area of research, with the goal of improving NKT cell immunotherapy in cancer patients.

1.5.3 Clinical trials with NKT cell immunotherapy

The promising preclinical data demonstrating the ability of NKT cells to reduce tumor growth and metastasis in several cancer models has resulted in several clinical trials being conducted. An early clinical trial found that direct administration of α-GalCer was well tolerated, having no dose-limiting toxicity; however, it failed to yield a discernable clinical response and only 7/24 patients achieved stable disease.[212] Various factors likely

contributed to this low efficacy, including varying numbers of NKT cells in patients, low NKT cell numbers at baseline, and induction of anergy by repeated administration of free α-GalCer.[212] Multiple strategies have been tested to overcome the limitations of α-GalCer administration. Multiple clinical studies have used autologous DCs, or APCs pulsed with α-GalCer. In clinical trials in myeloma, non-small cell lung cancer (NSCLC), and head and neck cancer, α-GalCer-loaded-DCs were well-tolerated with no serious adverse events.[213–216] Treatment resulted in increased IFNγ production and expansion of NKT cells, leading to stable disease in many patients and increased median survival times.[213–216] Although α-GalCer-loaded-DCs show a improved immune response relative to direct administration of α-GalCer, this strategy still faces hurdles that limits its efficacy. Treatment is still dependent on baseline levels of NKT cells, which are often decreased in cancer patients.[177,217–219] Furthermore, it is difficult to obtain a large number of autologous DCs from immunocompromised cancer patients. Another strategy is to expand and activate NKT cells *ex vivo* and adoptively transfer them into the patient. Clinical trials using NKT cell adoptive transfer in NSCLC and advanced melanoma increased circulating NKT cell number and IFNγ production with no serious adverse effects.[220,221] However, very few patients showed any reduction in tumor progression. While α-GalCer-loaded-DCs and NKT cell adoptive transfer improved the immune response relative to direct administration of α-GalCer, limited clinical responses were observed. To overcome this, NKT cell immunotherapy strategies were used in combination approaches. In head and neck cancer, a combination treatment of *ex vivo* expanded NKT cells and α-GalCer-loaded-DCs led to increased circulating NKT cells and IFNγ production, resulting in stable disease or tumor regression in a majority of

patients.[222] However, compared to monotherapy, the combination treatment led to more mild to serious adverse events.[222] To date, lenalidomide is the only chemotherapy to be combined with NKT cell immunotherapy in a clinical trial. Lenalidomide treatment has shown to modulate immune function, altering cytokine production, NK cytotoxicity, and T cell activation.[223] Similarly, pre-treatment with lenalidomide increases NKT cell expansion, activation, and IFNγ production in both *in vitro* and *ex vivo* making it a good candidate for combination therapy.[224] Furthermore, patients with myelodysplastic syndrome treated with lenalidomide have increased NKT cell cytokine production, demonstrating similar effects *in vivo.*[225] In a phase I clinical trial, patients with asymptomatic myeloma were treated with α-GalCer-loaded-DCs in combination with lenalidomide, followed by continuous treatment with lenalidomide. [213] Treatment was well-tolerated with only one patient having a grade 3 adverse event. Treatment led to increased activation of NKT cells, NK cells, monocytes, and eosinophils, demonstrating strong innate immune activation.[213] Furthermore, NKG2D was significantly increased on NK cells, indicating increased NK cytotoxic potential.[213] Overall, treatment led to decreased tumor associated-immunoglobulin in all but one patient, indicating decreased tumor burden.[213] Clinical trials using NKT cell immunotherapy in combination with other therapeutics, such as checkpoint inhibitors and oncolytic viruses, still need to be conducted.

Recent clinical trials have looked at the efficacy of chimeric antigen receptor (CAR)- expressing NKT cells. A CAR is composed of the transmembrane domain and endodomain of CD3ζ linked to a single chain variable fragment of a monoclonal antibody that recognizes a specific antigen. CAR-T cells engineered to target CD19 have seen

significant clinical success in targeting B cell malignancies and can provide curative outcomes for many patients.[226] However, there are many advantages to using a CAR-NKT cell over a CAR-T cell, including reduced cytokine release syndrome and risk of graft vs. host disease.[227] CAR-NKT cells targeting GD2 (NCT03294954) and CD19 (NCT03774654). have shown great efficacy in preclinical models and are now being tested in phase I clinical trials.[228–230] Initial results have shown the treatment is safe with no dose limiting toxicities, including cytokine release syndrome.[231,232] Initial results also indicate that treatment is at least partially effective: of the 15 patients enrolled between the two trials, there were three complete responses, four partial responses, and three patients with stable disease.[231,232] Furthermore, the anti-CD19 CAR-NKT cells used in were allogenic cells and showed no graft vs. host disease,[232] demonstrating the potential for an off-the-shelf therapy.

1.6 Immune checkpoint inhibitors

The suppression of the immune system is a critical mechanism for tumor growth. A major immunoregulatory mechanism employed by tumors is the use of immune checkpoint receptors. Immune checkpoints receptors are immunoregulatory receptors on leukocytes that are critical in maintaining self-tolerance and modulating immune responses to diminish collateral damage.[233,234] Cancer cells often upregulate expression of immune checkpoint ligands to inhibit immune cells from targeting them;[122] therefore, blocking this interaction can be an effective way to increase immune targeting of tumor cells.

Immune checkpoint inhibitors (ICIs) are monoclonal antibodies that block the interaction between an immune checkpoint receptor and its ligand, stopping the inhibitory

signal and increasing the immune response towards the tumor. Common ICIs are ipilimumab (anti-CTLA-4);[235] nivolumab and pembrolizumab (anti-PD-1);[236,237] and avelumab and atezolizumab (anti-PD-L1).[238] All have been approved by the FDA for treatment of melanoma, bladder cancer, or NSCLC.

Ipilimumab (anti-CTLA-4) was the first ICI to be approved by the FDA. It was approved in 2011 for treatment in melanoma.[239] CTLA-4 is constitutively expressed on Tregs but only expressed on activated T cells.[233] CTLA-4 binds to the costimulatory receptors CD80/CD86 on APCs, displacing the costimulatory receptor CD28 that signals to support T cell activation and survival. In contrast, when CTLA-4 binds CD80/CD86 on APCs, it results in the activation of immunoregulatory Src homology 2 domain-containing protein tyrosine phosphatase-1/2 (SHP-1 and SHP-2), inhibiting T cell function.[240] CTLA-4 binding to CD80/CD86 on tumor-associated APCs limits the anti-tumor immune response in the TME, allowing tumor cells to escape targeting by the immune system.[241,242] Contrary to traditional T cells, CTLA-4 engagement on Tregs enhances their suppressive function.[243,244] Preclinical models showed blocking CTLA-4 with ipilimumab induced regression in many tumor models.[245,246] This action was mediated by $CD8^+$ T cells overcoming tolerance and maintaining activity against the tumor. The prolonged $CD8^+$ T cell response was dependent on $CD4^+$ T cells and IL-2, as mice who were deficient in either CD4 or IL-2 did not have an increased $CD8^+$ T cell response.[247] Additionally, anti-CTLA-4 antibodies have been observed in mouse models to deplete Treg cells by ADCC, by engaging with FcγR (CD16) on effector cells such as monocytes, macrophages, and NK cells that mediate Treg killing.[248,249]

PD-1 is expressed on activated innate and adaptive immune cells and binds to PD-L1 on tumor cells and activated APCs, leading to activation of SHP-1 and SHP-2 and decreased immune cell function.[234] Cancer cells often upregulate PD-L1 to inhibit immune cells from targeting them.[68,69,250] Furthermore, PD-1 is highly expressed on Tregs, where binding PD-L1 can induce Treg proliferation and function, further increasing immune suppression.[137] In diseases with chronic antigen exposure like cancer, this continuous exposure leads to high expression of PD-1 that induces an anergic state. Indeed, PD-1^+ T cells have decreased cytokine expression compared to PD-1^- T cells in many cancer models.[251] Blockade of PD-1 can partially reverse this anergic state.

PD-1 blockade in preclinical tumor models has shown significant promise. In both murine and humanized melanoma models, PD-1 blockade decreased tumor growth by increasing infiltration and activation of $CD4^+$ and $CD8^+$ T cells; enhancing $CD8^+$ T cell cytotoxicity; as well as increasing production of IFNγ, CXCL10, and IL-2 .[252,253] PD-1 blockade was shown to be more effective in tumors with a higher accumulation of $CD8^+$, $PD1^+$ and PD-$L1^+$ cells.[254] Patients with metastatic melanoma who were given pembrolizumab had a strong increase in $Ki67^+$ granzyme B^+ $CD8^+$ T cells in the blood and tumor, indicating a strong T cell response.[255] Pembrolizumab also led to a 6 month progression-free survival of 47.3 % compared to ipilimumab's 26.5%.[256] Additionally, pembrolizumab caused less grade 3-4 adverse advents (13.3% to 19.9%), indicating that pembrolizumab may be better at treating advanced melanoma then ipilimumab.[256] PD-1 and CTLA-4 blockade have often been used in combination. Combination treatment with anti-PD-1 and CTLA-4 increased the response rate and tumor regression in melanoma

patients compared to CTLA-4 monotreatment.[257,258] However, this came with more serious adverse effects.[259]

Blockade of PD-L1 has similar outcomes to blocking PD-1, resulting in increased $CD8^+$ T-cell cytotoxicity, IL-2 and IFNγ production, and reduced tumor progression.[255] Similar to PD-1 blockade, anti-PD-L1 has been shown to be more effective when there is higher tumor expression of PD-L1.[254] PD-L1 blockade has been successful in cancers with high expression of PD-L1, such as NSCLC. Treatment of NSCLC with atezolizumab increased median overall survival by 4.2 months when compared to the chemotherapy docetaxel.[260]

While blockade of CTLA-4 and the PD-1/PD-L1 axis have seen the most clinical success, other immune checkpoint receptor blockades are starting to emerge as promising therapeutics. Treatment with anti-PD-1 increases expression of T cell immunoglobulin-3 (TIM-3), LAG-3, and T cell immunoglobulin and ITIM domain (TIGIT) which leads to ICI acquired resistance.[261] Similar to PD-1, T cells that express TIM-3, LAG-3, and/or TIGIT are anergic and have reduced anti-tumor function compared to T cells that are negative for these receptors.[262] Blockade of TIM-3, LAG-3, and/or TIGIT have shown promising results in preclinical models, especially when used in combination with anti-PD-1.[263–266] While no ICI targeting TIM-3, LAG-3, or TIGIT has been approved for treatment, many are currently in clinical trials.[267,268]

1.7 Oncolytic viruses

Immunotherapy is emerging as a safe and effective way to treat cancer. However, a major roadblock facing immunotherapy is its ineffectiveness in “cold” tumors lacking immune cell infiltration, or tumors that have mainly immunosuppressive immune populations.[269]

Converting a cold tumor to a hot tumor is an ongoing theme of immunotherapy research, in an effort to improve outcomes and widen the patient population. One strategy is to combine immunotherapy with therapies that boost tumor immune infiltration, with one example being oncolytic viruses (OVs).

OVs are either wildtype or genetically-modified viruses that selectively infect, replicate in, and lyse malignant cells by taking advantage of dysregulated signaling pathways, altered receptor expression, or metabolism.[270–272] The selectivity of the OV can be accomplished in several ways: 1) the receptor the OV uses is upregulated on cancer cells, 2) genetic modification of the OV making it unable to replicate in normal cells, or 3) natural sensitivity to a protein found in normal cells that is absent or downregulated in tumor cells. This increased selectivity of OVs is meant to ensure that they have minimal adverse effects, giving them a distinct advantage over traditional treatments such as chemotherapy and radiation. OVs can kill tumor cells by three mechanisms: 1) direct lysis of tumor cells, 2) destruction of the TME and, 3) stimulation of the immune system towards the tumor by release of both viral pathogen-associated molecular patterns and tumor DAMPs (Figure 3).[273,274]

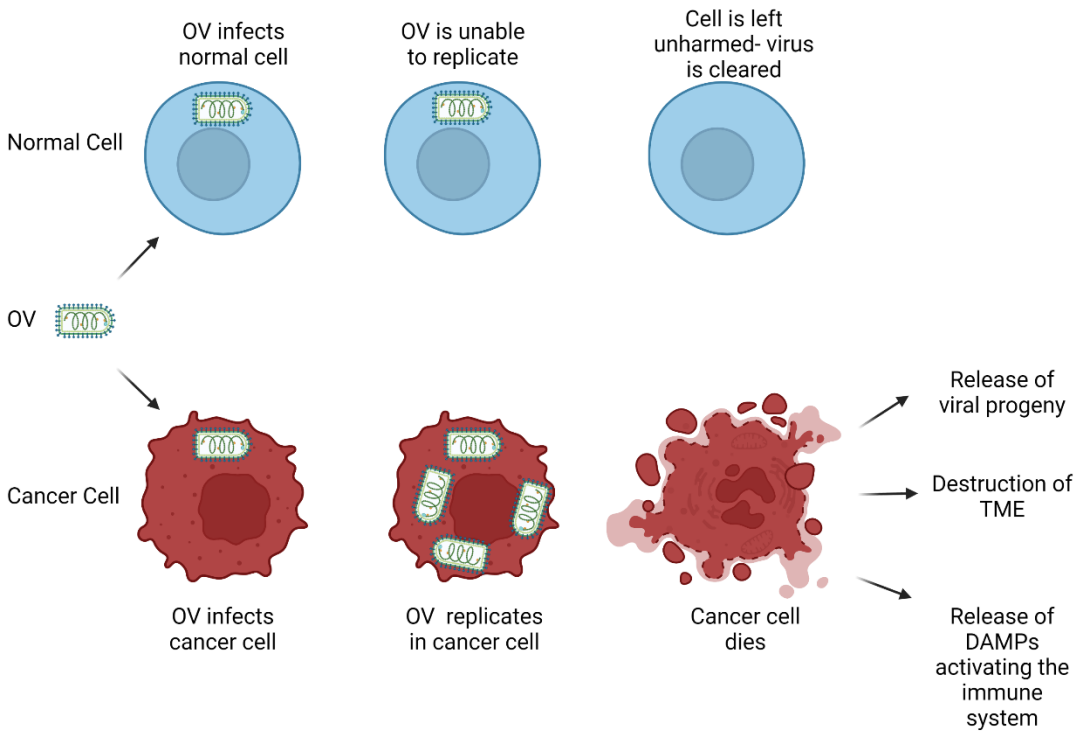


Figure 3: Schematic of oncolytic virus infection. Oncolytic viruses preferentially infect cancer cells by taking advantage of altered metabolism, signalling pathways or receptor expression. When an oncolytic virus infect a normal cell, it is unable to replicate due to the cells anti-viral response. The virus is cleared and left unharmed. When an oncolytic virus infects a cancer cell, it is able to replicate due to the cancer cells aberrant anti-viral response. Viral replication leads to cancer cell death, leading to release of viral progeny, destruction of the tumor microenvironment and release of damage associated molecular patterns that can activate anti-tumor immunity. DAMPS = damage associated molecular patterns OV= oncolytic virus, TME= tumor microenvironment,

After infection, the OV replicates, leading to cell lysis and viral spread to other cancer cells. While previous work has focused on the ability of OVs to directly lyse tumor cells, there is increasing evidence pointing to the importance of OVs in activating anti-tumor immunity.[275–277] The TME contains many cell types, including endothelial cells, cancer-associated fibroblasts, Tregs, immature DCs, and MDSCs, all working together to create an environment that promotes tumor growth and immune suppression.[278] OVs can destroy the TME by lysing cancer-associated fibroblasts and vascular endothelial cells, promoting proinflammatory cytokine release and decreasing immune suppression by Tregs and MDSCs, resulting in inflammation of the tumor site and increased anti-tumor immune activity.[279]

Talimogene laherparepvec (T-VEC) is an oncolytic herpes simplex virus-1 expressing GM-CSF. It is currently the only oncolytic virus approved by the FDA. In a phase I clinical trial, intratumoral injections of melanoma lesions with T-VEC led to necrosis and pyroptosis-induced cell death.[280] T-VEC injections increased expression of perforin and granzyme B by tumor infiltrating $CD8^+$ T cells, indicating an increase in anti-tumor immunity.[281] Furthermore, there were decreased numbers of $CD4^+$ and $CD8^+$ Tregs in the tumor and peripheral blood, demonstrating decreased immunosuppression.[281] This increased anti-tumor immunity led to an increase in median survival of 23.2% compared to GM-CSF-treated melanoma patients; however, only 26.4% of patients responded to T-VEC.[281] To improve the response rate and median survival, T-VEC is being studied in combination with ICIs. T-VEC in combination with ipilimumab achieved a response rate of 50% compared to 26.4% with T-VEC alone, though higher toxicity was observed.[281,282] The total number of $CD8^+$ T cells as well as the proportion of

activated $CD8^+$ T cells were increased in the peripheral blood in response to combination treatment.[282] $CD4^+$ T cells expressing inducible T cell costimulator were also increased in the peripheral blood.[282,283] A phase III clinical trial is currently ongoing to evaluate the efficacy of T-VEC combined with other immune checkpoint inhibitors.[284] The success of T-VEC demonstrates a place for OVs in cancer treatment. However, more research needs to be done to bring other OVs to patients.

1.7.1 Vesicular stomatitis virus

Vesicular stomatitis virus (VSV) is an enveloped, negative-strand RNA virus from the *Rhabodoviridae* family. VSV has a 11 kilobase genome that codes for five genes: the glycoprotein (G), large protein (L), matrix protein (M), nucleoprotein (N), and phosphoprotein (P). The L and P protein combine to make the viral RNA polymerase and replicate viral mRNA.[285] The N protein initiates VSV genome replication and encapsulates its RNA to protect it from nucleases.[286] The VSV G protein mediates viral entry into the cell by binding to the host low density lipid (LDL) receptor.[287] The LDL receptor is broadly upregulated on many types of cancer cells, making VSV preferentially infect cancer cells.[288] Furthermore, the LDL receptor is upregulated on many cancer subtypes including TNBC and pancreatic cancer.[289,290] Following binding to the LDL receptor, VSV is endocytosed and fuses with an endosome membrane leading to the release of the VSV genome into the cytoplasm. The VSV M protein plays a major role in viral assembly and budding. Importantly, VSV M protein also inhibits type I interferon (IFN) mRNA nuclear export by interfering with the host RAE1-NUP98 complex, inhibiting the host anti-viral response.[291] To induce preferential infection of cancer cells, a mutant VSV virus was generated with a deletion of a methionine in the M protein

(VSV-ΔM51), inhibiting the ability of VSV to block type I IFN nuclear export and making it unable to replicate in type I IFN-producing cells.[292] Cancer cells often have defects in type I IFN signalling, allowing VSV-ΔM51 to replicate in cancer cells, but not non-transformed cells.[293]

VSV-ΔM51 has shown great success in a variety of preclinical models including breast and pancreatic cancer.[275,294–296] While VSV-ΔM51 can infect and lyse a wide range of cancer types, there is growing evidence that VSV-ΔM51's enhances the anti-tumor immune response.[276,297] VSV-ΔM51 infection increased anti-tumor function of $CD8^+$ T cells, NK, and NKT cells causing increased tumor regression in multiple cancer models.[275,298–300] Furthermore, VSV-ΔM51 oncolysis mobilizes DAMPs, inducing markers of immunogenic cell death (ICD) such as membrane-bound calreticulin (CALR), and the release of adenosine triphosphate (ATP), CXCL10, and high mobility group box 1 (HMGB1).[275] These ICD mediators lead to increased immune infiltration, activation, and DC maturation, increasing immune activation towards the tumor and promoting tumor regression.[301] Furthermore, the increase inflammatory signalling can increase subsequent immunotherapy, including NKT cell immunotherapy.[275]

The VSV-ΔM51 genome is amenable to reverse genetics, allowing for genes that increase virulence or immunogenicity to be added to the VSV-ΔM51 genome and expressed at the site of infection.[302] VSV-ΔM51 variants engineered to express different immunomodulating proteins increase NK and T cell infiltration and IFNγ production leading to increased tumor regression compared to VSV-ΔM51.[303–306] VSV-ΔM51 engineered to express TAAs increased $CD8^+$ T cell activation, tumor regression, and overall survival in B16 tumor models.[307] VSV-ΔM51 engineered to express IFNβ

increased $CD8^+$ T cell tumor infiltration and decreased immune suppression in NSCLC.[308] Furthermore, this virus induced immune memory formation, slowing tumor growth when mice were rechallenged.[308] The preclinical success of VSV-ΔM51 expressing IFNβ has led to its use in clinical trials. The virus is currently being used in phase I/II clinical trials in various cancers either as a monotherapy (NCT01628640) or combined with chemotherapy (NCT03120624) or ICIs (NCT03647163).

1.8 Interleukin-15

Interleukin-15 (IL-15) is a key signalling molecule in the anti-tumor immune response. IL-15 is a common γ chain (γc) cytokine produced by myeloid and intestinal epithelial cells with pluripotent effects on both innate and adaptive immune cells.[309] The IL-15 receptor is expressed on a wide range of immune cells. The IL-15 receptor is a heterotrimeric receptor compared of three subunits: a α subunit specific for IL-15 (IL-15Rα), a β subunit shared with interleukin-2 (IL-2Rβ), and the common γc shared with the γc cytokine family.[310] IL-15 can bind its receptor and signal via two mechanisms: cis-presentation and trans-presentation (Figure 4). Cis-presentation is when free IL-15 binds to the trimeric IL-15 receptor complex on an immune cell, leading to Janus kinase-signal transducer and activator of transcription (JAK/STAT) signalling and gene transcription.[310,311] Trans-presentation is when IL-15Rα and IL-15 are produced by the same dendritic cell allowing for intercellular binding of IL-15 to the IL-15Rα in the endoplasmic reticulum, before transporting the complex to the cell surface.[312,313] There it binds the IL-2Rβ and γc subunit complex on another immune cell, leading to JAK/STAT signalling and gene transcription. While both cis and trans-presentation lead to the same gene transcription, cis-presentation produces a faster, transient response, whereas trans-

presentation produces a slower, more persistent response.[314] IL-15 signalling is conserved across species, as human IL-15 can induce IL-15 signalling in murine cells, however the reverse is not true.[315,316] IL-15 signalling in immune cells leads to transcription of genes associated with increased survival, proliferation, and function, leading to enhanced accumulation and anti-tumor function of NKT, NK, and T cells.[317–319] IL-15 is also essential for proper NK cell development.[320] DCs cultured with IL-15 increase CD86, CD40, and MHC II expression, increasing their ability to stimulate T cells.[321] IL-15 signalling may also inhibit the immunosuppression of the TME. IL-15 makes T cells resistant to Treg immunosuppression and IL-15 does not enhance survival of Tregs.[322–324] However, the effects of IL-15 on tumor-associated immunosuppression need to be further elucidated.

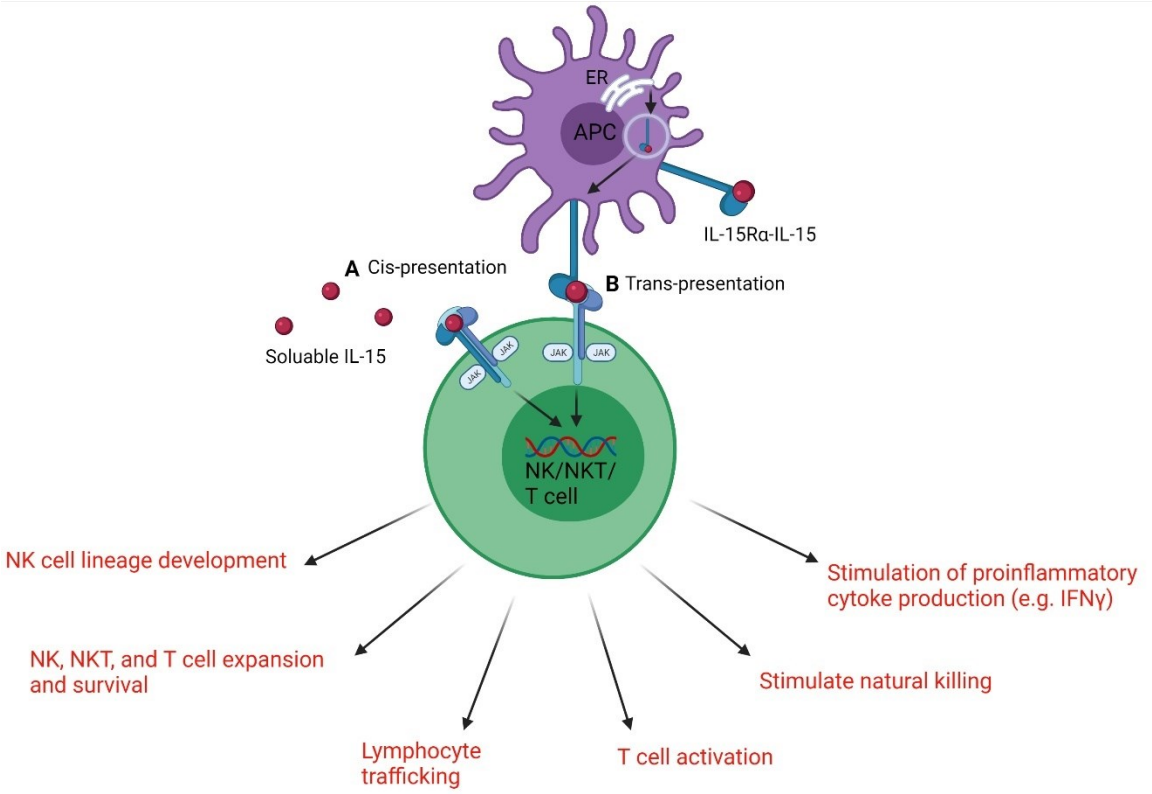


Figure 4: Cis and trans presentation of IL-15 to lymphocytes leads to increased anti-tumor immune activity. IL-15 has two mechanisms of signaling: A) cis-presentation and B) trans-presentation. A) Soluble IL-15 binds to IL-15 receptor alpha (IL-15Rα) which in turn associates with the IL-2 receptor beta (IL-2Rβ) and common gamma chain (γc) on the same cell. B) IL-15 and IL-15Rα are produced and bound together in the ER of an antigen presenting cell. The IL-15Rα-IL-15 complex is then trafficked to the cell surface where it binds to the IL-2Rβ and γc of another cell. Both cis-presentation and trans-presentation lead to the same downstream signaling and gene transcription, resulting in increased expansion, survival, and anti-tumor function of NK, NKT, and T cells.

1.8.1 IL-15 in immunotherapy

Due to the far-reaching effects of IL-15 on immune cells with important anti-tumor functions, its potential as an immunotherapeutic has been an area of great research interest. The direct administration of soluble IL-15 enhances NK and $CD8^+$ T cell anti-tumor function in a wide range of mouse tumor models.[325–328] However, direct administration of soluble IL-15 induces immune checkpoint receptor expression and increased IL-10 production by $CD8^+$ T cells, dampening the immune response in turn.[326] Administration of ICIs with IL-15 increases the efficacy of IL-15 immunotherapy, demonstrating that free IL-15 immunotherapy can be effectively combined with other immunotherapies.[326]

A major limitation of soluble IL-15 is its short half-life, limiting its efficacy.[329] To increase the efficacy of IL-15 immunotherapy, IL-15 linked to IL-15Rα (IL-15 superagonists) have been developed. These IL-15 superagonists have greater potency and stability compared to free IL-15, extending its half-life and therapeutic potential.[330,331] Furthermore, IL-15 superagonists do not require trans-presentation or cell-to-cell contact to induce IL-15-mediated responses. In multiple preclinical models, IL-15 superagonists improved survival and reduced tumor progression.[332–336] Their antitumor effects were mediated by increased T cell and NK cell infiltration and production of IFNγ, TNF, CD107a, granzyme B, and perforin.[332–336] Furthermore, some IL-15 superagonists have been linked to a IgG1 Fc domain, increasing ADCC and further increasing granzyme B and perforin release from NK cells.[337] Similar to soluble IL-15, IL-15 superagonists

increase the efficacy of ICI therapy, leading to clinical studies examining combination therapy of ICIs and IL-15 superagonists.[334,335]

OVs have been engineered to express IL-15 and IL-15 superagonists,[304,338,339] allowing for localized expression of the cytokine at the site of the tumor. In a CT26 lung metastasis model, VSV expressing IL-15 (VSV-IL-15) improved survival and the anti-tumor immune response.[304] Importantly, VSV-IL-15 out performed VSV expressing green fluorescent protein (GFP) (VSV-GFP) in combination with systemic administration of IL-15,[304] demonstrating that local delivery of IL-15 was superior. Adenovirus and vaccina virus expressing IL-15 superagonists increased overall survival and the anti-tumor immune response in models of hepatocellular carcinoma and colon carcinoma, respectively.[338,339] Interestingly, the adenovirus expressing an IL-15 superagonists lead to better overall survival and anti-tumor response when directly compared to adenovirus expressing IL-15,[339] indicating that an OV IL-15 superagonists are more effective than OVs expressing IL-15. Vaccina virus expressing a IL-15 superagonist also increased the efficacy of subsequent anti-PD-1 compared to vaccina virus with no genetic insert,[338] indicating the ability of OVs expressing IL-15 or IL-15 superagonists to increase subsequent immunotherapies.

Both free IL-15 and IL-15 superagonists have been tested in clinical trials. Free IL-15 increased $CD8^+$ T cell and NK cell proliferation, IFNγ production, and killing, leading to stable disease.[329,340] However, dose-limiting toxicities were observed at therapeutic doses.[329,340] IL-15 superagonists are currently in multiple phase I and II clinical trials, either as monotherapies or in combination with ICIs. The IL-15 superagonist N-803 increased NK and $CD8^+$ T cell numbers and serum IFNγ and TNF in

relapsing blood cancer and solid tumors.[341–343] In a phase I trial of NSCLC patients, N-803 in combination with nivolumab (anti-PD-1) led to tumor reduction in 43% of patients, and 76% of patients achieved stable disease, further demonstrating the ability of IL-15 immunotherapy to be combined with ICIs.[343] Currently, N-803 is the only IL-15 superagonist with published clinical data, however there are multiple IL-15 superagonists currently undergoing clinical trials.[344]

1.9 Fusion associated small transmembrane proteins

Respiratory enteric orphan virus (reovirus) fusion associated small transmembrane (FAST) proteins are non-structural, accessory proteins from the *Reoviridae* genera *Aquareovirus* and *Orthoreovirus* that mediate membrane fusion.[345] FAST proteins are trafficked through the endoplasmic reticulum-Golgi secretory pathway to the plasma membrane where they induce cell-to-cell fusion, resulting in the formation of multi-nucleated syncytium.[346] FAST proteins are the smallest viral membrane fusion proteins (~100-150 amino acids) and are the only examples of non-enveloped virus membrane fusogens that induce syncytium formation.[347] Fusogenic *Aquareoviruses* and *Orthoreoviruses* encode six different FAST proteins with limited sequence similarity (p10, p13, p14, p15, p16, p22).[345] All contain three domains essential for fusion: an ectodomain, a transmembrane domain, and an endodomain. The transmembrane domain serves as a reverse signal anchor sequence to direct proper positioning in the plasma membrane with a N-terminal ectodomain and C-terminal endodomain.[345] The N-terminal functions as the fusion peptide, with myristolylation being required for fusion activity.[348] The endodomain is responsible for interaction with multiple cytoplasmic proteins, transport to the plasma membrane, and pore formation.[346,349,350] The endodomains of

FAST proteins contain a polybasic motif which is essential for cell-to-cell fusion and can function as a Golgi export signal.[346,347,351] The endodomains of FAST proteins also contain an amphipathic α-helix that promotes pore formation.[350]

1.9.1 FAST proteins in cancer treatment

FAST proteins do not bind to specific cell receptors and therefore can fuse most cells, including cancer cells.[352,353] Syncytium formation allows for the rapid, cell-cell transmission of the virus followed eventually by cell lysis.[354] Therefore, FAST proteins have been incorporated into OVs to enhance viral dissemination and cell lysis in the tumor. The FAST protein p14 has been cloned into the VSV-ΔM51 genome, where it has been observed to increase viral spread and oncolytic activity *in vitro*.[303] In mouse models of breast cancer and colon carcinoma, VSV-ΔM51 expressing p14 (VSV-p14) slowed tumor progression and increased overall survival compared to mice treated with VSV-ΔM51, demonstrating that p14 can increase the efficacy of VSV-ΔM51 virotherapy in multiple *in vivo* models.[303] Mice bearing 4T1 mammary carcinoma tumors treated with VSV-p14 had increased numbers of $CD69^+$ splenic $CD4^+$, $CD8^+$, NK, and NKT cells, and increased numbers of $CD69^+$ $CD4^+$ and $CD8^+$ T cells in tumors compared to VSV-GFP, indicating that p14 can increase the ability of VSV-ΔM51 to induce anti-tumor immunity.[303] Similarly, p14 increased a replication-defective adenovirus oncolytic efficacy, however this was model dependent.[355] The effects of other FAST proteins on the efficacy of VSV-ΔM51 will be discussed later in this dissertation.

1.10 Immunogenic Cell Death

ICD is an immunostimulatory cell death process induced by organelle stress and accompanied by the release of DAMPs (Figure 5).[356] The DAMPs produced in the

process of ICD bind to pattern recognition receptors (PRRs) on DCs, initiating downstream activation of both innate and adaptive immune cells.[357] ICD can be induced by different stressors, including oncolytic viruses, radiation therapy, and certain types of chemotherapies.[275,358,359] Furthermore, the ability of these conventional therapies to induce an anti-tumor immune response allows them to be effectively combined with immunotherapies in both preclinical and clinical settings.[296,360–362] However, different therapies cause the release of different mediators of ICD leading to differing abilities to activate an anti-tumor immune response. This thesis will discuss some of the main mediators of ICD (CALR, HMGB1, and CXCL10) and how they activate the anti-tumor immune response. However, there are other mediators including ATP, Annexin A1, heat shock protein 70, and type I IFNs that also have important roles in ICD but will not be discussed.[363]

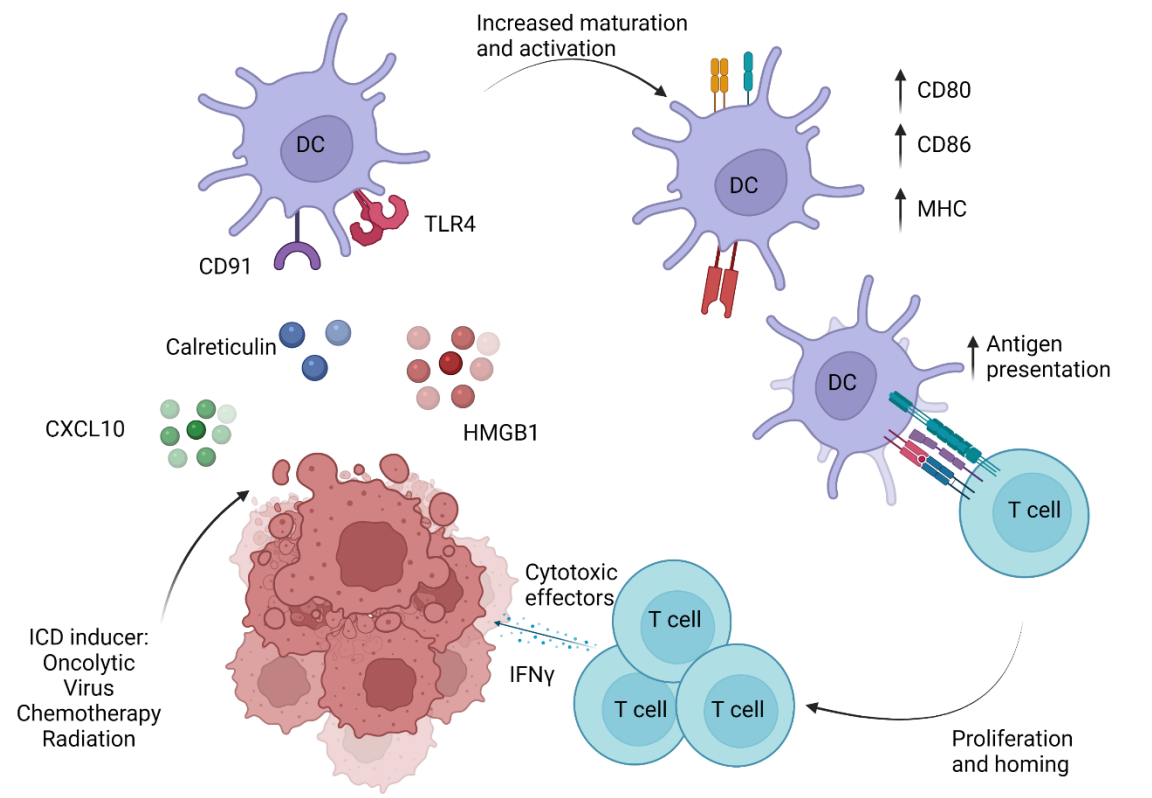


Figure 5: Mechanisms of immunogenic cell death. Cancer cells are treated with an anti-cancer therapy that induces ICD. Cancer cells undergoing ICD release damage associated molecular patterns that bind to receptors on immature DCs leading to activation and maturation. DCs travel to lymph nodes to prime T cells leading to T cell activation and proliferation, culminating in an anti-tumor response towards the tumor. DC= dendritic cell,HMGB1= high mobility group box protein 1, ICD= immunogenic cell death, IFNγ= interferon gamma, MHC= major histocompatibility complex TLR4= toll-like receptor 4.

1.10.1 Calreticulin

An important mediator of ICD is surface mobilization of CALR which functions as an "eat me" signal, enhancing uptake of tumor antigens for presentation by APCs.[364] CALR is mobilized from the ER to the plasma membrane in response to stress.[365] Surface CALR on dying cells binds to CD91 on DCs, leading to phagocytosis of the dying cells.[366] In preclinical cancer models, the use of CALR-blocking antibodies or cells engineered to not express CALR decreased anti-tumor $CD8^+$ T cell function.[364,367] Ovarian carcinoma patients with higher expression of surface CALR had increased $CD8^+$ T cell response, with higher levels of degranulation and IFNγ production compared to patients with low CALR surface expression, indicating that CALR is important for induction of an anti-tumor immune response in a clinical setting.[368] CALR surface mobilization increases NK cell activation as well. CALR surface expression in myeloid leukemia patients is also associated with IL-15 trans-presentation to NK cells, increasing their activity toward the tumor.[369] Taken together, CALR surface expression can stimulate both an innate and adaptive anti-tumor immune response.

1.10.2 High Mobility Group box 1

Cells undergoing ICD release HMGB1 into the extracellular space. Extracellular HMGB1 binds to multiple PRRs on APCs, including TLR4 and receptor for advanced glycation end-products (RAGE).[370,371] HMGB1 binding to TLR4 and RAGE on DCs induces pro-inflammatory cytokine release, DC migration, and increased antigen presentation to T cells.[370,372] However, TLR4 signalling is required for HMGB1 release upon cell death to be immunogenic.[356] In preclinical models, antibody-mediated neutralization of TLR4 or HMGB1 limited the therapeutic effect of ICD-inducing

chemotherapies.[370,373] Furthermore, HMGB1-deficient tumors have reduced ICD.[374] Patients with TLR4 loss-of-function mutations have worse disease outcomes when receiving ICD-inducing chemotherapies, indicating that HMGB1 binding TLR4 is important in both preclinical and clinical settings.[375] HMGB1 can also be released in non-immunogenic forms of cell death but is not sufficient for immune stiumlation,[356] making HMGB1 detection alone unreliable for indication of ICD. Furthermore, HMGB1 binding to TLR4 or RAGE on tumor cells can increase tumorigenesis by promoting angiogenesis, chemoresistance, cell proliferation, and metastasis, making its role in immunotherapy more complicated.[376–378]

1.10.3 CXCL10

CXCL10 is a potent chemoattractant for NK, NKT, and T cells.[379,380] CXCL10 binds to CXCR3 on NK, NKT, and T cells, directing them to tumor sites. Newer work has identified CXCL10 as another critical marker for ICD.[381] Anthracyclines, an ICD-inducing chemotherapy, increase CXCL10 production through type I IFN signalling.[381] Tumors deficient in type I IFN signalling lost their chemosensitivity, which could be restored through intratumoral inoculations of CXCL10.[381] Furthermore, tumors failed to respond to anthracyclines in the presence of antibodies blocking the CXCL10 receptor, CXCR3, further demonstrating the importance for CXCL10 in ICD induction.[381] Other ICD-inducing cancer therapies, such as OVs and radiation therapy, also increase CXCL10 production,[275,382] indicating that it could be an important mediator in multiple ICD-inducing treatments. The relevance of the other CXCR3 ligands, CXCL9 and CXCL11 in ICD, still need to be further elucidated.

1.11 Animal Models of Cancer

Murine tumor models are important systems for studying the efficacy of new immunotherapies and their effects on immune cell interactions. The work in this thesis uses two syngeneic murine cancer models: Panc02 pancreatic ductal adenocarcinoma cells on the C57BL/6 background, and 4T1 mammary adenocarcinoma cells on the BALB/c background.

Panc02 cells were developed by administration of 3-methyl-cholanthrene to the pancreas of male C57BL/6 mice.[383] Panc02 cells are the most widely-used pancreatic cancer cell line and effectively establish both subcutaneous and orthotopic tumors. Similar to human PDAC, Panc02 tumors have a low immune cell infiltration and do not respond well to ICI monotherapy.[384,385] Orthotopic tumors establish extensive fibrotic stroma, similar to human PDAC patients.[386] Unlike human PDAC, Panc02 tumors are highly susceptible to gemcitabine and radiation therapy, whereas human PDAC only see modest benefits from these therapies.[384,385,387] Furthermore, Panc02 form limited metastatic disease, while human PDAC aggressively metastasizes to the liver. To overcome this limitation, highly metastatic Panc02-H7 cells have been established.[388] Alternatively, Panc02 cells can be injected intrasplenically or intravenously (iv) to establish liver or lung metastasis, respectively.[384,389]

4T1 cells accurately mimic stage IV human breast cancer, with many characteristics that make it a good murine model for studying human breast cancer. 4T1 cells can be easily injected into the 4th mammary gland of a mouse, allowing the primary tumor to grow in the anatomically correct site. 4T1 cells aggressively metastasize to lungs, draining lymph nodes, and bone marrow, similar to human breast cancer.[390] 4T1

tumors are highly immunosuppressive, with a high number of tumor infiltrating MDSCs, similar to human breast cancer.[391] Lastly, 4T1 cells are resistant to 6-thioguanine, allowing for the examination of metastatic burden using clonogenic assays.[390]

1.12 Thesis Overview

Pancreatic cancer is one of the leading causes of cancer death worldwide, with a 5-year survival rate of less than 10%.[18,19] This is largely attributed to poor rates of early diagnosis, a high metastasis rate, and intrinsic or acquired resistance to chemotherapy.[20–22] Despite improvements in treatment options that have increased survival rates in other cancers, survival rates in pancreatic cancer have stayed relatively similar for 20 years, highlighting the need for new, effective treatment options.[18]

Metastatic breast cancer is the leading causes of cancer death in woman.[1] Since 1991, the breast cancer death rate has been declining, largely due to increased screening and new treatment options.[1] However, many of these treatments are associated with severe adverse advents, highlighting the need for safer and more effective treatments.

While NKT cell therapy has seen some success in phase I/II studies, most patients only experience stable disease, leaving considerable room for improvement.[212–216,222] One way to improve NKT cell immunotherapy is by combining it with other therapies. Previous work has demonstrated that chemotherapy and OVs can increase NKT cell immunotherapy efficacy.[275,392] However, the OVs used did not express recombinant genes that can further enhance treatment. NKT cell immunotherapy and combination therapy has also not been tested in pancreatic cancer. *I hypothesize that combining NKT cell immunotherapy with the oncolytic virus VSV-ΔM51 expressing either IL-15 or FAST proteins will increase the efficacy of NKT cell immunotherapy in mouse models of*

pancreatic and breast cancer, respectively. **To test the hypothesis in the pancreatic cancer model, I: 1) determined whether combination treatment can decrease tumor progression compared to single treatments; 2) assessed whether combination treatment increased immune infiltration and function of anti-tumor immune cells; 3) examined the combination treatment effect on immunosuppression. To test the hypothesis in the breast cancer model, I: 1) determined whether FAST proteins can increase VSV-ΔM51 oncolytic efficacy; 2) determined the ability of each FAST protein to augment VSV-ΔM51 induced ICD and immune infiltration; 3) assessed whether VSV expressing a FAST protein could increase subsequent NKT cell immunotherapy.**

The results section of this thesis is broken into two chapters. The first chapter details my work examining VSV-IL-15 in combination with NKT cell immunotherapy and anti-PD-1 checkpoint blockade in pancreatic cancer. The second chapter details my work examining VSV expressing FAST proteins in a primary 4T1 model and in combination with NKT cell immunotherapy in a 4T1 metastatic model. Overviews of each chapter are provided below.

Chapter 3: Natural killer T cell activation in combination with oncolytic vesicular stomatitis virus expressing IL-15 and anti-PD-1 induces pancreatic tumor regression in syngeneic mice

Pancreatic cancer is one of the leading causes of cancer death, with a 5-year survival rate of less than 10%. Using experimental mouse models of pancreatic ductal adenocarcinoma, NKT cell activation therapy was examined in combination with a

recombinant VSV-ΔM51 engineered to express the cytokine IL-15 and checkpoint anti-PD-1 blockade. This triple therapy enhanced immune cell activation and cytotoxicity against pancreatic cancer cells compared to single treatments and combined treatment with NKT cell activation and VSV-IL-15. Triple therapy increased survival time, with 20% of mice clearing their tumor, and slowed tumor growth upon tumor rechallenge. Therefore, combined NKT cell immunotherapy, VSV-IL-15, and anti-PD-1 blockade present a promising treatment strategy for pancreatic cancer.

Chapter 4: Natural killer T cell activation in combination with oncolytic vesicular stomatitis virus expressing fusion associated small transmembrane proteins clears breast cancer metastases

Metastatic Breast cancer is the leading cause of cancer death in woman. Using a 4T1 experimental mouse model of metastatic triple-negative breast cancer, we examined NKT cell activation therapy in combination with recombinant oncolytic VSV-ΔM51 reovirus fusion associated small transmembrane proteins p10ARV, (VSV-p10ARV), p10NBV (VSV-p10NBV), p14 (VSV-p14), p15 (VSV-p15), p14/p15 chimera (VSV-p14/p15), and mutated p14 (VSV-Δp14). Treatment with VSV-p14 or VSV-p15 enhanced overall survival time and increased immunogenic cell death compared to untreated mice and mice treated withVSV-ΔM51expressing GFP. VSV-p14 or VSV-p15 in combination with NKT cell immunotherapy cleared lung metastatic burden, with 100% of mice being metastasis-free, and inhibited tumor growth upon 4T1 rechallenge. Therefore, combined NKT cell immunotherapy with either VSV-p14 or VSV-p15 presents a promising treatment strategy for metastatic breast cancer.

CHAPTER 2: MATERIALS AND METHODS

2.1 Mice

Female C57BL/6 and BALB/c mice were purchased form Charles River Laboratories. Jα18$^{-/-}$ mice were obtained from Dr. Michell Kronenberg, La Jolla Institute for Allergy & Immunology.[393] Female and male Jα18$^{-/-}$ mice were bred at Dalhousie University. Mice were maintained in the Carleton Animal Care Facility at Dalhousie University and used at 8–12 weeks of age. Mice were group housed in temperature-controlled biocontainment rooms with a 12-hour light/dark cycle and free access to food and water. All experimental procedures were approved by the University Committee on Laboratory Animals following the guidelines of the Canadian Council on Animal Care.

2.2 Cell lines and culture

Panc02, pancreatic adenocarcinoma cells (RRID: CVCLD627) were acquired from Dr. John Bell (University of Ottawa). Panc-1 (RRID:CVCL_0480), AsPC1 (RRID:CVCL_0152), and Capan-2 (RRID:CVCL_0026) cells were acquired from Dr. Jeanette Boudreau (Dalhousie University). Vero kidney epithelial cells (RRID: CVCL_0059), 4T1 mammary carcinoma cells (RRID: CVCL_0125) and B16-F10 melanoma cells (RRID: CVCL_0159) were purchased from ATCC. All cell lines were cultured at 37ºC, 5% CO_2, in Dulbecco's Modified Eagle Medium (DMEM) (VWR Life Science, Mississauga, ON) supplemented with 10% fetal bovine serum (FBS) and 100 µg/mL streptomycin, 100 units/mL penicillin (Fisher-HyClone, Ottawa, ON). Cells were harvested in the logarithmic growth phase using trypsin-EDTA (ethylenediamine-tetraacetic acid) (Corning) treatment. Washed cells were re-suspended in phosphate buffered saline (PBS) for *in vivo* experiments.

2.3 Cell isolation

Mice were anesthetized using 2-3% isoflurane and euthanized by cervical dislocation. Liver, spleen, tumor and lymph node lymphocytes were isolated by mechanical dispersion through 70-micron wire mesh. Cells were pelleted by centrifugation at 300 x g for 8 minutes at 4°C. Liver and tumor lymphocytes were isolated by centrifugation through a 33% Percoll gradient (GE Healthcare, Baie d'Urfe, QC). Red blood cells were lysed with 5 mL of lysis buffer [150 mM NH_4Cl (Sigma-Aldrich), 10 mM $KHCO_3$ (J.T. Baker, Montreal, QC), and 0.1 mM EDTA (Sigma-Aldrich)] for 5 minutes, followed by addition of an equal volume of phosphate buffered saline (PBS; HyClone, Ottawa, ON) with 2% FBS to inhibit further lysis. Cells were centrifuged at 300 x g for 8 minutes, resuspended in PBS supplemented with 2% FBS and counted using a hemocytometer.

2.4 Bone marrow derived dendritic cells

To generate bone marrow derived dendritic cells, bone marrow was extracted from the femur and tibia of C57BL/6 or BALB/c mice and cultured in 6-well plates in complete RPMI-1640 (10% FBS, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 1X non-essential amino acids, 1mM sodium pyruvate, 100 μg/mL streptomycin, and 100 units/mL penicillin) with 40ng/mL of GM-CSF and 10ng/mL of IL-4. Cells were given fresh complete media, with 40ng/mL of GM-CSF and 10ng/mL of IL-4 on day 3. Non-adherent cells were collected and re-plated in complete RPMI-1640 with 20ng/mL of GM-CSF on day 6. α-GalCer (KRN7000; Funakoshi Ltd.) was sonicated for 20 minutes at 50ºC before being added to the DCs at 0.4μg/mL. DCs were collected the next day and 6 x 10^5 (C57BL/6) or 2x10^5 (BALB/c) were injected intravenously to induce NKT cell activation.

2.5 Antibodies and flow cytometry

The following antibodies were obtained from BD Bioscience, eBioscience, or BioLegend: fluorescein isothiocyanate-labeled NK1.1 (clone PK136), Ly6C (clone HK1.4), TCRβ (clone H57-597), CD44 (clone Im7) and CD62L (clone MEL-14); phycoerythrin-labeled TCRβ (clone H57-597), and Ly6G (clone 1A8); Peridinin-chlorophyll-protein Complex: CY5.5 conjugate-labeled CD11c (clone N418), CD8α (clone 53-6.7), CD49b (clone DX5), F4/80 (BM8), TCRβ (clone H57-597), and NK1.1 (clone PK136); Phycoerythrin: Cy-7 tandem conjugate-labeled CD4 (clone RM4-5), and CD11b (clone M1/70) CD45 (30-F11) TCRβ (clone H57-597);. Allophycocyanin-labelled F4/80 (clone BM8), CD80 (clone 16-10A1), CD11c (clone N418), CD62L (clone MEL-14), Annexin V, and TCRβ (clone H57-597). Allophycocyanin-eflour 700-labelled CD4 (clone RM4-5) and MHC II (clone M5/114.15.2); Allophycocyanin-eflour 780-labelled MHC II (clone M5/114.15.2), CD11c (clone N418), CD69 (clone H1.2F3), and CD8α (clone 53-6.7); Brilliant Violet 510 conjugate-labeled CD19 (clone 6D5); Brilliant Violet 605 conjugate-labeled CD8α (53-6.7) Brilliant Violet 650 conjugate-labeled CD25 (clone pc61) and CD11b (M1/70); Brilliant Violet 785 conjugate-labeled Allophycocyanin-labeled PD-1 (29F.1.A12); 7-Aminoactinomycin D (7AAD); Brilliant Violet 450 conjugate-labeled viability dye. Allophycocyanin-labeled and phycoerythrin-labeled CD1d tetramers loaded with the synthetic glycolipid PBS57 were obtained from the NIH Tetramer Core Facility. Cells were incubated with the viability dye for 20 minutes in the dark at room temperature. Cells were washed and incubated for 30 min at 4°C with antibody panels to stain cell subsets. Cells were then washed, and fixed in 2% paraformaldehyde (Fisher Scientific) for 20 minutes. If multiple Brilliant violet

antibodies were used, panels were prepared in brilliant violet buffer before incubation with cells. Acquisition was performed using a two-laser FACSCalibur or three-laser FACSCanto or FACSCelesta with FACS Diva software (BD Biosciences). Analysis was done using Flowjo software (BD Biosciences).

2.6 Intracellular staining

For intracellular staining, cells were fixed with 4% paraformaldehyde after staining cell-surface antigens. Cells were resuspended in permeabilization buffer (BD Biosciences) and incubated for 20mins at 4°C with phycoerythrin-labeled FoxP3 (FJK-16s) or IFNγ (XMG1.2). Samples were acquired using a three-laser FACSCanto or FACSCelesta using FACS Diva (BD Biosciences) and analyzed using FlowJo (BD Biosciences).

2.7 Cell sorting

Spleen lymphocytes were isolated by mechanical dispersion through 70-micron wire mesh. Cells were pelleted by centrifugation at 300 x g for 8 minutes at 4°C. Red blood cells were lysed with 5 mL of lysis buffer [150 mM NH_4Cl (Sigma-Aldrich), 10 mM $KHCO_3$ (J.T. Baker, Montreal, QC), and 0.1 mM EDTA (Sigma-Aldrich)] for 5 minutes, followed by addition of an equal volume of phosphate buffered saline (PBS; HyClone, Ottawa, ON) with 2% FBS to inhibit further lysis. Cells were centrifuged at 300 x g for 8 minutes, resuspended in 1 mL of PBS. 5µl of FITC-TCRβ (clone H57-597), PerCP-Cy5.5-NK1.1(clone PK136), APC-CD8α (clone 53-6.7) and PE-CD1d Tetramer were added. Cells were incubated for 30mins at 4°C. Cells were washed by addition of 10 mLs of PBS with 2% FBS and centrifuged at 300 x g for 8 mins. Cells were resuspended at 40-50 million cells per mL. A two-laser FACSAriaII cell sorter with FACSDiva software

(BD Biosciences) was used to sort cell populations (>90% purity) for *ex vivo* cytotoxicity assays.

2.8 Panc02 subcutaneous model

Panc02, pancreatic adenocarcinoma cells were cultured at 37°C, 5% CO2 in DMEM supplemented with 10% FBS, 100 units/mL of Penicillin (Hyclone), and 100µg/mL of Streptomycin (Hyclone). Cells were harvested in the logarithmic growth phase using trypsin-ethylenediaminetetraacetic acid (Corning). Cells were resuspended in saline and 2 × 10^6 cells (100 µL volume) were injected subcutaneously. Panc02 tumors were measured every second day using electronic calipers and tumor volume was calculated using (W^2 X L)/2. Tumors (~200mm^3) were injected intratumorally on days 26, 28, and 30 with either PBS, VSV-GFP or VSV-IL-15 at 5 x 10^8 plaque forming units/mouse (pfu/mouse). On Day 31 mice were injected intravenously with saline or bone marrow derived dendritic cells loaded with α-GalCer (6 x 10^5) to activate and proliferate the NKT cells. Survival and tumor volume were monitored over time. Tissues were harvested on day 38 to examine immune populations.

2.9 Anti-PD-1 treatment

Anti-PD-1 (clone RMPI-14) was purchased from Biocell. Anti-PD-1 treatment was started 3 days after NKT cell activation via dendritic cells loaded with α-GalCer on day 34 of the Panc02 subcutaneous model. Anti-PD-1 treatment was delivered intraperitoneal (ip.) weekly at 300ug per injection for 3 weeks. Tumor size was measured every second day using electronic calipers and tumor volume was calculated using (W^2 X L)/2. Overall survival was monitored. Tissues were harvested on day 38 and 55 to examine immune populations.

2.10 Panc02 orthotopic model

Panc02, pancreatic adenocarcinoma cells were cultured at 37°C, 5% CO2 in DMEM supplemented with 10% FBS, 100 units/mL of Penicillin (Hyclone), and 100µg/mL of Streptomycin (Hyclone). Cells were harvested in the logarithmic growth phase using trypsin-ethylenediaminetetraacetic acid (Corning). The surgical region was wiped with chlorhexidine followed by 70% ethanol, followed by iodine and again with 70% ethanol. An incision was made in the abdomen above the spleen. The spleen was lifted out to expose the pancreas. Cells were resuspended in saline, mixed at a 1:1 ratio with Matrigel. $5x10^5$ cells (50µL volume) were injected into the pancreas. The abdomen was sutured together using 5-0 vicryl suture (Ethicon, Somerville, NJ) and the skin was sutured together using a 5-0 polypropylene suture (Ethicon, Somerville, NJ). On days 14, 16, and 18, PBS, VSV-GFP or VSV-IL-15 (5×10^8 pfu/mouse) were injected intravenously. On Day 19 mice were injected intravenously with saline or bone marrow derived dendritic cells loaded with α-GalCer (6×10^5) to activate and proliferate the NKT cells. Survival was monitored over time. Spleens and tumors were harvested on day 26 to examine immune populations.

2.11 4T1 primary tumor model

4T1 cells were harvested in the logarithmic growth phase using trypsin-EDTA (Corning). Cells were resuspended in saline and $2x10^5$ cells (100 µl volume) were injected orthotopically into the fourth mammary fat pad of female BALB/c mice. On days 10, 12, and 14, PBS, VSV-GFP, VSV-p10ARV, VSV-p10NBV, VSV-p14, VSV-Δp14, VSV-p15, or VSV-p14/p15 (1×10^7 or 1×10^8 pfu/mouse) intratumorally. Tumor size was measured every second day using electronic calipers and tumor volume was calculated

using (W^2 X L)/2. Tumor volume and overall survival was monitored. Tissues were harvested on day 15 to examine immune populations.

2.12 4T1 metastatic model

4T1 cells were harvested in the logarithmic growth phase using trypsin-EDTA (Corning). Cells were resuspended in saline and $2x10^5$ cells (50 µl volume) were injected orthotopically into the fourth mammary fat pad of female BALB/c mice. Primary mammary tumors were resected 12 days after tumor cell injection when the primary tumors reached ~200 mm^3 in size. Primary subcutaneous tumor excision was performed aseptically in anesthetized mice (2-3% inhaled isoflurane). The surgical region was wiped with chlorhexidine followed by 70% ethanol, followed by iodine and again with 70% ethanol. The tumor and subcutaneous fat tissue were surgically resected, and the skin was sutured together using a 5-0 polypropylene suture (Ethicon, Somerville, NJ). Mice received a subcutaneous treatment of 0.1 mg/kg buprenorphine (BCM Corporation; Bloomingdale, NJ) as analgesic. Animals were transferred to clean cages and allowed to recover overnight on a 42°C heating pad. On days 13, 15, and 17, PBS, VSV-GFP, VSV-p14 or VSV-p15 (5×10^7 or 5×10^8 pfu/mouse) intravenously. On day 19 mice received α-GalCer-loaded DCs ($2x10^5$) intravenously to active NKT cells. Overall survival was monitored. Spleens and lungs were harvested for immune phenotyping and clonogenic assays on day 26.

2.13 Clonogenic assay

Clonogenic assays were caried out as previously described.[201] Lungs were harvested and dissociated by mechanical dispersion through a sterile 40-micron nylon mesh. Cells were washed, centrifuged at 300 x g for 8 minutes, and resuspended in 5 mL of clonogenic

assay media (RPMI-1640 supplemented with 10% FBS, 100 µg/mL streptomycin, and 100 units/mL penicillin, 20 nM HEPES, 60 µM 6-Thioguanine). In a 6 well plate, different dilutions (400µl, 200µl, 100µl) of the lung cell suspension were added to a final volume of 2 mLs. After 10 days in culture, cell colonies were fixed with 1 mL 95% methanol for 5 minutes, washed twice with 1 mL of distilled water, and stained with 1mL of 0.03% methylene blue. The number CFU were counted. The number of CFU in the whole lung tissue was calculated as: (5 x # colonies in 400µl plate + 10 x # colonies on 200µl plate + 20 x # colonies on 100µl plate)/3.

2.14 Oregon green staining

2 x 10^7 naïve splenocytes, Panc02, B16, and 4T1 cell in PBS were stained with 5 µM Oregon green (Life Technologies) for 8 minutes at room temperature. Dye was quenched using RPMI-1640 (supplemented with 100 µg/mL streptomycin, and 100 units/mL penicillin, 10% FBS and 1% HEPES) and was washed two more times to remove extra dye. Cells were recounted on a hemocytometer to account for cell death. Oregon green-labeled cells were then used in suppression and cytotoxicity assays.

2.15 T cell suppression assay

50µL of blood was drawn via submandibular puncture from naïve and tumor bearing mice. Cells were resuspended in 200µL of RPMI-1640 media (supplemented with 100 µg/mL streptomycin, and 100 units/mL penicillin, 10% FBS and 1% HEPES) following red blood cell lysis, and 50µL was added to each well in a round bottomed 96 well plate. Responder splenocytes were isolated from the spleens of naïve mice by mechanical dispersion through wire mesh followed by red blood cell lysis. Responder cells were labeled with 5 µM Oregon green (Life Technologies) as previously described.

Naïve splenocytes (2×10^5) were combined in wells with blood leukocytes. Proliferation of T cells were induced by adding T activator anti-CD3/28 Dynabeads (Gibco Life Technologies) in a ratio of 1 bead:2 splenocytes. Cocultures were incubated at 37°C 5% CO2 for 72 hrs. Samples were then stained with Allophycocyanin-labelled TCRβ (clone H57-597) as described in 2.6. $TCR\beta^+$ T cell proliferation was assessed by organ green dilution using flow cytometry.

2.16 Cytotoxicity assay

$CD8^+$ $TCR\beta^+$ T cells, $TCR\beta^-$ $NK1.1^+$ NK cells, and CD1d $tetramer^+$ $TCR\beta^+$ were isolated from the spleen of treated mice by bead separation or FACS sorting and co-cultured at a 1:1 ratio with Oregon green-labelled Panc02, B16, or 4T1 cells for 18hrs in complete DMEM. After incubation supernatant was collected to examine IFNγ and TNF production. Oregon green-labelled Panc02, B16 or 4T1 cells were examined by flow cytometry using allophycocyanin-labeled annexin V (BioLegend) and 7-amino-actinomycin D (BioLegend) using a three-laser FACSCanto.

2.17 ELISAs

Supernatants were collected 18 hours after immune cell coculture with Panc02, B16 or 4T1 cells. Supernatants were collected 24 hours after viral infection with UV-VSV, VSV-GFP, or VSV-IL-15. Tumors were isolated and homogenized 24 hours post final viral infection. IFNγ, TNF, and IL-15 production was measured by ELISA using Ready-Set-Go ELISA kits (invitrogen) as per manufacturer protocol. Briefly, capture antibodies were coating on an ELISA plate (Corning) over night at 4°C. Plates were washed and blocked before adding samples and standard in duplicate. Plates were incubated with samples and standard overnight at 4°C. Plates were then washed, followed by the

addition of the detection antibody, and incubated for 2 hours. Plates were then washed, before adding streptavidin-HRP and incubated for 1 hour. Plates were washed and TMB substrate was added for 15 mins followed by stop solution. Absorbance was measured at 450nm using an Epoch microplate spectrophotometer (BioTek).

2.18 Immunogenic cell death

Tumors and blood were isolated 24 hours post final virus injection. Tumors were dissociated through a 70-micron wire mesh into a single cell suspension by mechanical dispersion. Tumors were stained with anti-calreticulin (ab2907, Abcam) for 30 mins followed by Alexflour647 secondary (A21244, Life technologies) for 30 mins. Samples were read using a three-laser FACSCelesta with FACSDiva and analysis was performed using Flowjo software (BD Biosciences). Serum was isolated from blood using serum separator tubes (Sarstedt) and span at 10,000 RPMs for 20 mins. Serum concentration of HMGB1 and CXCL10 was determined by ELISA (Elabscience and eBioscience, respectively).

2.19 MTT assay

Panc02, Panc-1, ASPC1, and Capan-2 cells were infected at a multiplicity of infection (MOI) of 10, 1, or 0.1 with VSV-GFP, VSV-IL-15, or UV inactivated VSV (UV-VSV). At 24, 48, and 72 hours post infection. 0.5mg/mL MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma) diluted in PBS was added and incubated for 2 hours at 37°C. MTT reagent was removed and 100ul of DMSO was added. Plates were shook for 5 mins to dissolve the formazan. Viability was examined on a plate reader (Epoch, Bioteck) at 570nm.

2.20 Generation of recombinant VSV

Generation of recombinant VSV has been previously described.[292,303,304] Briefly human IL-15, p10ARV, p10NBV, p14, p15, P14/15, Δp14, and GFP were subcloned into the XhoI and Nhe1 sites located between the G and L gene in pVSVΔM51-XN. QM5 cells were infected with vaccina virus expressing T7 RNA polymerase, modified pVSVΔM51-XN, and plasmids expressing VSV N, P, and L proteins. After 48 hours cell culture supernatants were collected, filtered and used to infect Vero cells. Vero cells supernatants were isolated by plaque purification before sequencing and amplification.

2.21 Virus production

VSV-GFP and VSV-IL-15 were provided by Dr. Brian Lichty (McMaster University). VSV-p10ARV, VSV-p10NBV, VSV-p14, VSV-p15, VSV-Δp14, and VSV-p14/15 were provided by Dr. Roy Duncan (Dalhousie University). Vero cells at ~95% confluency was infected with VSV at a MOI of ~0.1 in serum free DMEM for 2 hours, followed by complete DMEM for 46 hours. Supernatant was collected and filtered using a 45µm filter. The supernatant was layered on 1.1mLs of 20% sucrose in PBS and centrifuged at 36,000 rpms, for 90 minutes at 4°C. Collected virus was resuspended in 15% glucose in PBS and stored in at -80°C. Virus titers were determined by plaque assay using Vero cells.

2.22 Plaque assay

Vero cells were plated in 12 well plates at $2x10^5$ cells per well in complete DMEM. The next day when vero cells were 100% confluent, media was aspirated and 200µl 10-fold serial dilutions (10^{-7} to 10^{-12}) of the virus stock in DMEM was added to the wells in duplicate. Cells were incubated at 37ºC, and 5% CO_2 for 2 hours with gentle shaking

every 30 minutes. Infection media was aspirated and 1mL of 1% agarose overlay (3.75 mL of 4% agarose in DMEM, 0.75 mL of FBS, and 10.5 mL of DMEM for a 12 well plate) was added to each well. Cells were incubated undisturbed for 48 hours at 37ºC, and 5% CO_2. The agarose was removed, and the cells fixed in 10% formaldehyde (Fisher Scientific) for 1 hour and stained with 1% crystal violet (Sigma Aldrich). Plaques were counted for each dilution and virus titer was determined by multiplying the number of plaques by the dilution factor.

Virus titre = (number of plaques in duplicate wells/2)/ (inoculum volume x dilution used)

2.23 Statistical analysis

Data are expressed as mean ± SEM unless otherwise stated. A non-parametric two-tailed Mann–Whitney U-test was used to compare between two data groups. Comparisons between more than two data groups were made using a Kruskal–Wallis non-parametric analysis of variance with Dunnett's post-test. Tumor growth data were analyzed using area under curve, followed by a Kruskal- Wallis non-parametric analysis with Dunn's post-test. Survival data were analyzed by log-rank (Mantel–Cox) significance test. Significance was set at $P < 0.05$. Statistical computations were carried out using GraphPad Prism 8.4.

CHAPTER 3: NATURAL KILLER T CELL IMMUNOTHERAPY COMBINED WITH IL-15 EXPRESSING ONCOLYTIC VIROTHERAPY AND PD-1 BLOCKADE INDUCES PANCREATIC TUMOR REGRESSION

This work appears in part in publication:

Nelson A, Gebremeskel S, Lichty BD, et al Natural killer T cell immunotherapy combined with IL-15-expressing oncolytic virotherapy and PD-1 blockade mediates pancreatic tumor regression Journal for ImmunoTherapy of Cancer 2022;10:e003923. doi: 10.1136/jitc-2021-003923

Contributions:

Conceptualization, A.N, B.J.; Methodology, A.N., S.G.; Formal Analysis, A.N.; Investigation, A.N; Resources, A.N., B.L.; Writing - Original Draft, A.N.; Writing – Review & Editing, A.N., B.L., B.J. Visualization, A.N., B.J.; Supervision, B.J.; Funding Acquisition, A.N., B.J.

3.1 Introduction

Pancreatic cancer is one of the leading causes of cancer death worldwide, with a 5-year survival rate of less than 10%.[18,19] This is largely attributed to poor rates of early diagnosis, a high metastasis rate, and intrinsic or acquired resistance to chemotherapy.[20–22] Despite improvements in treatment options that have increased survival rates in other cancers, survival rates in pancreatic cancer have only increased modestly over 20 years, highlighting the need for new and effective treatment options. Recent clinical studies have shown that immunotherapies such as checkpoint inhibitors, adoptive cell transfers, and vaccines can provide curative outcomes in cancer patients.[394–396] However, limited clinical success has been observed in pancreatic cancer using current immunological approaches.[397–399] While NKT cell activation prior to implantation of Panc02 pancreatic adenocarcinoma cells impaired tumor growth, the potential therapeutic benefit of NKT immunotherapy in pancreatic cancer has not been thoroughly examined.[400] Here we examined the therapeutic benefit of NKT cell activation alone, in combination with a recombinant oncolytic VSV-ΔM51 expressing IL-15, and in a triple therapy incorporating checkpoint anti-PD-1 blockade.

While many studies have focused on the ability of oncolytic viruses to directly kill cancer cells, there is increasing evidence that oncolytic viruses work, at least in part, by stimulating anti-tumor immunity.[275–277] VSV-ΔM51 is a single strand, negative sense RNA virus with oncolytic activity that is currently being used in preclinical[275,294–296] and clinical studies.[401] IL-15 is a proinflammatory cytokine essential for the survival and function of many anti-tumor immune cells, including NKT cells, and has been shown to increase immune targeting of cancer.[317–319] Importantly, VSV-ΔM51 engineered to

deliver IL-15 induced responses that were superior to treatment with parental VSV-ΔM51 and/or systemic delivery of recombinant IL-15.[304]

Immune checkpoint antibodies that block inhibitory signaling receptors on immune cells have seen success in both preclinical and clinical settings.[256,402,403] PD-1 blockade has been shown to preserve the anti-tumor functions of NKT cells.[404,405] Therefore, blocking PD-1/PD-L1 interactions may enhance NKT cell-based combined immunotherapies.

Using subcutaneous and orthotopic mouse models of pancreatic cancer, we show that NKT cell activation in combination with VSV-IL-15 enhances the anti-tumor immune response against pancreatic cancer cells leading to increased tumor regression and overall survival time. However, all tumors relapsed over time. While anti-PD-1 therapy was ineffective on its own, supplementation of our combined immunotherapy with anti-PD-1 blockade further enhanced and prolonged the immune response, leading to extended tumor control and increased survival.

3.2 Results

3.2.1 VSV-IL-15 infection and cytokine production *in vitro*

To test whether VSV-IL-15 can effectively infect and kill pancreatic cancer cells, we infected mouse Panc02 pancreatic ductal carcinoma cells, and human pancreatic ductal carcinoma cell lines Panc-1, AsPC1, and Capan-2 with VSV-GFP, VSV-IL-15, or UV inactivated VSV (UV-VSV) and examined cell viability. VSV-GFP and VSV-IL-15 significantly reduced Panc02, Panc-1, AsPC1, and Capan-2 cell viability demonstrating VSV's ability to infect and lyse both mouse and human pancreatic cancer cell lines (Figure 6A). There was no difference between VSV-GFP and VSV-IL-15 in terms of

their ability to lyse the pancreatic cancer cell lines, indicating that the addition of IL-15 into its genome, did not negatively affect VSV's oncolytic ability (Figure 6A). To verify that VSV-IL-15 can produce IL-15, we infected Panc02 cells and the human pancreatic cancer cell lines at an MOI of 1 for 24 hours before collecting the supernatant and examining IL-15 production. As expected, pancreatic cancer cell lines infected with VSV-IL-15 secreted significantly higher quantities of IL-15 compared to cells infected with either UV-VSV or VSV-GFP, demonstrating proper bioactivity in both human and mouse pancreatic cancer cells (Figure 6B).

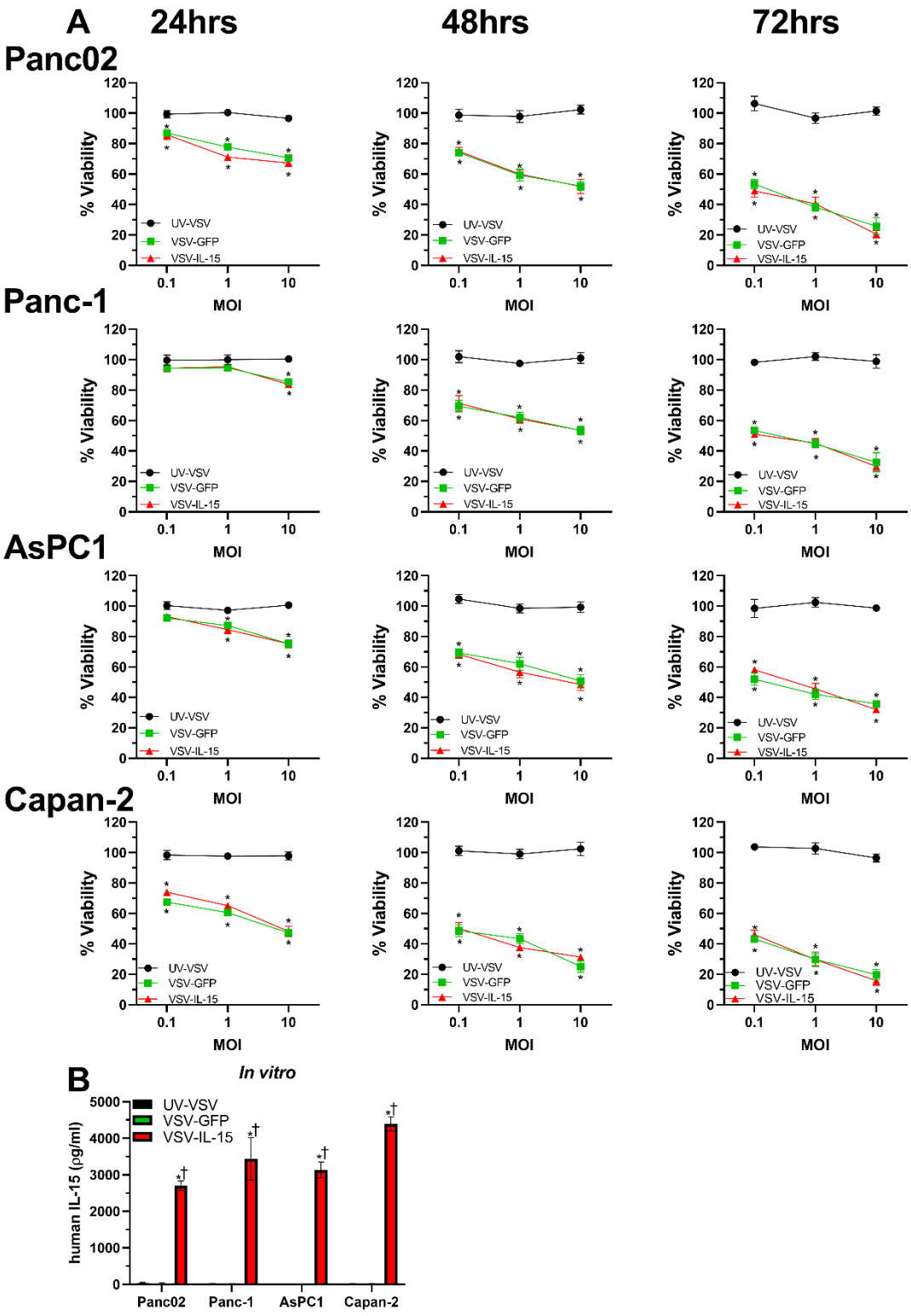

A
24hrs
48hrs
72hrs
Panc02
Panc-1
AsPC1
Capan-2
% Viability
MOI
0.1
1
10
UV-VSV
VSV-GFP
VSV-IL-15
B
In vitro
human IL-15 (pg/ml)
Panc02
Panc-1
AsPC1
Capan-2

Figure 6: VSV-IL-15 infection and IL-15 production of pancreatic cancer *in vitro*. A) Murine Panc02 and human Panc-1, AsPC1, and Capan-2 pancreatic adenocarcinoma lines were treated with VSV-IL-15, VSV-GFP, or UV-inactivated VSV. Cell viability was analyzed at different multiplicities of infection (MOI) using an MTT cell viability assay at 24, 48- and 72-hours following infection. Cell viability is expressed relative to untreated media conditions for each time point (n=3 per group). *$p < 0.05$ compared to UV-inactivated VSV. B) Murine Panc02 and human Panc-1, AsPC1, and Capan-2 pancreatic adenocarcinoma lines were treated with VSV-IL-15, VSV-GFP, or UV-inactivated VSV at an MOI of 1 for 24 hours. Culture supernatants were collected for measurement of IL-15 by ELISA (n=3 per group). *$p < 0.05$ compared to UV-inactivated VSV. $^{\dagger}p<0.05$ compared to VSV-GFP.

3.2.2 VSV-IL-15 infection and cytokine production *in vivo*

To examine whether VSV-IL-15 can effectively infect and kill pancreatic cancer cells *in vivo*, we injected Panc02 tumor bearing mice with either VSV- GFP or VSV-IL-15 (it. 5 x 10^8 PFUs on days 26, 28, and 30). Tumors were isolated 24 hours post virus injection, and cytokine production and viral titers were examined by ELISA and plaque assay, respectively. VSV-IL-15 treatments significantly increased localized production of IL-15 in homogenates of established Panc02 tumors compared to treatments with VSV-GFP (Figure 7A). There was no difference in viral titers between treatments with VSV-GFP and VSV-IL-15, further indicating that the addition of IL-15 does not impact VSV's oncolytic ability (Figure 7B). In summary, VSV-IL-15 can infect and produce IL-15 cytokine in Panc02 tumors, *in vivo.*

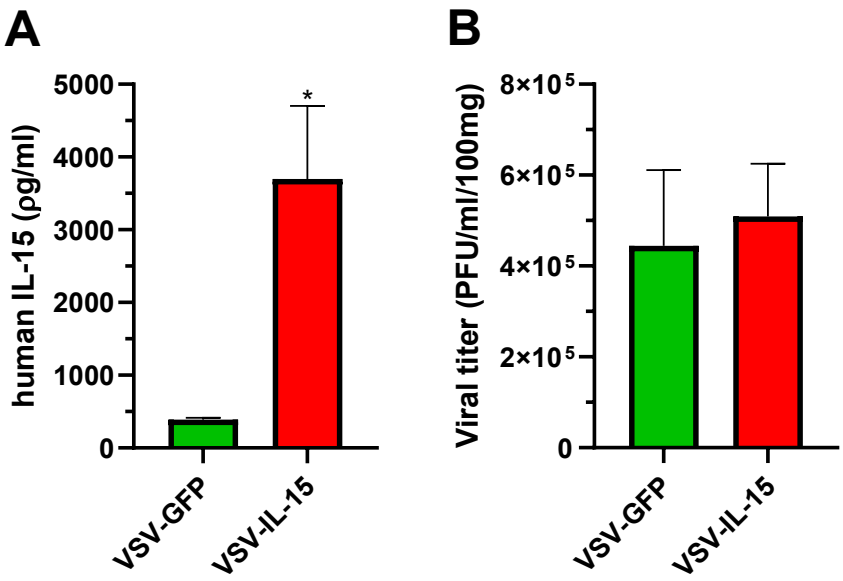


Figure 7: VSV-IL-15 infection and cytokine production of pancreatic cancer *in vivo*. Mice with subcutaneous Panc02 tumors were treated with VSV-GFP or VSV-IL-15 Tumors were isolated 24 hours after final virus injection. A) IL-15 production was measured by ELISA. B) Tumor viral titers were measured by plaque assay on Vero cells. (n=4-5). Viral titers and IL-15 concentration in tumors were normalized per 100mg of tumor weight. $^{*}p<0.05$ compared to VSV-GFP.

3.2.3 Combination of VSV-ΔM51 and NKT cell activation induces pancreatic tumor regression in syngeneic mice

We established a tumor model in which Panc02 pancreatic ductal carcinoma cells are implanted subcutaneously (2 x 10^6 in 100µl of saline) into syngeneic C57BL/6 mice (Figure 8A). Tumor-bearing mice were treated with VSV-GFP or VSV-IL-15 (it. 5 x 10^8 PFUs on days 26, 28, and 30) and/or NKT cell activation therapy via delivery of α-GalCer-loaded-DCs (iv. 6X 10^5 on day 31). Individual therapies induced modest decreases in tumor growth and increased survival time. VSV-ΔM51 treatments combined with NKT cell activation induced superior tumor regression compared to individual therapies, with the combination of VSV-IL-15 and NKT cell activation leading to the greatest tumor regression (Figure 8B) and significantly longer overall survival time (Figure 8C). Overall, the combination of VSV-IL-15 and NKT cell activation effectively decreased pancreatic tumor burden and increased survival time.

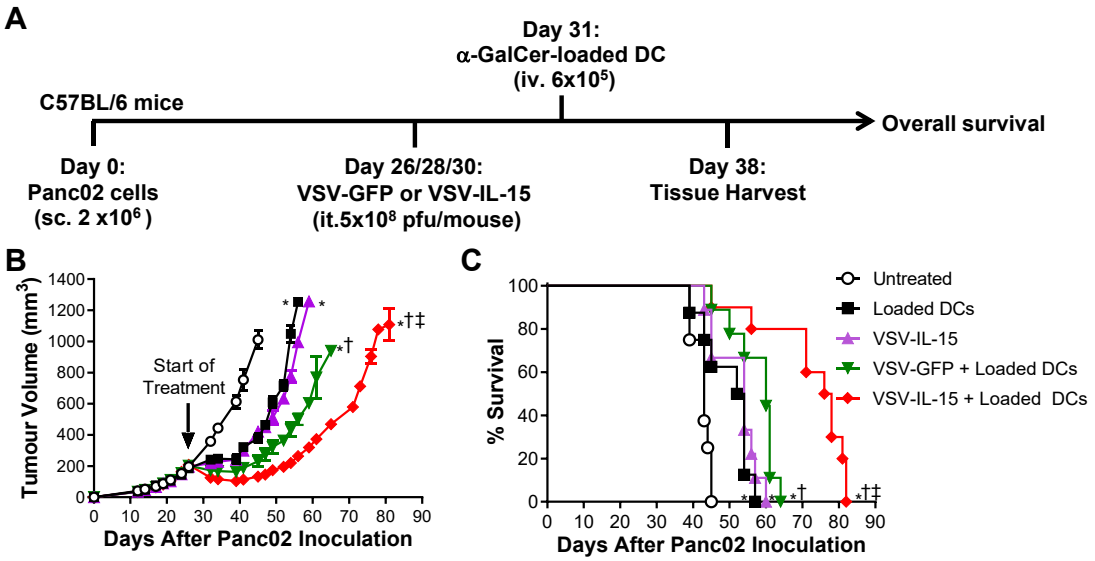


Figure 8: Combination of VSV-ΔM51 and NKT cell activation induces pancreatic tumor regression in syngeneic mice. A) Schematic of the pancreatic cancer model and treatment timeline. Subcutaneous Panc02 tumor volume (B) and survival (C) were assessed in untreated mice and mice receiving VSV-IL-15 treatments, glycolipid-loaded DCs, combined treatment with VSV-GFP plus glycolipid-loaded DCs, or combined treatment with VSV-IL-15 plus glycolipid-loaded DCs (n= 8-12 per group). *$p<0.05$ compared to untreated. $^{\dagger}p<0.05$ compared to single treatments. $^{\ddagger}P<0.05$ compared to combined treatment with VSV-GFP plus glycolipid-loaded DCs.

3.2.4 Combination of VSV-ΔM51 and NKT cell activation increases pancreatic tumor immune infiltration in syngeneic mice

To examine the effect of our treatments on the anti-tumor immune response, mice were treated as described in Figure 8A. Tumors and spleens were harvested on day 38 to assess immune cell accumulation and activation (Figure 9). As expected, NKT cells expanded in the spleen following delivery of α-GalCer-loaded DCs (Figure 9A). Glycolipid treatment also increased NKT cell infiltration into the tumor (Figure 9B). Oncolytic virus treatments did not expand NKT cells or cause NKT cell accumulation, but combined treatment with VSV-IL-15 and NKT cell activation therapy markedly enhanced NKT cell infiltration into the tumor. None of our individual or combined treatments altered the number of splenic NK cells, DCs, Tregs, $CD4^+$ or $CD8^+$ T cells (Figure 9A). In contrast, accumulation of NK cells, DCs, $CD4^+$ and $CD8^+$ T cells in the tumor was greatly enhanced by combined therapy with VSV-IL-15 and NKT cell activation (Figure 9B). NKT cell therapy trended towards increased $CD8^+$ T cell infiltration, which was significantly enhanced by VSV-IL-15 but not VSV-GFP. The number of Tregs in the tumor were not altered at this time point by our individual or combined therapies (Figure 9B). Overall, the combination of VSV-IL-15 and NKT cell activation effectively increased infiltration of immune cells in the tumors.

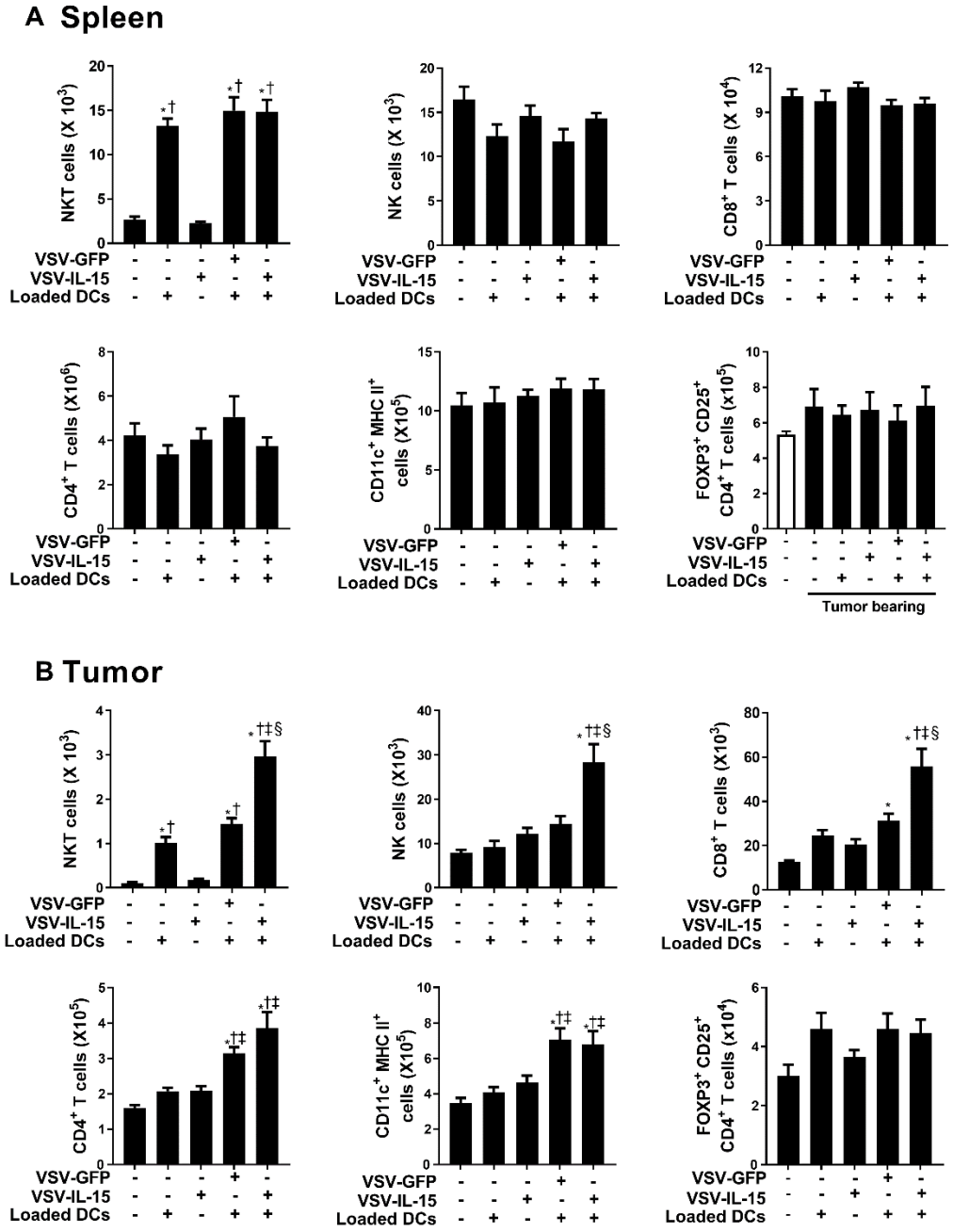


Figure 9: Combination of VSV-ΔM51 and NKT cell activation increases pancreatic tumor immune infiltration in syngeneic mice. Mice were treated as described in Figure 8A. A) Spleens and B) tumors of mice were isolated and dispersed into single cell suspensions. Flow cytometry was used to assess NKT cells (CD1d tetramer$^+$ TCRβ^+), NK cells (NK1.1$^+$ TCRβ^-), CD8$^+$ T cells (TCRβ^+ CD8α^+), CD4$^+$ T cells (CD4$^+$ TCRβ^+), T regulatory cells (FoxP3$^+$ CD25$^+$ CD4$^+$) and dendritic cells (MHC II$^+$ CD11c$^+$) (n= 7-12 per group). Immune cell populations in tumors were normalized per 100mg of tumor weight. *p<0.05 compared to untreated. †p<0.05 compared to VSV-IL-15. ‡P<0.05 compared to glycolipid-loaded DCs. §P<0.05 compared to combined treatment with VSV-GFP plus glycolipid-loaded DCs.

3.2.5 Combination of VSV-ΔM51 and NKT cell activation induces pancreatic tumor regression in an orthotopic model

To test our combination treatment in a more physiologically relevant microenvironment, Panc02 cells were injected orthotopically into the pancreas of C57BL/6 mice. This model has been shown to better recapitulate the desmoplasia and immunosuppression seen in pancreatic cancer patients.[386,406] Panc02 pancreatic ductal carcinoma cells were implanted into the pancreas of C57BL/6 mice (5×10^5 in 50µl of saline and Matrigel) (Figure 10A). Mice were treated with VSV-GFP or VSV-IL-15 (iv. 5×10^8 PFUs on days 14, 16, and 18) and/or NKT cell activation therapy via delivery of via α-GalCer-loaded-DCs (iv. $6X\ 10^5$ on day 19). Individual therapies increased survival time (Figure 10B) and decreased tumor weights at day 26 (Figure 10C). Combination therapy with VSV-ΔM51 and NKT cell activation significantly increased overall survival time and tumor control, with VSV-IL-15 exhibiting better protection than VSV-GFP (Figure 10B-C). Overall, the combination of VSV-IL-15 and NKT cell activation effectively decreased pancreatic tumor burden and increased survival in a more physiologically relevant model.

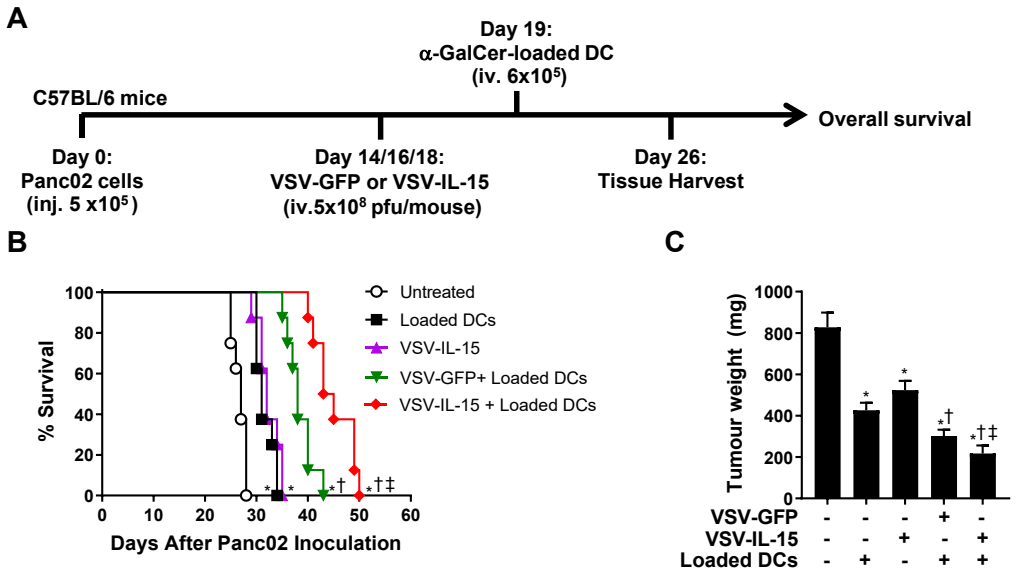


Figure 10: Combination of VSV-ΔM51 and NKT cell activation induces pancreatic tumor regression in an orthotopic pancreatic cancer model. A) Schematic of the orthotopic pancreatic cancer model and treatment timeline. B) Survival was assessed in untreated mice and mice receiving VSV-IL-15 treatments, glycolipid-loaded DCs, combined treatment with VSV-GFP plus glycolipid-loaded DCs, or combined treatment with VSV-IL-15 plus glycolipid-loaded DCs (n= 8 per group). *$p<0.05$ compared to untreated. †$p<0.05$ compared to single treatments. ‡$P<0.05$ compared to combined treatment with VSV-GFP plus glycolipid-loaded DCs. C) Tumors from untreated and treated mice were isolated and weighed at day 26 (n= 6 per group). *$p<0.05$ compared to untreated. †$p<0.05$ compared to single treatments. ‡$P<0.05$ compared to combined treatment with VSV-GFP plus glycolipid-loaded DCs.

3.2.6 Combination of VSV-ΔM51 and NKT cell activation increases pancreatic tumor immune infiltration in an orthotopic pancreatic cancer model

To examine the effect of our treatments on the anti-tumor immune response in the orthotopic model, mice were treated as described in Figure 10A. Tumors and spleens were harvested on day 26 to examine immune cell populations. The patterns of immune cell expansion and infiltration were similar to the subcutaneous model. NKT cell activation therapy increased NKT cell expansion in the spleen (Figure 11A), while NK cell, DC, Treg, $CD4^+$ or $CD8^+$ T cell numbers were not altered. NKT cell activation induced infiltration of NKT cells and $CD8^+$ T cells into the tumor, while VSV-IL-15 on its own significantly increased $CD8^+$ T cell infiltration (Figure 11B). Combination therapy with VSV-GFP and NKT cell activation slightly increased NKT cell and $CD8^+$ T cell infiltration over individual treatments, while combination therapy with VSV-L-15 caused further increases in NKT cell, NK cell, and $CD8^+$ T cell infiltration. However, combination therapy of VSV and NKT cell therapy increased DC and CD4+ T cell tumor infiltration (Figure 11B). The orthotopic pancreatic model has a compressed timeline due to tumor-induced loss of pancreatic function, but the microenvironment appeared to have minimal impact on the pattern of response to therapy in comparison to the subcutaneous model. Overall, the combination of VSV-IL-15 and NKT cell activation effectively increased infiltration of immune cells in the tumors. Importantly, responses were also similar when the virus was delivered intratumorally (subcutaneous) or intravenously (orthotopic).

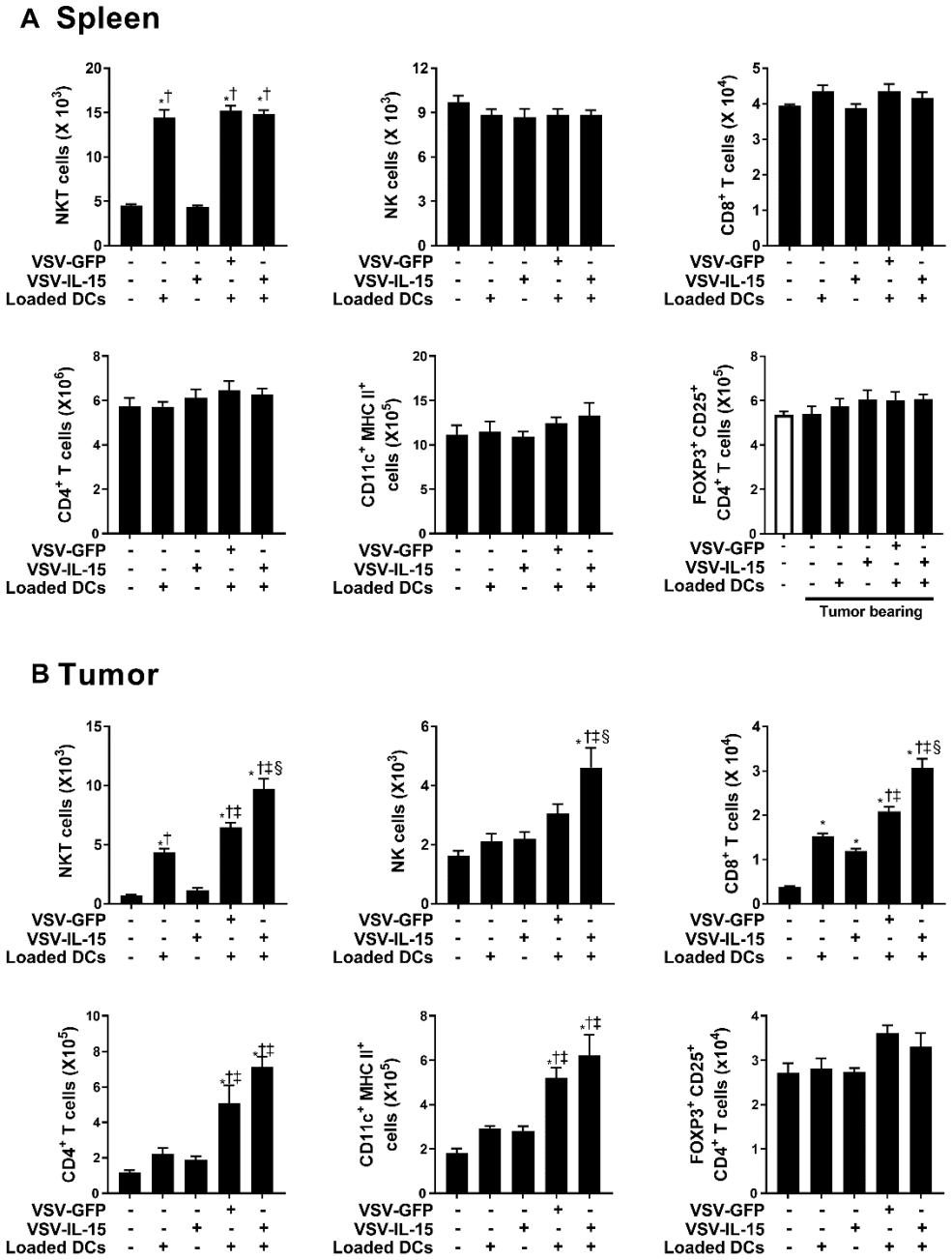


Figure 11: Combination of VSV-ΔM51 and NKT cell activation increases pancreatic tumor immune infiltration in an orthotopic pancreatic cancer model. Mice were treated as described in Figure 10A. A) Spleens and B) tumors of mice were isolated and dispersed into single cell suspensions. Flow cytometry was used to assess NKT cells (CD1d tetramer$^+$ TCRβ$^+$), NK cells (NK1.1$^+$ TCRβ$^-$), CD8$^+$ T cells (TCRβ$^+$ CD8α$^+$), CD4$^+$ T cells (CD4$^+$ TCRβ$^+$), T regulatory cells (FoxP3$^+$ CD25$^+$ CD4$^+$) and dendritic cells (MHC II$^+$ CD11c$^+$) (n= 6 per group). Immune cell populations in tumors were normalized per 100mg of tumor weight. *p<0.05 compared to untreated. †p<0.05 compared to VSV-IL-15. ‡P<0.05 compared to glycolipid-loaded DCs. §P<0.05 compared to combined treatment with VSV-GFP plus glycolipid-loaded DCs.

3.2.7 Combined VSV-IL-15 and NKT cell activation increases immune cell cytotoxicity and cytokine production in subcutaneous and orthotopic models

Since NKT cell activation, VSV, and IL-15 can increase the function of anti-tumor immune cells including NK cells, NKT cells, and $CD8^+$ T cells,[275,304,318,319,407] we examined functional immune responses in the subcutaneous and orthotopic models. To examine the effect of our treatments on the cytotoxic activity of immune cells, we sorted NK ($NK1.1^+$, $TCR\beta^-$), NKT (CD1d $tetramer^+$, $TCR\beta^+$) and $CD8^+$ T cells ($TCR\beta^+$, $CD8^+$) from the spleens of untreated and treated mice and co-cultured them with Oregon green-labelled Panc02 cells. After 18hours the supernatant and cells were isolated to examine cytokine release and cytotoxicity towards Panc02 cells, respectively (Figure 12). NKT cells, NK cells, and $CD8^+$ T cells from mice that had received NKT cell activation therapy exhibited increased cytotoxicity against Panc02 cells (increased $7AAD^+AnnexinV^+$ Panc02 cells), with the combination of VSV-IL-15 and NKT cell activation further increasing cytotoxicity compared to other groups (Figure 12A). Treatments with VSV-IL-15 on their own did not significantly impact cytotoxicity. In addition to increased cytotoxicity, NKT cell therapy increased production of the anti-tumor cytokines IFNγ (Figure 12B) and TNFα (Figure 12C) in co-culture. Cytokine release was further enhanced using cells from mice that received combination therapy with NKT cell activation and VSV-IL-15. VSV-IL-15 therapy alone did not increase cytokine production in any of the co-cultures. The patterns of functional responses were the same in the subcutaneous and orthotopic models at seven days post NKT cell activation.

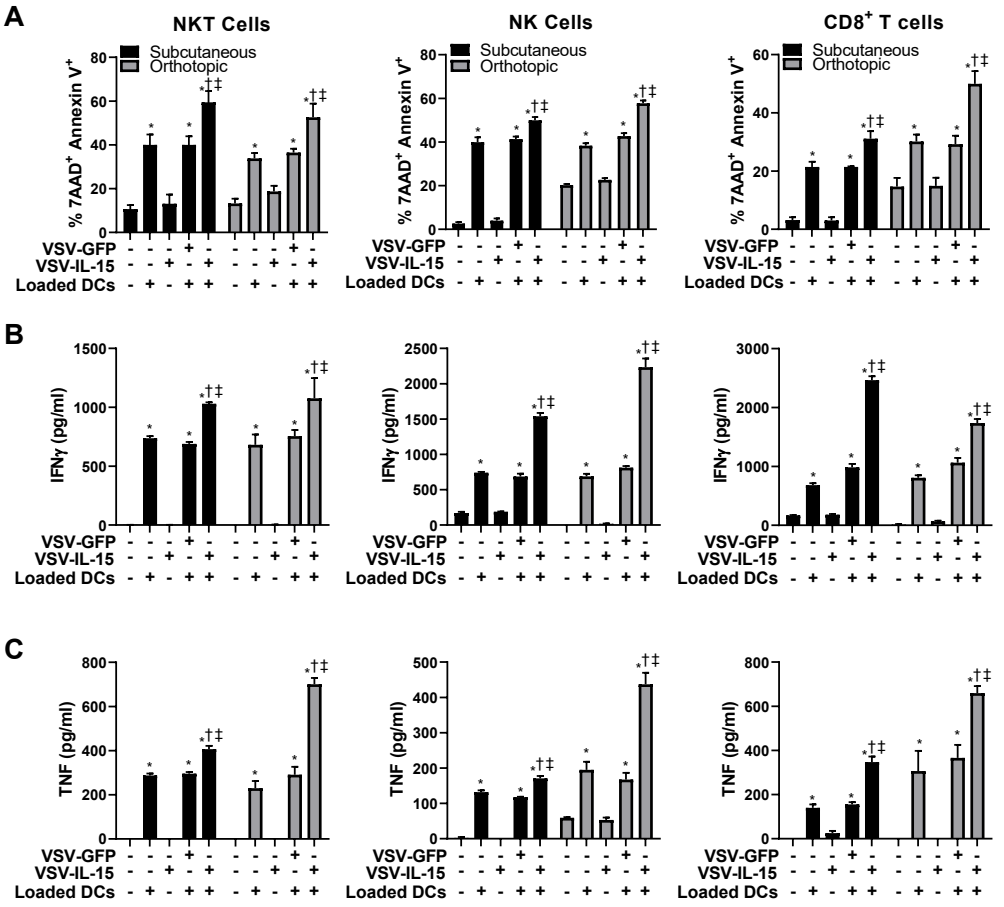
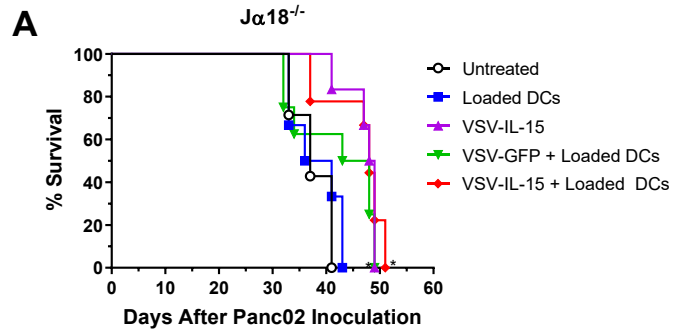
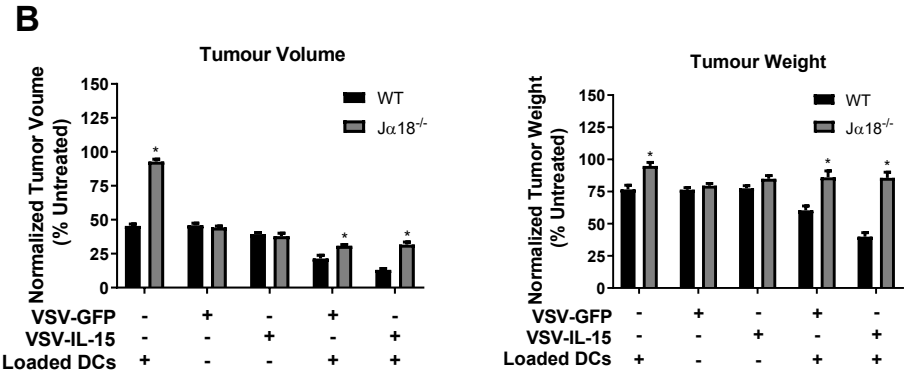


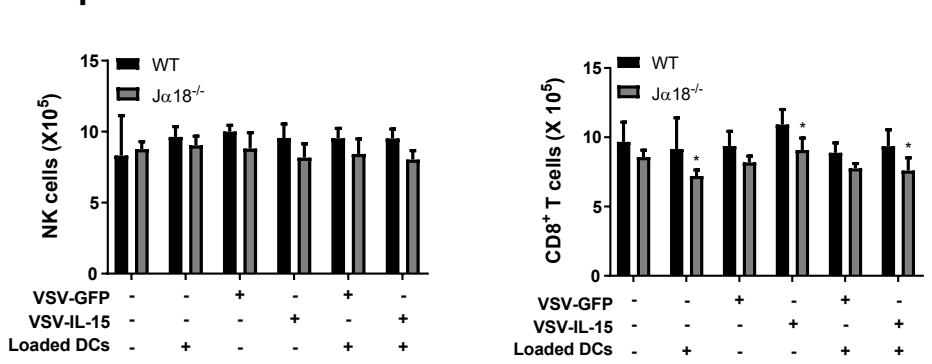
Figure 12: Combined VSV-IL-15 and NKT cell activation increased NKT cell, NK cell, and CD8+ T cell cytotoxicity and cytokine production following *in vitro* restimulation with Panc02 cells. Mice with subcutaneous or orthotopic Panc02 tumors were treated as in Figure 8A and Figure 10A, respectively. NKT cells (CD1d tetramer$^+$ TCRβ$^+$), NK cells (NK1.1$^+$ TCRβ$^-$) and CD8$^+$ T cells (TCRβ$^+$ CD8α$^+$) were sorted and cocultured with Oregon green labelled Panc02 cells at a 1:1 ratio. After 18 hours, Panc02 cells were stained with Annexin V and 7AAD to assess cytotoxicity. Culture supernatants were collected for measurement of IFNγ and TNF by ELISA. (n=3-4 per group). $^*p<0.05$ compared to untreated. $^\dagger p<0.05$ compared to glycolipid-loaded DCs. $^\ddagger P<0.05$ compared to combined treatment with VSV-GFP plus glycolipid-loaded DCs.

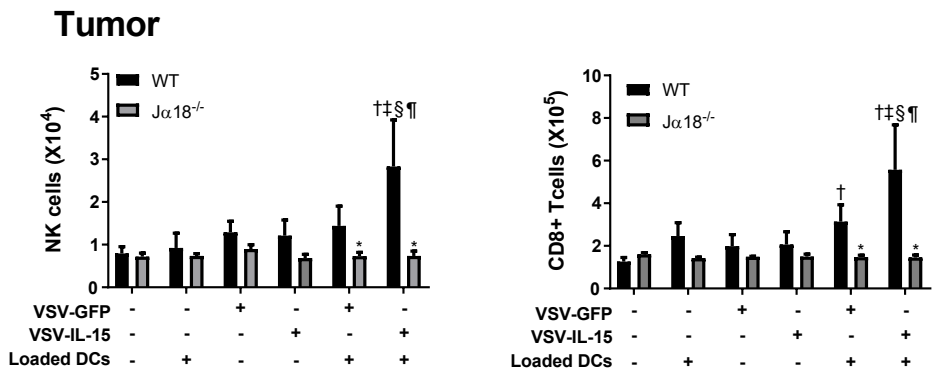
3.2.8 Jα18$^{-/-}$ mice lacking NKT cells have impaired tumor regression and anti-tumor immune response.

Immune profiling and functional assays suggested that NKT cell activation is the critical component to initiate robust immune activation in our combination therapy. To confirm the central contribution of NKT cells, we tested our therapies in Jα18$^{-/-}$ mice that lack NKT cells but have no alterations in CD8^{+} T cells or NK cells.[393] Jα18$^{-/-}$ mice exhibited impaired tumor control following NKT cell activation therapy and glycolipid-loaded DCs were not able to synergize with VSV-ΔM51 treatments (Figure 13A-B). Furthermore, Jα18$^{-/-}$ mice had reduced numbers of tumor infiltrating NK and CD8^{+} T cells (Figure 13C). Mice deficient in NKT cells exhibited no increases in cytotoxicity (Figure 13D) or cytokine production (Figure 13E-F) of NK or CD8^{+} T cells in cocultures with Panc02 cells, confirming that the NKT cell activation was essential for the therapeutic benefit of the combination therapy. Although VSV-ΔM51 treatments did not enhance immune responses in wild-type or Jα18$^{-/-}$ mice, they did increase survival and reduce tumor weights (Figure 13A-C). Mice treated with VSV-GFP alone exhibited similar reduction in tumor weights and immune cell infiltration to mice treated with VSV-IL-15 alone (Figure 13B-C). This suggests that VSV-ΔM51 is operating largely through oncolysis and that IL-15 supports the NKT cell activation therapy.


A
Jα18-/-
% Survival
Days After Panc02 Inoculation
Untreated
Loaded DCs
VSV-IL-15
VSV-GFP + Loaded DCs
VSV-IL-15 + Loaded DCs


B
Tumour Volume
Normalized Tumor Voume (% Untreated)
WT
Jα18-/-
VSV-GFP
VSV-IL-15
Loaded DCs
Tumour Weight
Normalized Tumor Weight (% Untreated)


C Spleen
NK cells (X10^5)
CD8+ T cells (X 10^5)
WT
Jα18-/-
VSV-GFP
VSV-IL-15
Loaded DCs


Tumor
NK cells (X10^4)
CD8+ Tcells (X10^5)
WT
Jα18-/-
VSV-GFP
VSV-IL-15
Loaded DCs

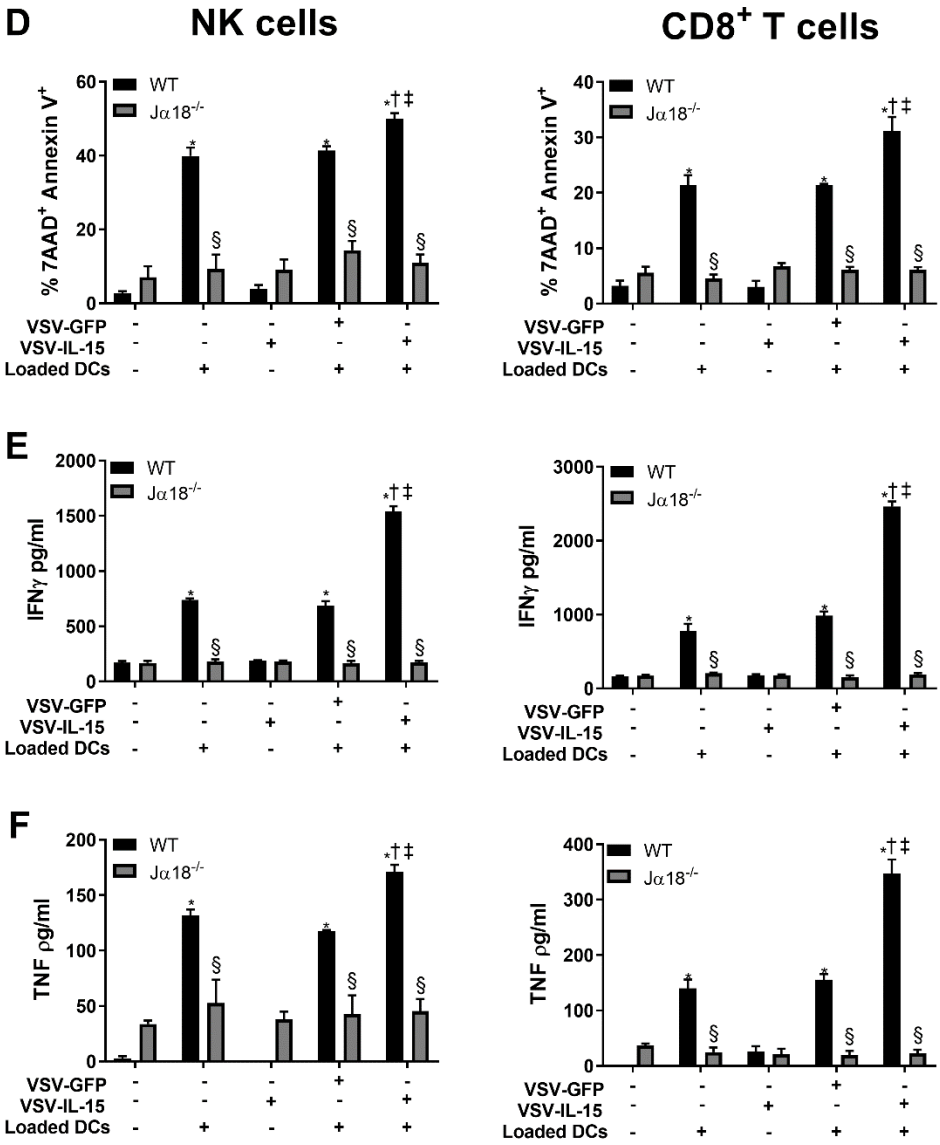


Figure 13: Therapy-induced survival, tumor regression, and immune cell recall responses are impaired in NKT deficient (Jα18-/-) mice. Wild-type and NKT cell-deficient Jα18$^{-/-}$ mice were inoculated with Panc02 tumor cells and treated as in Figure 8A. A) Survival was assessed in Jα18$^{-/-}$ mice inoculated with Panc02 cells and treated with NKT cell activation, VSV-IL-15, VSV-GFP + NKT cell activation, or VSV-IL-15 + NKT cell activation. (n=6-9 per group). *p<0.05 compared to untreated. B) Tumor volumes and weights were measured at day 38. Measurements were normalized to the untreated group of each mouse strain (n=4-11 per group). *p<0.05 compared to wild-type. C) Spleens and tumors of mice were isolated and dispersed into single cell suspensions. Flow cytometry was used to assess NK (NK1.1$^+$ TCRβ$^-$) and CD8$^+$ T cells (TCRβ$^+$ CD8α$^+$). Immune cell populations in tumors were normalized per100mg of tumor weight. *p<0.05 compared to wild-type. †p<0.05 compared to untreated. ‡P<0.05 compared to glycolipid-loaded DCs. §p<0.05 compared to VSV-IL-15. ¶P<0.05 compared to combined treatment with VSV-GFP plus glycolipid-loaded DCs. Panc02

cells were incubated for 18 hours at a 1:1 ratio with NK cells, $CD8^+$ T cells isolated from the spleens of wild-type and $J\alpha18^{-/-}$ mice receiving different therapies. D) Cytotoxicity was measured by staining Panc02 cells with Annexin V and 7AAD. E) IFNγ and F) TNF were determined in culture supernatants by ELISA (n=3 per group). *$p<0.05$ compared to untreated. †$P<0.05$ compared to glycolipid-loaded DCs. ‡$P<0.05$ compared to combined treatment with VSV-GFP plus glycolipid-loaded DCs. $^{§}p<0.05$ compared to wild-type.

3.2.9 Combined therapy supplemented with PD-1 blockade increases survival and enhances tumor clearance

While our therapies induced tumor regression and increased anti-tumor immunity, the Panc02 tumors all relapsed and progressed over time. We hypothesized that this could be due to upregulation of immunosuppressive mechanisms that impair the immune response. The immunosuppressive checkpoint molecule PD-1 and its ligand PD-L1 are upregulated in response to pro-inflammatory cytokines,[69] which are generated in response to our NKT cell and combination therapies. Indeed, Panc02 cells cultured *in vitro* with IFNγ (1000pg/mL for 72 hours) exhibited increased PD-L1 expression (Figure 14A). Similarly, Panc02 tumor cells isolated from untreated tumor-bearing mice exhibited increased PD-L1 expression, which was increased further after combination therapy with VSV-IL-15 and NKT cell activation (Figure 14B). Similarly, the expression of PD-1 was increased on NKT cells, NK cells, and $CD8^+$ T cells isolated from the tumor and spleen (Figure 14C-D). We therefore examined the therapeutic benefit of adding PD-1 blockade (RMPI-14) to our combined VSV-IL-15 and NKT cell activation regimen (Figure 14E). Three days after NKT cell activation, mice were given anti-PD-1 (ip. 300μg/ week) for three weeks (Figure 14E). The addition of anti-PD-1 increased overall tumor regression and survival time, resulting in 20% of the mice experiencing complete tumor clearance (Figure 14F-G). Mice treated with anti-PD-1 alone had no benefit compared to untreated mice, indicating that immune activation is required to establish anti-tumor immunity. Therefore, anti-PD-1 can be effectively added to our combination therapy to increase therapeutic benefit in pancreatic cancer.

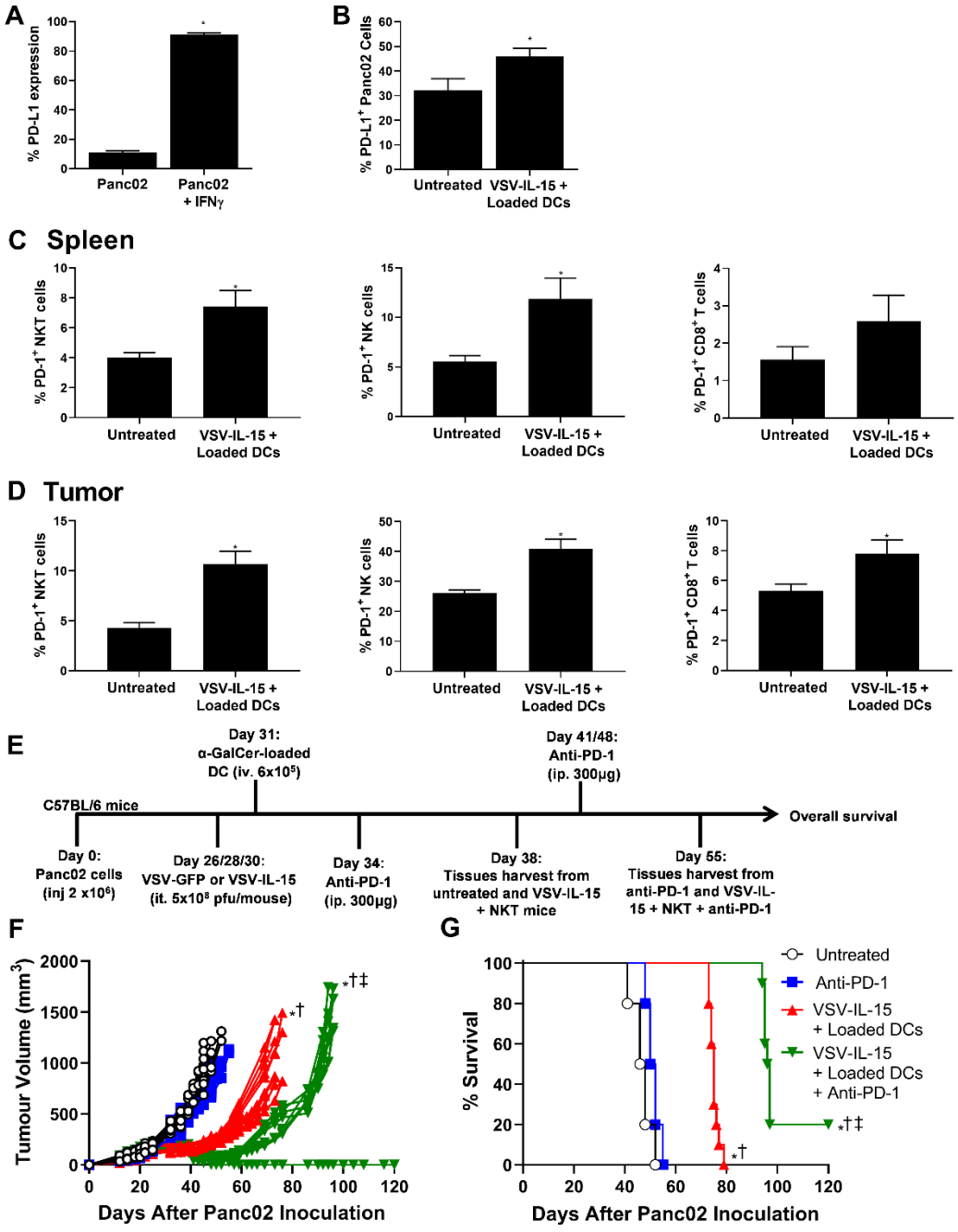

A
% PD-L1 expression
Panc02
Panc02 + IFNγ
B
% PD-L1+ Panc02 Cells
Untreated
VSV-IL-15 + Loaded DCs
C Spleen
% PD-1+ NKT cells
% PD-1+ NK cells
% PD-1+ CD8+ T cells
D Tumor
% PD-1+ NKT cells
% PD-1+ NK cells
% PD-1+ CD8+ T cells
E
C57BL/6 mice
Day 0: Panc02 cells (inj 2 x10^6)
Day 26/28/30: VSV-GFP or VSV-IL-15 (it. 5x10^8 pfu/mouse)
Day 31: α-GalCer-loaded DC (iv. 6x10^5)
Day 34: Anti-PD-1 (ip. 300μg)
Day 38: Tissues harvest from untreated and VSV-IL-15 + NKT mice
Day 41/48: Anti-PD-1 (ip. 300μg)
Day 55: Tissues harvest from anti-PD-1 and VSV-IL-15 + NKT + anti-PD-1
Overall survival
F
Tumour Volume (mm3)
Days After Panc02 Inoculation
G
% Survival
Untreated
Anti-PD-1
VSV-IL-15 + Loaded DCs
VSV-IL-15 + Loaded DCs + Anti-PD-1
Days After Panc02 Inoculation

Figure 14: PD-1 blockade increases therapeutic benefit of combined VSV-IL-15 and NKT cell activation therapy against Panc02 tumors. A) PD-L1 expression was measured by flow cytometry on cultured Panc02 cells supplemented with 1 ng/mL of IFNγ for 72 hours (n= 3 per group). *p<0.05 compared to unstimulated Panc02 cells. B) PD-L1 expression was measured on Panc02 tumor cells isolated from untreated mice and mice treated with VSV-IL-15 plus glycolipid-loaded DCs (n= 5 per group). *p<0.05 compared to untreated Panc02 tumors. PD-1 expression on NKT cells (CD1d tetramer$^+$ TCRβ$^+$), NK cells (NK1.1$^+$ TCRβ$^-$) and CD8$^+$ T cells (TCRβ$^+$ CD8α$^+$) isolated from C) spleens and D) tumors of untreated mice or mice treated with VSV-IL-15 plus glycolipid-loaded DCs (n= 5 per group). *p<0.05 compared to untreated mice. E) Schematic of pancreatic cancer model timeline with PD-1 blockade. F) Panc02 tumor volume in individual mice and G) overall survival and were assessed in untreated mice and mice receiving anti-PD-1 treatment alone, combined treatment with VSV-IL-15 plus glycolipid-loaded DCs, or combined treatment with VSV-IL-15, glycolipid-loaded DCs and anti-PD-1 treatments (n= 10 per group). *p<0.05 compared to untreated. P<0.05 compared to anti-PD-1. ‡p<0.05 compared to VSV-IL-15 plus glycolipid-loaded DCs

3.2.10 Mice rechallenged with Panc02 cells demonstrate tumor immune memory

Mice that survived the initial tumor challenge were rechallenged with Panc02 cells. Rechallenged mice exhibited slower tumor growth and lower tumor weight at harvest compared to naïve controls, indicating that an immune memory response had formed (Figure 15A-B). Furthermore, tumors from rechallenged mice had increased immune infiltration of effector memory ($CD44^+$, $CD62L^-$) $CD4^+$ and $CD8^+$ T cells, demonstrating an increased immune memory response to the tumor (Figure 15C). While it didn't reach significance, there was an increase in $CD62L^+$ memory NKT cells, central memory ($CD44^+$, $CD62L^+$) $CD4^+$ and $CD8^+$ T cells, and DC infiltration (Figure 15C). Spleens from rechallenged mice had significant increases in the number of DCs, NKT cells, $CD4^+$ and $CD8^+$ T cells (Figure 16). Furthermore, there was an increased number of $CD69^+$ NKT cells, $CD4^+$ and $CD8^+$ T cells demonstrating increased activation of these cells (Figure 16). Rechallenged mice had increased immune infiltration of effector memory and central memory $CD4^+$ and $CD8^+$ T cells, further demonstrating an increased immune memory response (Figure 16). Taken together, mice who survived initial tumor challenge formed an immune memory response towards Panc02 cells, leading to slower tumor growth upon rechallenge.

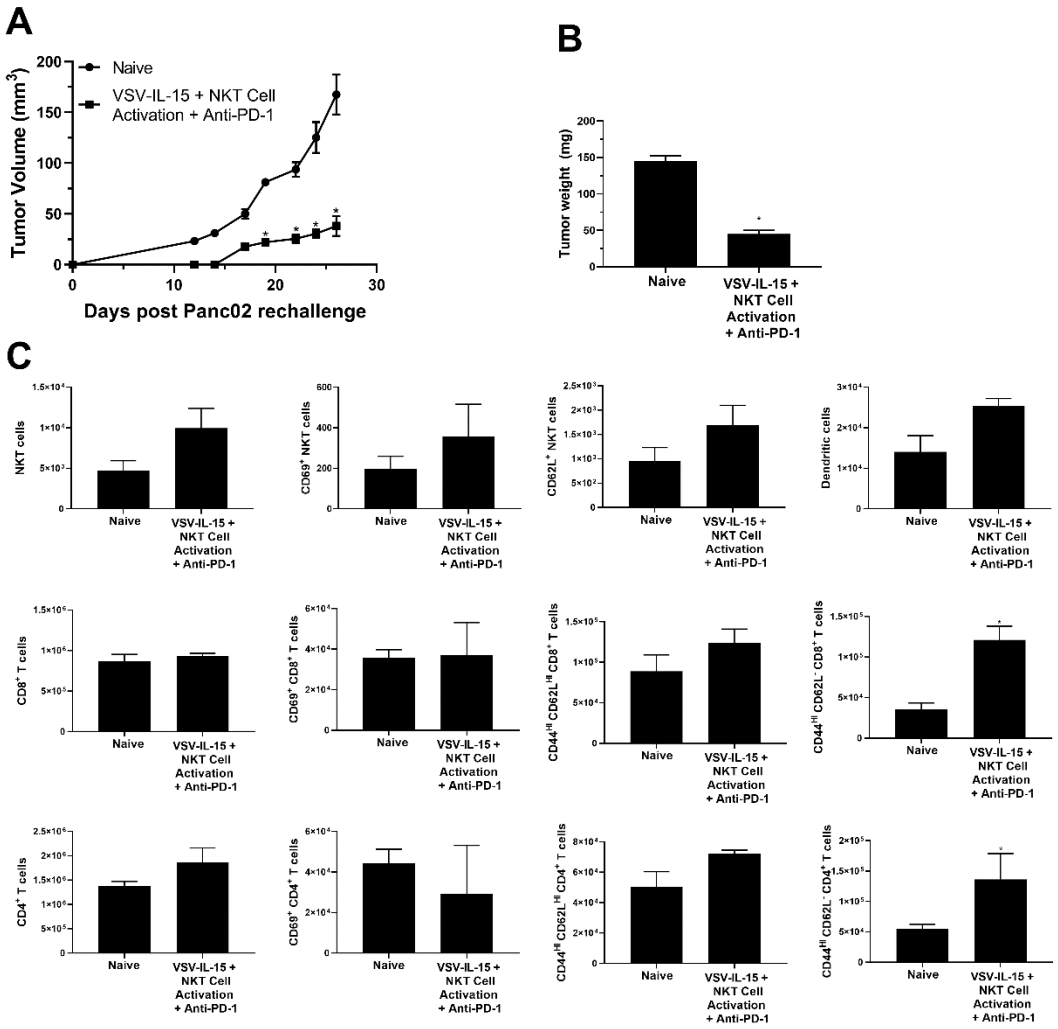


Figure 15: Mice rechallenged with Panc02 cells demonstrate tumor immune memory. Panc02 cells ($2x10^6$) were inoculated subcutaneously into naïve mice and mice who survived original tumor challenge following treatment with VSV-IL-15, glycolipid-loaded DCs and PD-1 blockade as in Figure 14E. A) Tumor volume was assessed over time and B-C) Tumors of mice were isolated and weighted 26 days after Panc02 cell injection and dispersed into single cell suspensions. Flow cytometry was used to assess NKT cells (CD1d tetramer$^+$ TCRβ$^+$), CD8$^+$ T cells (TCRβ$^+$ CD8α$^+$), CD4$^+$ T cells (TCRβ$^+$ CD4$^+$), their activation (CD69$^+$), memory NKT cells (CD62L$^+$ CD1d tetramer$^+$ TCRβ$^+$) dendritic cells (MHC II$^+$ CD11c$^+$), effector memory CD4$^+$ T cells (CD44^{HI} CD62L^{neg} TCRβ$^+$ CD4$^+$), Effector memory CD8$^+$ T cells (CD44^{HI} CD62L^{neg} TCRβ$^+$ CD8$^+$), central memory CD4$^+$ T cells (CD44^{HI} CD62L^{HI} TCRβ$^+$ CD8$^+$), central memory CD8$^+$ T cells (CD44^{HI} CD62L^{HI} TCRβ$^+$ CD8$^+$). Immune cell populations in tumors were normalized per 100mg of tumor weight. (n=2-6 per group). *$p<0.05$ compared to naive.

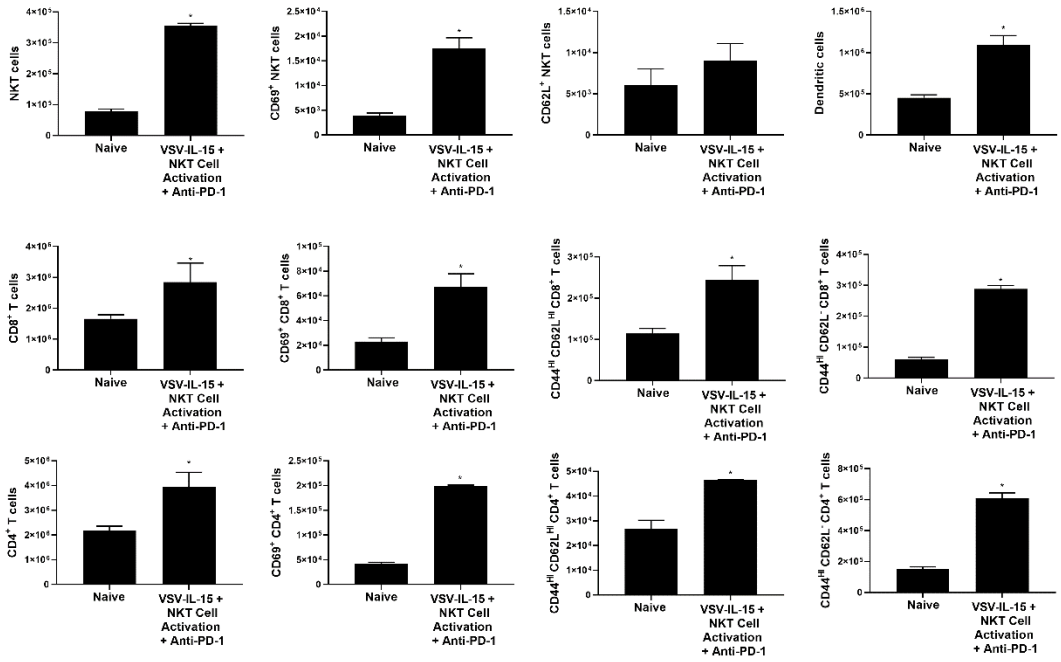


Figure 16: Mice rechallenged with Panc02 cells have increased splenic immune memory populations. A) Panc02 cells ($2x10^6$) were inoculated subcutaneously into naïve mice and mice who survived original tumor challenge following treatment with VSV-IL-15, glycolipid-loaded DCs and PD-1 blockade as in Figure 14E. Spleens of mice were isolated 26 days after Panc02 cell injection and dispersed into single cell suspensions. Flow cytometry was used to assess NKT cells (CD1d tetramer$^+$ TCRβ^+), CD8$^+$ T cells (TCRβ^+ CD8α^+), CD4$^+$ T cells (TCRβ^+ CD4$^+$), their activation (CD69$^+$), memory NKT cells (CD62L$^+$ CD1d tetramer$^+$ TCRβ^+) dendritic cells (MHC II$^+$ CD11c$^+$), effector memory CD4$^+$ T cells (CD44^{HI} CD62L^{neg} TCRβ^+ CD4$^+$), Effector memory CD8$^+$ T cells (CD44^{HI} CD62L^{neg} TCRβ^+ CD8$^+$), central memory CD4$^+$ T cells (CD44^{HI} CD62L^{HI} TCRβ^+ CD8$^+$), central memory CD8$^+$ T cells (CD44^{HI} CD62L^{HI} TCRβ^+ CD8$^+$). (n=2-6 per group). *$p<0.05$ compared to naive.

3.2.11 PD-1 blockade enhances and maintains NK, NKT, and T cell activation induced by combination therapy

To determine the impact of the PD-1 blockade on immune cell populations, we harvested spleens and tumors before PD-1 therapy (day 38) or 7 days after the final anti-PD-1 treatment (day 55). At day 55, tumors in mice that received VSV-IL-15 combined with NKT cell immunotherapy exhibited relapse and were larger than on day 38 (Figure 17A). In contrast, mice that received combined VSV-IL-15 and NKT cell immunotherapy plus anti-PD-1 therapy had significantly smaller tumors (Figure 17A).

While VSV-IL-15 combined with NKT cell activation increased the infiltration of NKT cells, NK cells, and $CD8^+$ T cells into tumors on day 38 (Figure 9 and 17B), by day 55 the number of immune cells in the tumor had returned to baseline (Figure 17B). Combined treatment that also incorporated anti-PD-1 therapy maintained increased immune cell infiltration at day 55. PD-1 therapy on its own had no significant effect. Strikingly, anti-PD-1 therapy appeared to enhance and prolong immune activation as the number of NKT cells, NK cells, and $CD8^+$ T cells expressing the activation marker CD69 was significantly increased compared to day 38 and day 55 groups that did not receive anti-PD-1 therapy (Figure 17).

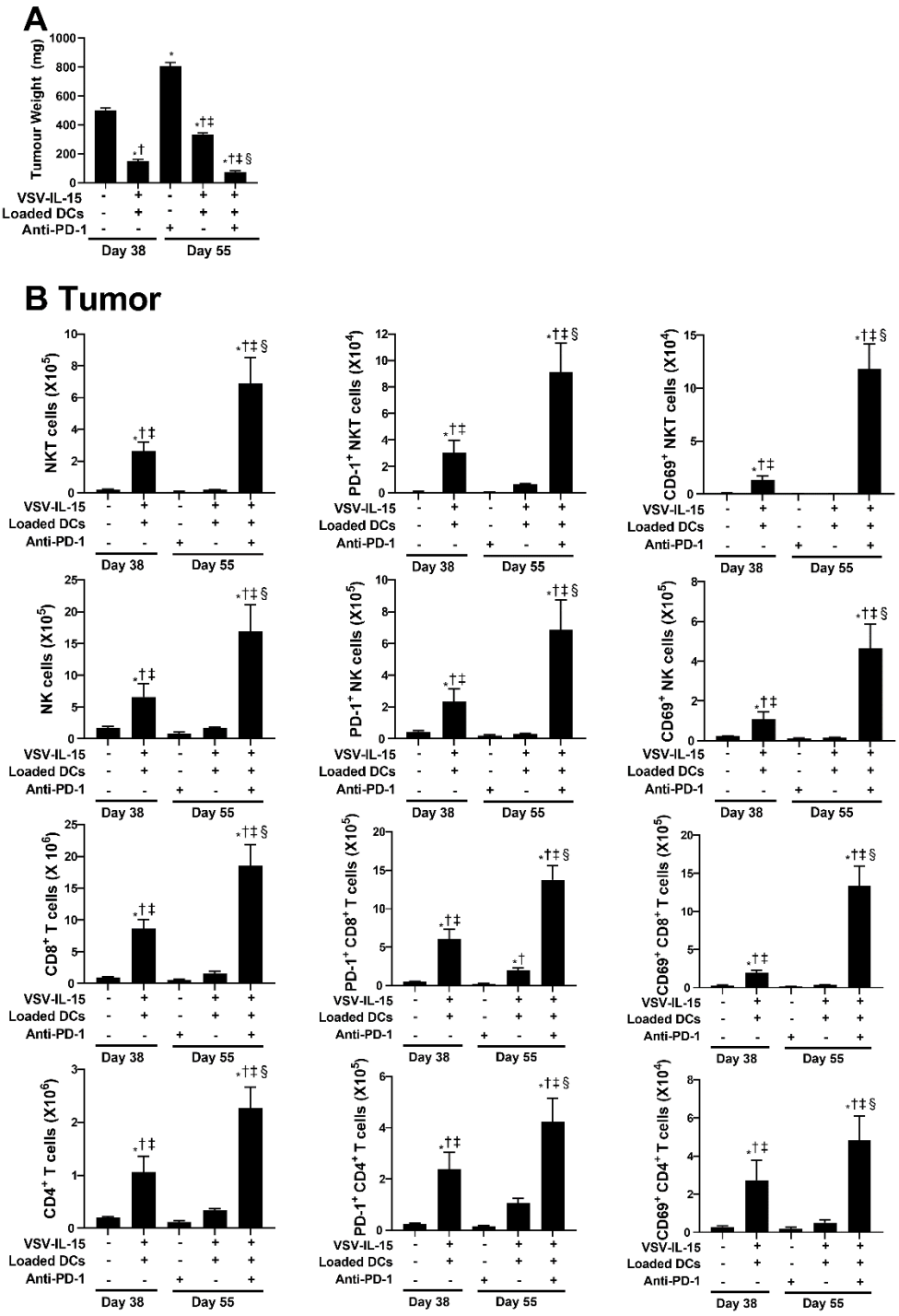

A
Tumour Weight (mg)
VSV-IL-15
Loaded DCs
Anti-PD-1
Day 38
Day 55
B Tumor
NKT cells (X10^5)
PD-1+ NKT cells (X10^4)
CD69+ NKT cells (X10^4)
NK cells (X10^5)
PD-1+ NK cells (X10^5)
CD69+ NK cells (X10^5)
CD8+ T cells (X 10^6)
PD-1+ CD8+ T cells (X10^5)
CD69+ CD8+ T cells (X10^5)
CD4+ T cells (X10^6)
PD-1+ CD4+ T cells (X10^5)
CD69+ CD4+ T cells (X10^4)

Figure 17: Additional PD-1 blockade increases activation of tumor infiltrating lymphocytes. Panc02 tumors were induced and treated as in Figure 143E. A) Tumors of untreated and treated mice were isolated and weighed at day 38 or 55 (n= 3-6 per group). B) Tumors were dispersed into single cell suspensions and flow cytometry was used to assess NKT cell (CD1d tetramer^{+} TCRβ^{+}), NK cell (NK1.1^{+} TCRβ^{-}) and CD8^{+} T cell (TCRβ^{+} CD8α^{+}) infiltration into tumors. Expression of CD69 and PD-1 were examined (n=3-6 per group). Immune cell populations in tumors were normalized per 100mg of tumor weight. *p<0.05 compared to untreated. †P<0.05 compared to anti-PD-1. ‡p <0.05 compared to VSV-IL-15 plus glycolipid-loaded DCs (Day 38). §p <0.05 compared to VSV-IL-15 plus glycolipid-loaded DCs (Day 55).

Similar results were observed in the spleen (Figure 18). Anti-PD-1 therapy was associated with increased PD-1 positive immune cells (Figure 17 and 18), consistent with enhanced immune activation.[408] This could account for tumor relapse after anti-PD-1 therapy ended. Taken together, triple therapy with VSV-IL-15, NKT cell activation and anti-PD-1 prolonged and increased immune cell activation and increased tumor regression.

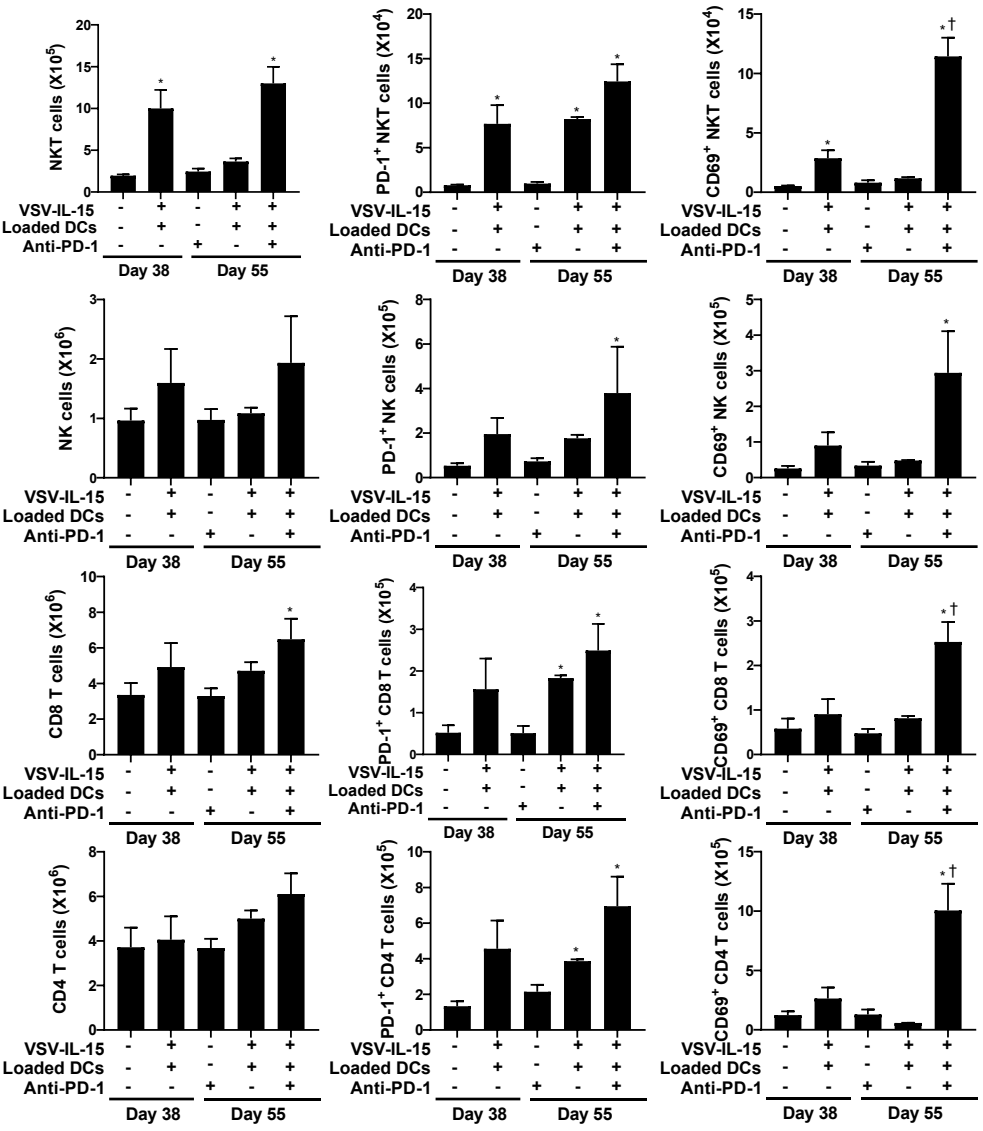


Figure 18: PD-1 blockade enhances and maintains tumor infiltrating NK, NKT, and T cell activation induced by combination therapy. Mice were inoculated subcutaneously with Panc02 cells ($2X10^6$). Mice were treated with VSV-GFP or VSV-IL-15 (it. $5X10^8$ PFU) on days 26, 28, and 30 and/or α-GalCer-loaded DCs (iv. $6X10^5$) on day 31. Mice were given anti-PD-1 (ip.300μg) weekly for three weeks starting on day 34. Spleens of untreated and treated mice were isolated and dispersed into single cell suspensions on day 38 or 55. Flow cytometry was used to assess NKT cells (CD1d tetramer$^+$ TCRβ$^+$), NK cells (NK1.1$^+$ TCRβ$^-$) and CD8$^+$ T cells (TCRβ$^+$ CD8α$^+$) (n=3-6 per group). *$p<0.05$ compared to untreated. †$p<0.05$ compared to VSV-IL-15 plus glycolipid-loaded DCs (Day 38).

3.2.12 Combination of VSV-IL-15 and NKT cell activation and PD-1 blockade increases immune cell cytotoxicity and cytokine production.

Due to differences in CD69 expression, we examined the effects of PD-1 blockade on cytotoxicity and cytokine production. Immune cells from mice treated with anti-PD-1 therapy alone exhibited no increase in cytotoxicity, IFNγ release, or TNF release compared to untreated mice (Figure 19). However, mice that received anti-PD-1 therapy in combination with VSV-IL-15 and NKT cell activation treatments exhibited modest but significant increases in NKT cell, NK cell, and $CD8^+$ T cell cytotoxicity, IFNγ and TNF (except for NKT cells) production at day 55 compared to peak activity at day 38 following VSV-IL-15 combined with NKT cell activation. Cells from mice that did not receive anti-PD-1 therapy were not functional at day 55, suggesting that they were anergic or specificity against Panc02 cells had been lost. Enhanced recognition of tumor cells by NK cells was not antigen-specific as NK cells from treated mice also responded to co-culture with B16-F10 melanoma cells. In contrast, the $CD8^+$ T cell response was antigen specific as there was no cytotoxicity or cytokine response to co-culture with B16. Therefore, the combination of VSV-IL-15, NKT cell activation, and anti-PD-1 therapies enhanced immune cytotoxicity against the immunizing tumor, and PD-1 therapy increased the duration of the functional anti-tumor immune response compared to combined treatment lacking PD-1 blockade.

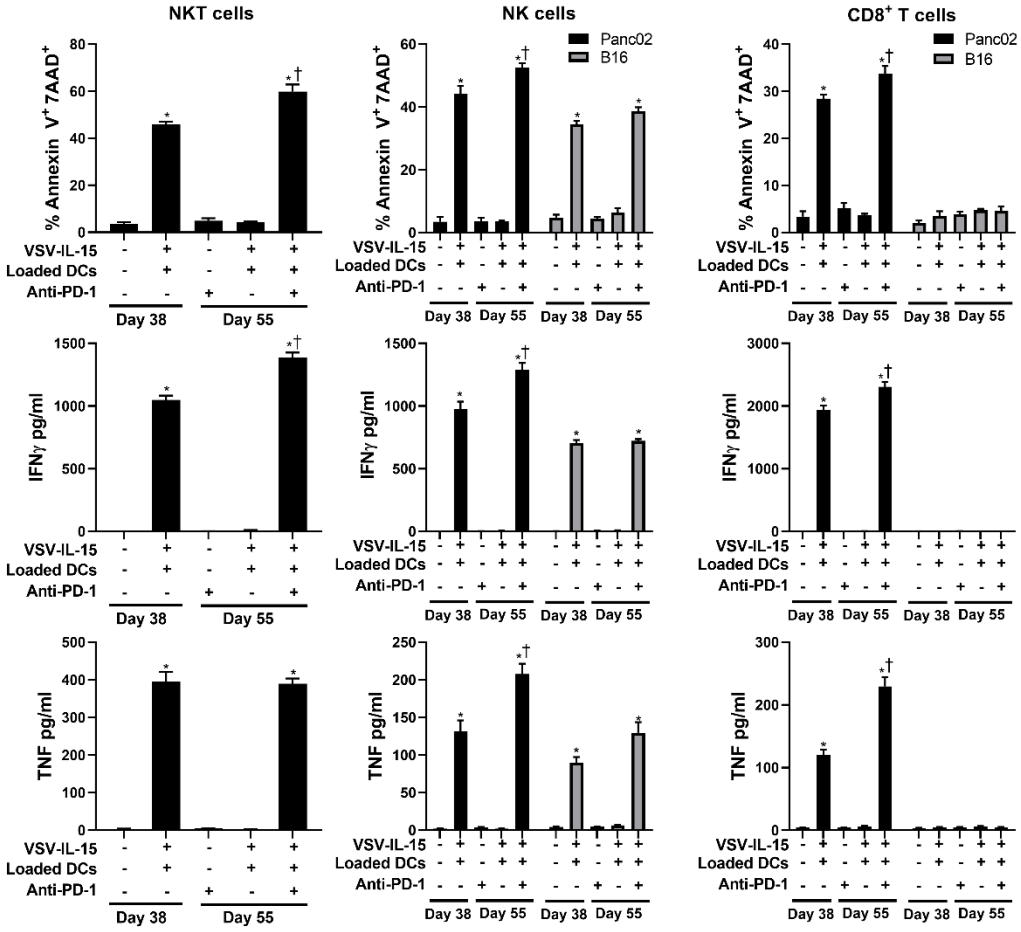


Figure 19: Combined VSV-IL-15, NKT cell activation, and PD-1 blockade increases NK, NKT and CD8+ T cell cytotoxicity and cytokine production following *in vitro* restimulation with Panc02 cells. Mice with subcutaneous Panc02 tumors were treated as in Figure 14E. NKT cells (CD1d tetramer$^+$ TCRβ$^+$), NK cells (NK1.1$^+$ TCRβ$^-$) and CD8$^+$ T cells (TCRβ$^+$ CD8α$^+$) were sorted and cocultured with Oregon green labelled Panc02 cell or B16-F10 melanoma cells at a 1:1 ratio. After 18 hours, Panc02 and B16-F10 cells were stained with Annexin V and 7-AAD to assess cytotoxicity. Culture supernatants were collected for measurement of IFNγ and TNF by ELISA (n=4 per group). $^*p<0.05$ compared to untreated. †$p<0.05$ compared to VSV-GFP plus glycolipid-loaded DCs.

3.2.13 NKT cell activation decreases MDSC immunosuppression in subcutaneous and orthotopic models of pancreatic cancer.

In addition to checkpoint-mediated immunosuppression, the tumor and microenvironment can promote other immunosuppressive mechanisms. We did not observe an accumulation of $FoxP3^+$ regulatory T cells in the spleen or tumor, nor an impact of our individual or combined therapies on regulatory T cells (Figure 9 and 11). Similarly, we did not see an impact of our induvial or combined therapies on $Ly6C^+$ $CD11b^+$ monocytic MDSCs (data not shown). Patients with pancreatic cancer exhibit elevated numbers of MDSCs,[409,410] a heterogeneous population of immature or altered myeloid cells that suppress immune functions.[409,410] Similarly, mice with subcutaneous and orthotopic Panc02 tumors had higher numbers of splenic $Ly6G^+$ $CD11b^+$ cells compared to naïve mice (Figure 20A). While NKT cell activation on its own did not significantly increase the number of $Ly6G^+$ $CD11b^+$ cells in the spleen or tumor, treatment with VSV-ΔM51 increased these cells in in both the subcutaneous and orthotopic Panc02 models (Figure 20A-B). In the tumor, this was further enhanced by combined VSV-ΔM51 and NKT cell activation therapies, with VSV-IL-15 inducing accumulation of more $Ly6G^+$ $CD11b^+$ cells than VSV-GFP (Figure 20A-B). This was further enhanced by the addition of anti-PD-1 (Figure 20C). Our lab has previously shown that NKT cell activation decreases MDSC-mediated immunosuppression in a 4T1 breast cancer model.[201] Therefore, we examined whether the same was true in pancreatic cancer. Blood cells from naïve, untreated, and treated mice were tested for their ability to suppress proliferation of Oregon green-labeled naïve T cells co-cultured with anti-CD3/CD28-coated stimulator beads. Blood $Ly6G^+$ $CD11b^+$ cells from untreated tumor-

bearing mice exhibited significant immunosuppression compared to cells from naive mice (Figure 20D-F). Blood leukocytes from mice that had received NKT cell activation therapy exhibited a partial inhibition of immunosuppressive activity (Figure 20D-E), resulting in increased T cell proliferation. Although VSV-IL-15 treatment increased the frequency of $Ly6G^+$ $CD11b^+$ cells, VSV-IL-15 treatment on its own did not alter tumor-induced immunosuppression (Figure 20D-E). Combination treatment with VSV-ΔM51 did not impair the effect of NKT cell activation on suppressive activity. The inhibition of suppression was NKT cell dependent as there was no rescue of T cell proliferation using cells from NKT cell-deficient $J\alpha18^{-/-}$ mice (Figure 20D).

As with the immune function assays, the benefit of NKT cell activation therapy against immunosuppression was overcome at day 55 (Figure 20F). The addition of anti-PD-1 therapy extended the ability of combined NKT cell activation and VSV-IL-15 therapy to impede immunosuppression (Figure 20F). Taken together, NKT cell activation can reduce immunosuppression in pancreatic cancer models and anti-PD-1 therapy can enhance this activity.

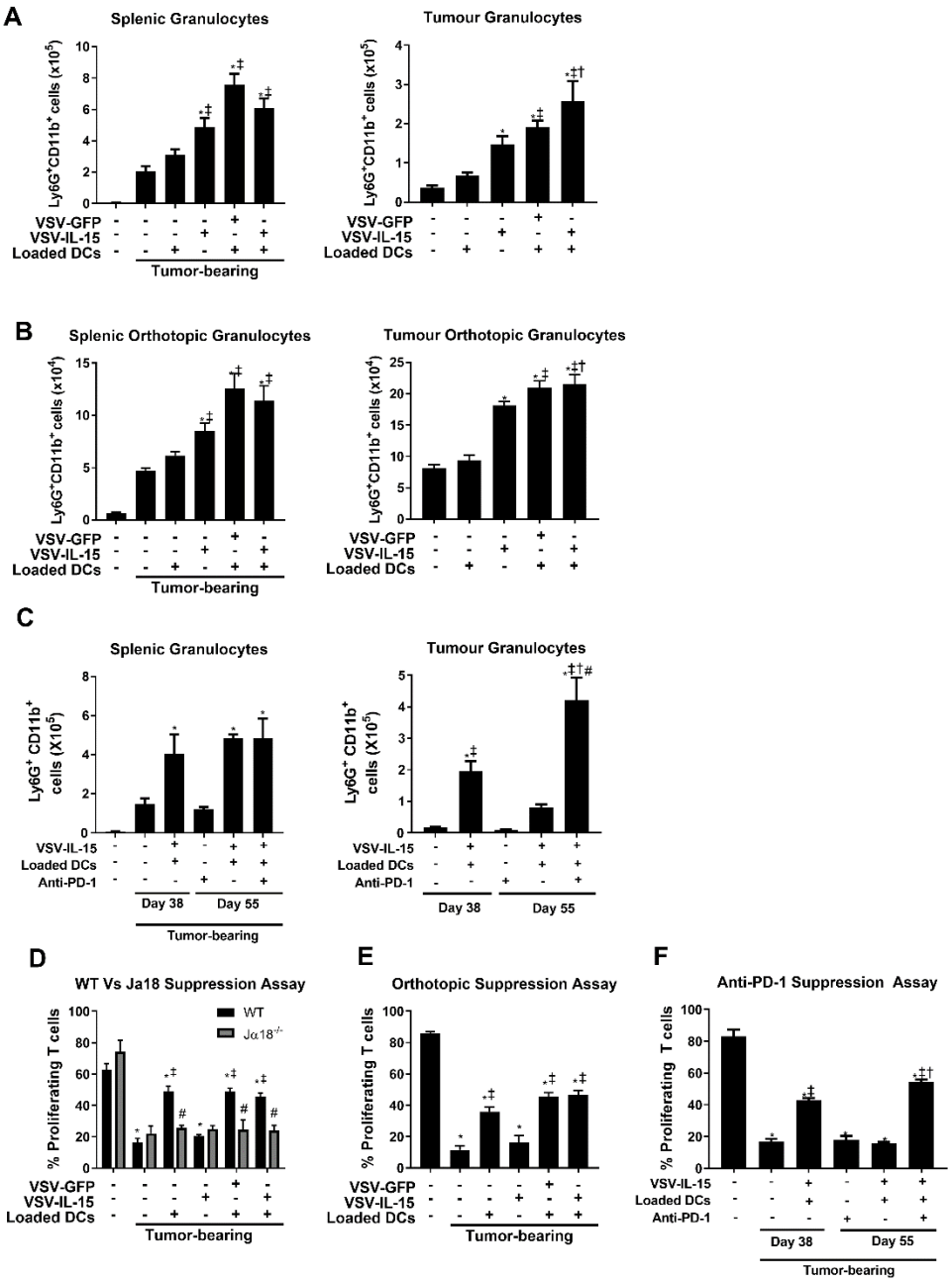

A
Splenic Granulocytes
Tumour Granulocytes
Ly6G+CD11b+ cells (x10^5)
VSV-GFP
VSV-IL-15
Loaded DCs
Tumor-bearing
B
Splenic Orthotopic Granulocytes
Tumour Orthotopic Granulocytes
Ly6G+CD11b+ cells (x10^4)
C
Splenic Granulocytes
Tumour Granulocytes
Ly6G+ CD11b+ cells (X10^5)
Anti-PD-1
Day 38
Day 55
D
WT Vs Ja18 Suppression Assay
% Proliferating T cells
WT
Jα18-/-
E
Orthotopic Suppression Assay
F
Anti-PD-1 Suppression Assay

Figure 20: VSV-ΔM51 induces granulocyte accumulation while NKT cell activation decreases MDSC-mediated immunosuppression in pancreatic cancer. A) Panc02 tumors were induced and treated with VSV-ΔM51 and glycolipid-loaded DCs as in Figure 8A. Spleens and tumors were isolated and dispersed into single cell suspensions. Flow cytometry was used to assess accumulation of granulocytic ($Ly6G^+$ $CD11b^+$) cells (n=5-9 per group). Granulocytic cells in tumors were normalized per 100mg of tumor weight. $^*p<0.05$ compared to untreated. $^\dagger p<0.05$ compared to glycolipid-loaded DCs. $^\ddagger p<0.05$ compared to VSV-IL-15. B) Panc02 tumors were induced and treated with VSV-ΔM51 and glycolipid-loaded DCs as in Figure 10A. Spleens and tumors were isolated and dispersed into single cell suspensions. Flow cytometry was used to assess accumulation of granulocytic ($Ly6G^+$ $CD11b^+$) cells (n=5-9 per group). Granulocytic cells in tumors were normalized per 100mg of tumor weight. $^*p<0.05$ compared to untreated. $^\dagger p<0.05$ compared to glycolipid-loaded DCs. $^\ddagger p<0.05$ compared to VSV-IL-15. C) Panc02 tumors were induced and treated with VSV-ΔM51, glycolipid-loaded DCs, and PD-1 blockade as in Figure 14E. Spleens and tumors were isolated and dispersed into single cell suspensions. Flow cytometry was used to assess accumulation of granulocytic ($Ly6G^+$ $CD11b^+$) cells (n=3-6 per group). Granulocytic cells in tumors were normalized per 100mg of tumor weight. *p<0.05 compared to untreated. †P<0.05 compared to anti-PD-1. $^\ddagger p<0.05$ compared to VSV-IL-15 plus glycolipid-loaded DCs (Day 38). $^\S p<0.05$ compared to VSV-IL-15 plus glycolipid-loaded DCs (Day 55). D-E) Naïve splenocytes from wild-type or $J\alpha18^{-/-}$ mice were stained with Oregon green and cultured with CD3/CD28 beads. Blood samples from untreated and treated mice were added to the cocultures for 72 hours. T cells ($TCR\beta^+$) were stained and examined for proliferation via Oregon green dilution (n=4-7 per group). $^*p<0.05$ compared to naïve. $^\dagger p<0.05$ compared to untreated. $^\ddagger p<0.05$ compared to WT. F) Naïve splenocytes from WT mice were stained with Oregon green and cultured with CD3/CD28 beads. Blood from untreated and treated mice were added to the coculture for 72 hours. T cells ($TCR\beta^+$) were stained and examined for proliferation (n=3-5 per group). $^*p<0.05$ compared to naïve. $^\dagger p<0.05$ compared to untreated. $^\ddagger p<0.05$ compared to VSV-IL-15 plus glycolipid-loaded DCs (Day 38).

CHAPTER 4: ONCOLYTIC VESICULAR STOMATITIS VIRUS EXPRESSING REOVIRUS MEMBRANE FUSION PROTEINS COMBINES WITH NATURAL KILLER T CELL IMMUNOTHERAPY TO CLEAR METASTATIC BREAST CANCER IN MICE

4.1 Introduction

One drawback of the Panc02 model is its lack of metastatic disease. Cancer metastasis is the leading cause of cancer-related deaths.[411] Current therapeutic treatments for cancer metastasis, including chemotherapy and radiation, are ineffective and cause many serious adverse events.[412,413] Furthermore, mutations to proteins involved in drug uptake and metabolism lead to acquired chemotherapeutic resistance, reducing a chemotherapeutic's efficacy over time.[414] Taken together, these factors highlight the need for treatments that effectively target cancer metastatic disease. Therefore, we look to test the efficacy of our recombinant VSV-ΔM51and NKT cell immunotherapy in a metastatic model. Breast cancer is the most common female cancer, and the second-leading cause of cancer death in women.[19] Triple negative breast cancer (Her2^{neg}, estrogen receptor^{neg}, progesterone receptor^{neg}) has the lowest survival rate out of any breast cancer subtype due to its lack of targeted therapy and its high rate of metastasis.[48,49] Previously, our lab showed that NKT cell immunotherapy was effective at targeting breast cancer metastasis, resulting in 40-50% of mice experiencing complete tumor clearance.[201] Combining NKT cell immunotherapy with chemotherapy or VSV-ΔM51 increased survival by a further 20-25%. [275,392] However, chemotherapy is associated with dose-limiting toxicities and severe adverse effects.[412,413] Therefore, we looked to improve our combination therapy with VSV-ΔM51.

The VSV-ΔM51 genome is amenable to modification via reverse genetics, allowing for insertion of exogenous genes to increase the virulence or immunomodulatory capabilities of the virus.[302] In the previous chapter, we used VSV-ΔM51 expressing the immunomodulatory protein IL-15. However, another strategy is to have VSV-ΔM51 express a protein that increases virulence, resulting in increased cancer cell lysis. Previously, it was shown that the reovirus FAST protein p14 increased the efficacy of VSV-ΔM51 virotherapy.[303] FAST proteins are small viral fusogens (~100-150 amino acids) that induce syncytium in a wide range of cells. [346,347] FAST proteins are trafficked to the cell membrane of infected cells where they induce fusion with neighboring cells, eventually causing membrane instability and cell death.[346] This allows the virus to infect neighboring cells without having to move out of the cell, allowing them to stay hidden from the immune system, increasing their virulence in cancer cells.[354] While p14 has been shown to increase the efficacy of VSV-ΔM51, there was limited work assessing the efficacy of other members of the FAST protein family. These other FAST proteins have limited sequence similarity and differing abilities to cause cell-to-cell fusion and cell death, which suggests that they may increase the efficacy of VSV-ΔM51 virotherapy to different extents.[345] Therefore, I compared the efficacy of VSV-p14 in the 4T1 model to VSV expressing the FAST proteins p10ARV (VSV-p10ARV), p10NBV (VSV-p10NBV), p15 (VSV-p15) and two mutated variants VSV-Δp14 and VSV-p14/15. VSV-Δp14 has three mutations in the p14 transmembrane domain (A41L, G42L, A45L) that significantly decreased surface expression of p14; however, syncytia levels were maintained at normal levels, demonstrating more efficient syncytia formation (data not shown). VSV-p14/15 expresses a chimeric p14 protein whose endodomain has

been replaced with the endodomain of p15.[347] The endodomain of the FAST protein is responsible for interaction with multiple cytoplasmic proteins, transport to the plasma membrane, and pore formation.[346,350,415] A plasmid containing the p14/15 protein caused more cell fusion than p14 or p15 in QM5 and Vero cells (data not shown), suggesting that the chimeric protein may increase the efficacy of VSV-ΔM51 more than p14 or p15.

Here I show that VSV-p15 increases VSV-ΔM51's efficacy in 4T1 breast cancer models compared to VSV-p14, VSV-p10ARV, VSV-p10NBV, and VSV-Δp14. VSV-p15 significantly increased immunogenic cell death markers as well as tumor immune infiltration, slowing tumor progression. VSV-p14, VSV-p14/15, and VSV-p15, when combined with NKT cell immunotherapy, cleared lung metastases in 100% of mice. However, combination therapy with VSV-p15 maintained the 100% clearance at a 10-fold dilution compared to VSV-p14 and VSV-p14/15.

4.2 Results

4.2.1 FAST proteins increase VSV-ΔM51 anti-tumor activity *in vivo*

We examined whether FAST proteins could increase the anti-tumor activity of VSV-ΔM51 in a 4T1 primary tumor model. $2x10^5$ 4T1 cells were injected into the 4th mammary pad of female BALB/c mice. Mice were treated with VSV-GFP, VSV-p10ARV, VSV-p10NBV, VSV-p14, VSV-Δp14 VSV-p15, or VSV-p14/15 (it. 1 x 10^8 PFUs) on days 10, 12, and 14 (Figure 21A). VSV-GFP significantly increased tumor regression and overall survival compared to untreated mice (Figure 21B-G). VSV-p14 and VSV-p15 significantly increased overall survival and tumor regression compared to untreated and VSV-GFP treated mice (Figure 21B-C). Furthermore, VSV-p15 infection caused the greatest tumor regression and overall survival, even at a 10-fold dilution

(Figure 21B-C). VSV-p10ARV did not increase overall survival time compared to VSV-GFP (Figure 21D-E). While VSV-p10NBV significantly increased tumor regression and overall survival (Figure 21D-E), it also displayed significant toxicity when delivered intravenously (data not shown). Therefore, VSV-p10ARV and VSV-p10NBV were dropped from further experiments, due to their toxicity or lack of efficacy *in vivo*. VSV-Δp14 and VSV-p14/15 significantly increased tumor regression and overall survival compared to VSV-GFP (Figure 21F-G). While VSV-p14/15 did show increased efficacy over VSV-p14, VSV-Δp14 did not, so VSV-Δp14 was dropped from future experiments (Figure 21F-G). Taken all together, FAST proteins increased the anti-tumor activity of VSV-ΔM51 *in vivo*, with p15 leading to the greatest increase in efficacy.

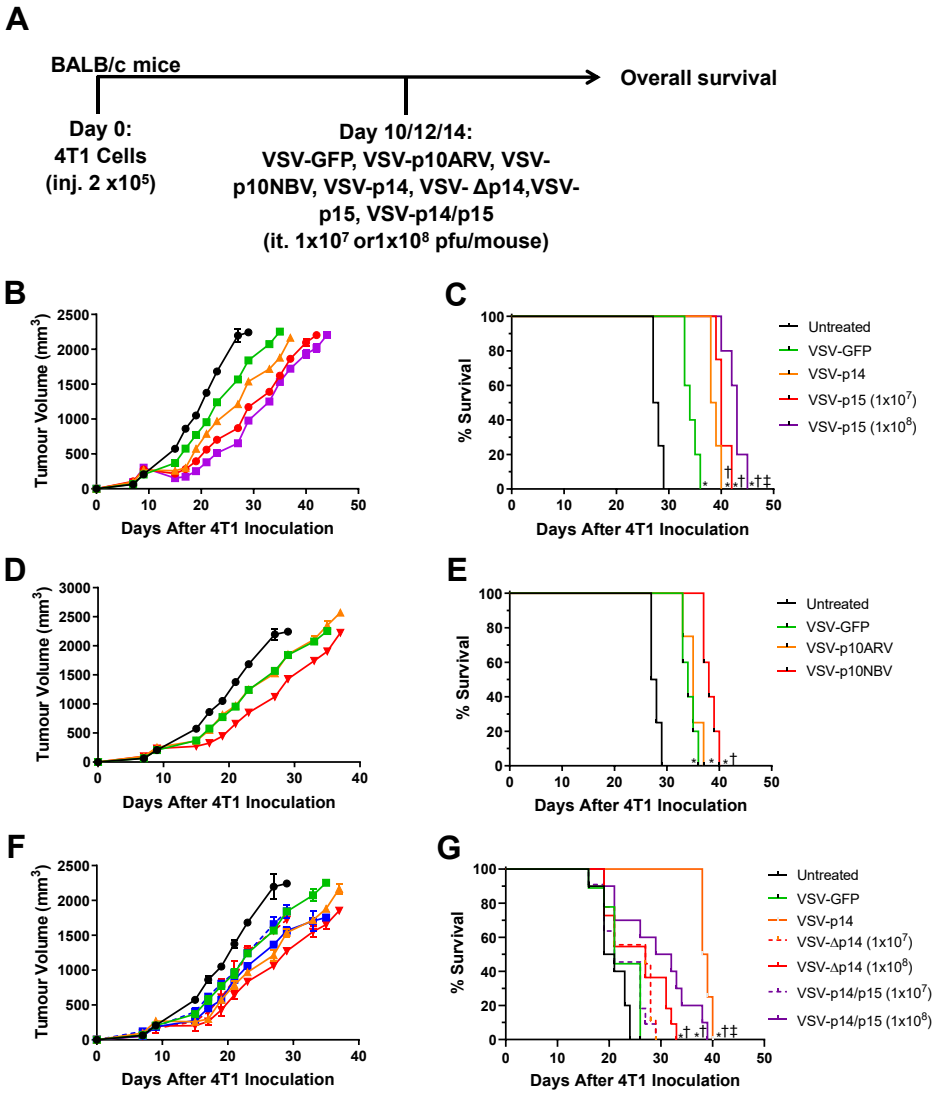


Figure 21: FAST proteins increase the anti-tumor activity of VSV-ΔM51 and overall survival in a primary 4T1 model. A) Schematic of 4T1 primary tumor model. 4T1 tumor volume (B) and survival (C) was assessed in untreated mice and mice treated with VSV-GFP, VSV-p14, VSV-p15 ($1X10^7$), or VSV-p15 ($1X10^8$) (n= 4-14 per group). $^*p<0.05$ compared to untreated. $^\dagger p<0.05$ compared to VSV-GFP. $^\ddagger p<0.05$ compared to VSV-p14. 4T1 tumor volume (D) and survival (E) was assessed in untreated mice and mice treated with VSV-GFP, VSV-p10ARV, and VSV-p10NBV (n= 4-14 per group). $^*p<0.05$ compared to untreated. $^\dagger p<0.05$ compared to VSV-GFP. $^\ddagger p<0.05$ compared to VSV-p14. 4T1 tumor volume (F) and survival (G) was assessed in untreated mice and mice treated with VSV-GFP, VSV-p14, VSV-Δp14, and VSV-p14/p15 (n= 9-14 per group). $^*p<0.05$ compared to untreated. $^\dagger p<0.05$ compared to VSV-GFP. $^\ddagger p<0.05$ compared to VSV-p14. $^*p<0.05$ compared to untreated. $^\dagger p<0.05$ compared to VSV-GFP. $^\ddagger p<0.05$ compared to VSV-Δp14.

4.2.2 FAST proteins increase VSV-ΔM51's ability to induce immunogenic cell death

An important mechanism by which oncolytic viruses carry out their anti-tumor effects is their ability to induce immunogenic cell death (ICD).[416] Previously our lab has shown that VSV-ΔM51 can induce ICD in 4T1 cells.[275] Therefore, we examined whether the expression of FAST proteins would increase VSV-ΔM51's ability to induce ICD. 24 hours after the final virus injection, tumors and blood were taken from treated and untreated mice to examine markers of ICD. Surface expression of CALR is a critical mediator of ICD, binding to CD91 on antigen presenting cells and inducing autophagy.[364] VSV-ΔM51 infection increased CALR surface expression compared to untreated mice (Figure 22A). Furthermore, VSV-p14, VSV-p15, and VSV-p14/15 significantly increased surface expression of CALR compared to VSV-GFP, demonstrating that FAST proteins increase ICD (Figure 22A). To further examine if cells were undergoing ICD, we looked at additional markers. HMGB1 binds to RAGE on DCs, increasing their maturation and homing to lymph nodes.[370] HMGB1also binds TLR4 on DCs, leading to their production of inflammatory cytokines.[370] Newer work identifies the CXCR3 ligand CXCL10 as another critical marker for ICD.[381] Therefore, we examined serum levels of HMGB1 and CXCL10. VSV-GFP increased serum levels of HMGB1 and CXCL10 compared to untreated mice (Figure 22B-C). However, VSV-p14, VSV-p15, and VSV-p14/15 significantly increased serum levels of HMGB1 and CXCL10 compared to untreated and VSV-GFP mice, further indicating that FAST proteins increase the ability of VSV to induce ICD (Figure 22B-C). Furthermore, VSV-p15 significantly increased serum

HMGB1 and CXCL10 compared to VSV-p14 at a 10-fold dilution, demonstrating that p15 was able to further increase ICD even at a lower concentration (Figure 22B-C).

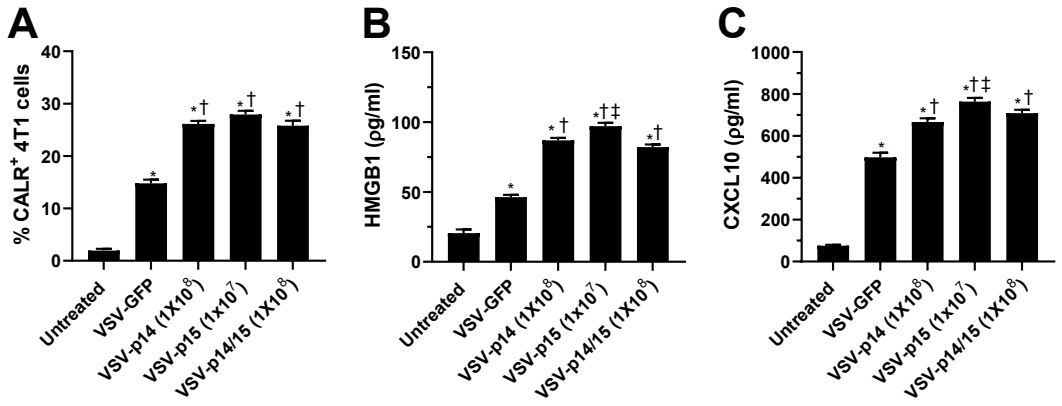


Figure 22: FAST proteins increase the ability of VSV-ΔM51 to induce immunogenic cell death. Mice were treated as in Figure 20A. Tumors and blood were harvested 24 hours from mice after final VSV-ΔM51 injection. A) Tumors were isolated and dispersed into single cell suspensions. Flow cytometry was used to assess surface expression of calreticulin. (n=4-9per group). Serum was isolated from blood of untreated and treated mice. B) HMGB1 and C) CXCL10 concentration was determined by ELISA (n=3-9per group). $^{*}p<0.05$ compared to untreated. $^{\dagger}p<0.05$ compared to VSV-GFP. $^{\ddagger}p<0.05$ compared to VSV-p14.

4.2.3 VSV-p14 and VSV-p15 increase 4T1 immune infiltration and activation

Oncolytic viruses work in part by increasing tumor immune infiltration.[276] To examine whether FAST proteins affect immune infiltration and activity towards the tumor, we examined immune populations in the spleen and tumor 24 hours post final virus injection. VSV-p14 and VSV-p15 increased NK, NKT, $CD4^+$ and $CD8^+$ T cells in the tumor (Figure 23). Furthermore, VSV-p14 and VSV-p15 increased the function of tumor infiltrating NK, NKT, $CD4^+$ and $CD8^+$ T cells, increasing CD69, PD-1, and IFNγ expression (Figure 23). VSV-P14 and VSV-p15 increased DC cell tumor infiltration and CD80 expression compared to VSV-GFP and untreated groups (Figure 23).

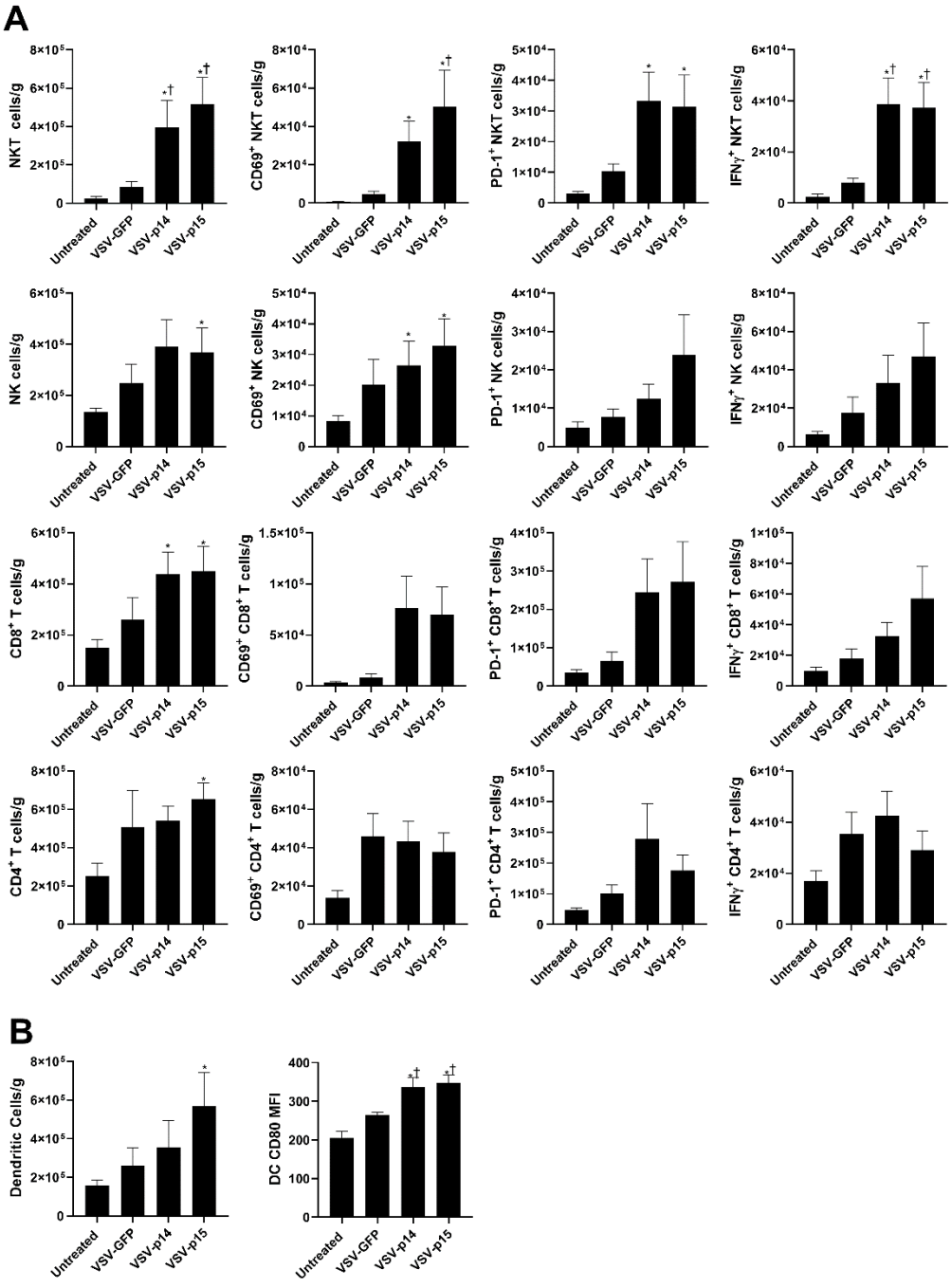


Figure 23: VSV-FAST increase 4T1 tumor immune infiltration and activation. Tumors of untreated and mice treated with VSV-GFP, VSV-p14 and VSV-p15 were isolated and dispersed into single cell suspensions. Flow cytometry was used to assess $CD8^+$ T cells ($TCR\beta^+$ $CD8\alpha^+$), $CD4^+$ T cells ($TCR\beta^+$ $CD4^+$), NK cells ($NK1.1^+$ $TCR\beta^-$), NKT cells (CD1d $tetramer^+$ $TCR\beta^+$), dendritic cells (MHC II^+ $CD11c^+$) and their expression of CD69, PD-1, IFNγ and CD80. (n= 8-9 per group). $^+$). Immune cell populations in tumors were normalized per 1g of tumor $^*p<0.05$ compared to untreated. $^\dagger p<0.05$ compared to VSV-GFP.

VSV-p14 and VSV-p15 also increased function of NKT, $CD4^+$ and $CD8^+$ T cells in the spleen, resulting in increased CD69, PD-1, and IFNγ expression (Figure 24). CD80 expression on splenic DCs was increased by VSV-p14 and VSV-p15 infection, indicating an increase in antigen presentation (Figure 24). Taken together, VSV-p14 and VSV-p15 increase immune infiltration and activity towards the tumor compared to VSV-GFP.

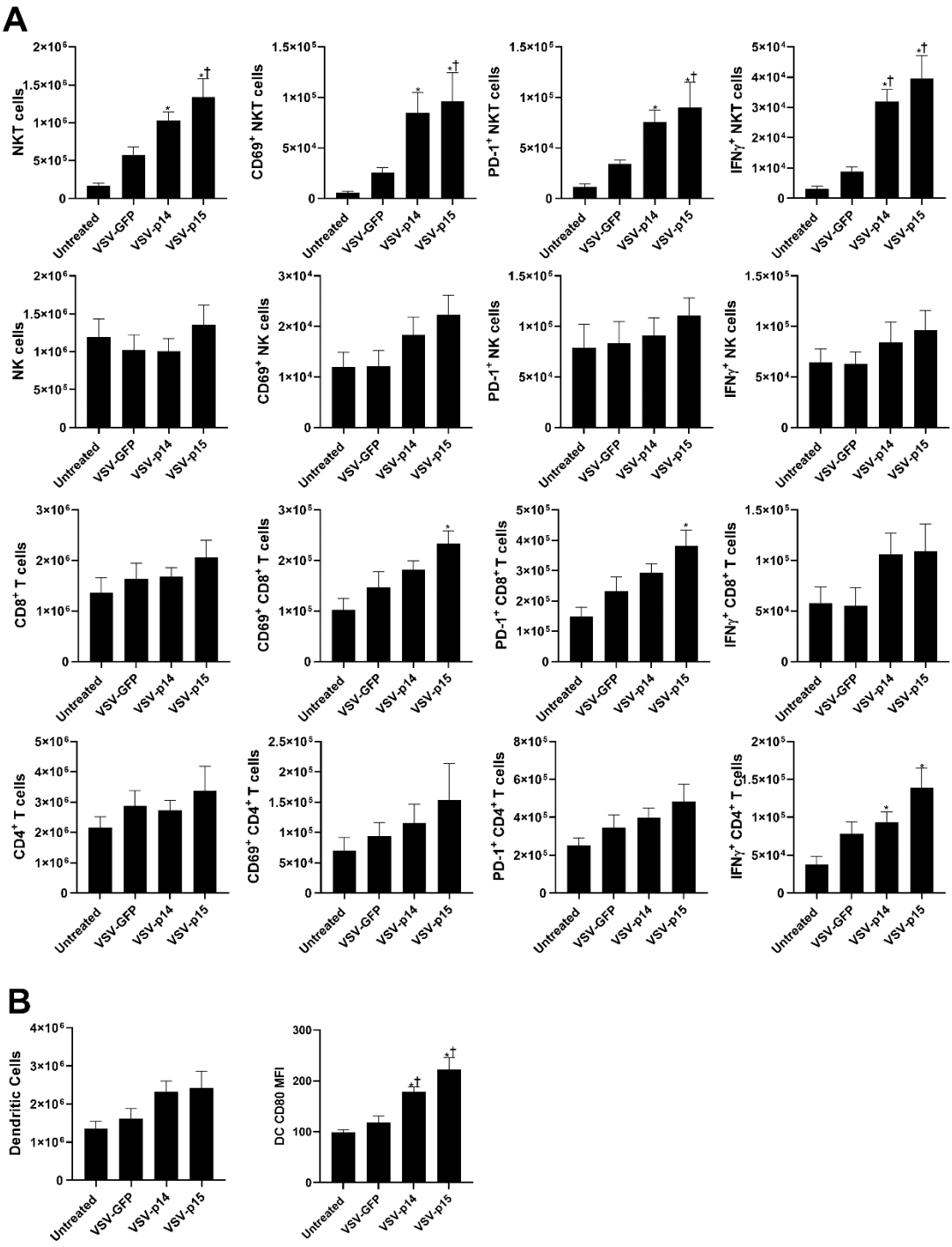


Figure 24: VSV-FAST increase immune activation in the spleen in a primary 4T1 tumor model. Spleens of untreated and mice treated with VSV-GFP, VSV-p14, VSV-p15, and VSV-p14/p15 were isolated and dispersed into single cell suspensions. Flow cytometry was used to assess $CD8^+$ T cells ($TCR\beta^+$ $CD8\alpha^+$), $CD4^+$ T cells ($TCR\beta^+$ $CD4^+$), NK cells ($NK1.1^+$ $TCR\beta^-$), NKT cells (CD1d $tetramer^+$ $TCR\beta^+$), dendritic cells (MHC II^+ $CD11c^+$) and their expression of CD69, PD-1, IFNγ and CD80. (n= 8-9 per group). $^*p<0.05$ compared to untreated. $^\dagger P<0.05$ compared to VSV-GFP.

4.2.4 VSV-p14 or VSV-p15 in combination with NKT cell activation clears lung metastasis and leads to 100% survival in a metastatic breast cancer model.

Metastasis is the leading cause of breast cancer-related death. [411] Earlier work has shown that VSV can increase the therapeutic benefit of NKT cell immunotherapy in a breast cancer metastasis model.[275] Therefore, we examined whether VSV-p14, VSV-p15, or VSV-p14/15 could increase the efficacy of NKT cell immunotherapy compared to VSV-GFP. $2x10^5$ 4T1 cells were injected into the 4^{th} mammary pad of female BALB/c mice. Tumors were surgically resected on day 12, when the tumors were ~200mm^3. Mice were treated with VSV-GFP, VSV-p14, VSV-p15, VSV-p14/15 (iv. 5 x 10^8 PFUs) on days 13, 15, and 17 followed by unloaded or α-GalCer loaded DCs (iv. 2x 10^5) on day 18 (Figure 25A). VSV-GFP and VSV-p14 alone increased overall survival time compared to untreated mice (Figure 25B). When VSV-GFP or VSV-p14 were used in combination with loaded DCs, 75 and 100% of mice survived, respectively (Figure 25B). VSV-p15 in combination with loaded DCs provided 100% protection when VSV-p15 was used at $5X10^7$, a 10-fold dilution lower than the dose delivered for VSV-p14 and VSV-GFP (Figure 25). When VSV-p14 was used at this lower concentration in combination with loaded DCs, the therapy lost its 100% protection (Figure 25C). VSV-p14/15 in combination with loaded DCs provided 100% protection when VSV-p14/15 was used at $5X10^8$, however similar to VSV-p14 it lost its 100% protection when used at $5X10^7$ (Figure 25C). Taken all together, VSV-ΔM51 expressing p14, p15, or p14/15 in combination with NKT cell immunotherapy provides 100% protection against breast cancer metastasis, and VSV-p15 can provide 100% protection at a 10-fold dilution compared to VSV-p14 and VSV-p14/15.

To examine how VSV-ΔM51 in combination with NKT cell immunotherapy provides increased protection, we isolated lungs on day 25 to examine metastatic burden. We examined lung metastatic burden by clonogenic plating assays.[390] VSV-ΔM51 treatment alone significantly decreased metastatic burden compared to untreated mice, with VSV-p14 and VSV-p15 significantly decreasing lung metastatic burden compared to VSV-GFP, demonstrating that the FAST proteins further decreased metastatic burden (Figure 25D). VSV-ΔM51 in combination with NKT cell immunotherapy significantly decreased metastatic burden compared to untreated and single treatments (Figure 25D). VSV-p14 or VSV-p15 combined with NKT cell immunotherapy completely cleared metastatic burden (Figure 25D). Furthermore, combination treatment with VSV-p15 was able to clear metastatic burden while using VSV-p15 at a 10-fold lower dilution compared to the other viruses. Whether VSV-p14/15 in combination with NKT cell immunotherapy clears metastatic burden still needs to be examined.

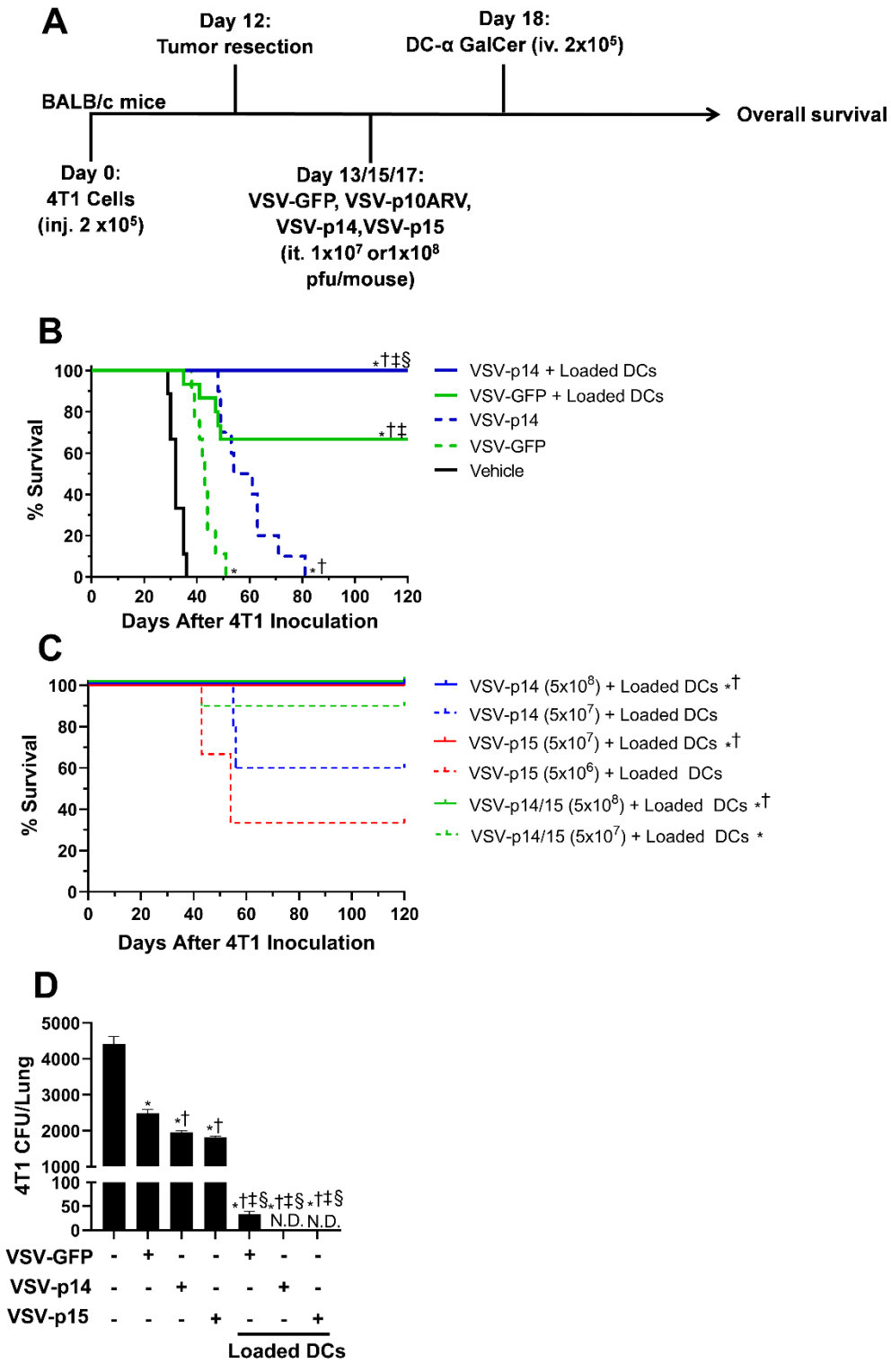

A
Day 12:
Tumor resection
Day 18:
DC-α GalCer (iv. 2x10⁵)
BALB/c mice
Overall survival
Day 0:
4T1 Cells
(inj. 2 x10⁵)
Day 13/15/17:
VSV-GFP, VSV-p10ARV,
VSV-p14,VSV-p15
(it. 1x10⁷ or1x10⁸
pfu/mouse)
B
% Survival
Days After 4T1 Inoculation
VSV-p14 + Loaded DCs
VSV-GFP + Loaded DCs
VSV-p14
VSV-GFP
Vehicle
C
% Survival
Days After 4T1 Inoculation
VSV-p14 (5x10⁸) + Loaded DCs
VSV-p14 (5x10⁷) + Loaded DCs
VSV-p15 (5x10⁷) + Loaded DCs
VSV-p15 (5x10⁶) + Loaded DCs
VSV-p14/15 (5x10⁸) + Loaded DCs
VSV-p14/15 (5x10⁷) + Loaded DCs
D
4T1 CFU/Lung
N.D. N.D.
VSV-GFP
VSV-p14
VSV-p15
Loaded DCs

Figure 25: VSV-p14, VSV-p15, or VSV-p14/15 in combination with NKT cell activation leads to 100% survival in a metastatic breast cancer model. A) Schematic of 4T1 metastatic model. B) Overall survival was assessed in untreated mice and mice receiving VSV-GFP or VSV-p14, alone and VSV-GFP, VSV-p14, in combination with NKT cell immunotherapy (n= 9-14 per group). $^{*}p<0.05$ compared to untreated. $^{\dagger}p<0.05$ compared to VSV-GFP. $^{\ddagger}p<0.05$ compared to VSV-p14. $^{\S}p<0.05$ compared to VSV-GFP + Loaded DCs. C) Overall survival was assessed in untreated mice and mice receiving VSV-p14, VSV-p15, or VSV-p14/15 in combination with NKT cell immunotherapy (n= 3-8 per group). $^{*}p<0.05$ compared to VSV-p15 ($5x10^{6}$) + Loaded DCs. $^{\dagger}p<0.05$ compared to VSV-p14 ($5x10^{7}$) + Loaded DCs. D) Spleens and lungs of untreated and treated mice were isolated and dispersed into single cell suspensions. A) Lung cells were cultured in media containing 6-thioguanine for 7 days, before fixation and staining with methylene blue. (n=4-7 per group). $^{*}p<0.05$ compared to untreated. $^{\dagger}p<0.05$ compared to VSV-GFP. $^{\ddagger}p<0.05$ compared to VSV-p10ARV. $^{\S}p<0.05$ compared to VSV-p14.

4.2.5 Re-challenged mice have an increased immune memory response towards 4T1 tumors

Mice who survived tumor challenge from Figure 5 were re-challenged with $2x10^5$ 4T1 cells. Mice who were treated upon initial tumor challenge with VSV-GFP, VSV-p14, or VSV-p15 in combination with NKT cell immunotherapy had reduced tumor growth upon re-challenge compared to naïve mice, demonstrating formation of immune memory (Figure 26A). There was no difference in tumor growth between the treated groups, indicating that the expression of a FAST protein did not lead to a stronger memory response (Figure 26A). Tumors from naïve and re-challenged mice were isolated on day 26 to examine immune infiltration. Treated mice had reduced tumor weight compared to naïve mice (Figure 26B); however, there was no difference in tumor weight between the different treatment groups (Figure 26B). Tumors from re-challenged mice had increased immune infiltration of NKT, DCs, $CD4^+$ and $CD8^+$ T cells (Figure 26C), indicating that re-challenged tumors were immunologically hotter compared to naïve tumors. Furthermore, the infiltrating immune cells expressed higher levels of CD69 (Figure 26C) demonstrating higher levels of activation in the tumor microenvironment. Re-challenged tumors also had increased memory NKT, $CD4^+$ and $CD8^+$ T cells, showing that a memory immune response had formed towards the tumor (Figure 26C). Similar results were seen in the spleens of treated mice (Figure 27). Taken together, re-challenged mice showed an increased immune memory response towards 4T1 cells compared to naïve mice, leading to slower tumor growth.

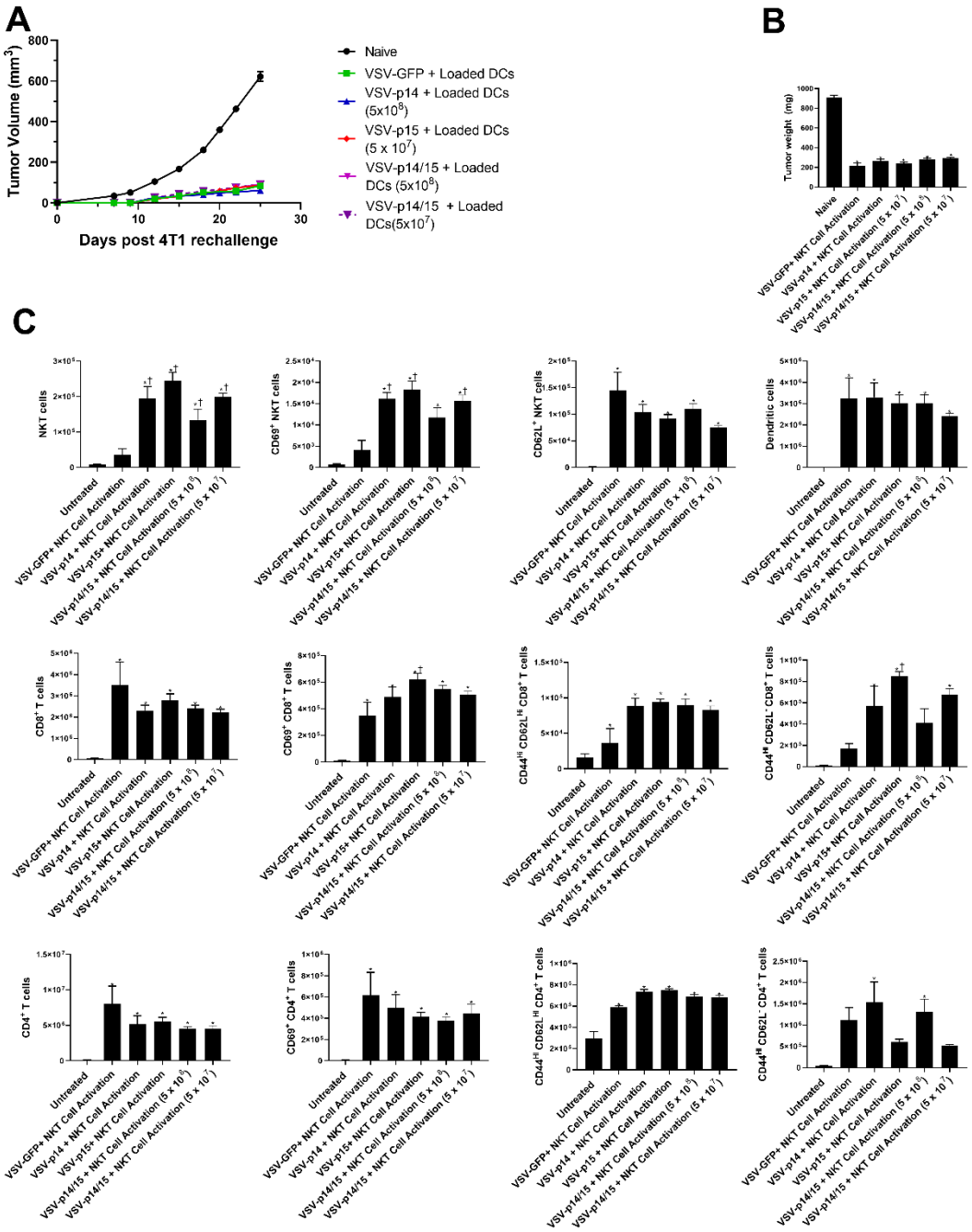

A
Tumor Volume (mm3)
Days post 4T1 rechallenge
Naive
VSV-GFP + Loaded DCs
VSV-p14 + Loaded DCs (5x10^8)
VSV-p15 + Loaded DCs (5 x 10^7)
VSV-p14/15 + Loaded DCs (5x10^8)
VSV-p14/15 + Loaded DCs(5x10^7)
B
Tumor weight (mg)
C
NKT cells
CD69+ NKT cells
CD62L+ NKT cells
Dendritic cells
CD8+ T cells
CD69+ CD8+ T cells
CD44HI CD62L HI CD8+ T cells
CD44HI CD62L- CD8+ T cells
CD4+ T cells
CD69+ CD4+ T cells
CD44HI CD62L HI CD4+ T cells
CD44HI CD62L- CD4+ T cells
Untreated
VSV-GFP+ NKT Cell Activation
VSV-p14 + NKT Cell Activation
VSV-p15+ NKT Cell Activation
VSV-p14/15 + NKT Cell Activation (5 x 10^8)
VSV-p14/15 + NKT Cell Activation (5 x 10^7)

Figure 26: Rechallenged mice have increased memory immune infiltration and reduced tumor growth compared to naïve mice. Tumor-resected mice that survived to day 120 (Figure 25) were re-challenged in the contralateral mammary fat pad with 4T1 cells. Tumor A) volume and B) weight was compared to naïve mice. C) Tumors of mice were isolated and dispersed into single cell suspensions. Flow cytometry was used to assess NKT cells (CD1d tetramer^{+} TCRβ^{+}), CD8^{+} T cells (TCRβ^{+} CD8α^{+}), CD4^{+} T cells (TCRβ^{+} CD4^{+}), their activation (CD69^{+}), memory NKT cells (CD62L^{+} CD1d tetramer^{+} TCRβ^{+}) dendritic cells (MHC II^{+} CD11c^{+}), effector memory CD4^{+} T cells (CD44^{HI} CD62L^{neg} TCRβ^{+} CD4^{+}), Effector memory CD8^{+} T cells (CD44^{HI} CD62L^{neg} TCRβ^{+} CD8^{+}), central memory CD4^{+} T cells (CD44^{HI} CD62L^{HI} TCRβ^{+} CD8^{+}), central memory CD8^{+} T cells (CD44^{HI} CD62L^{HI} TCRβ^{+} CD8^{+}). Immune cell populations in tumors were normalized per 1g of tumor (n = 3-12 per group). *$p < 0.05$ compared to naïve control. †$p<0.05$ compared to VSV-GFP + Loaded DCs.

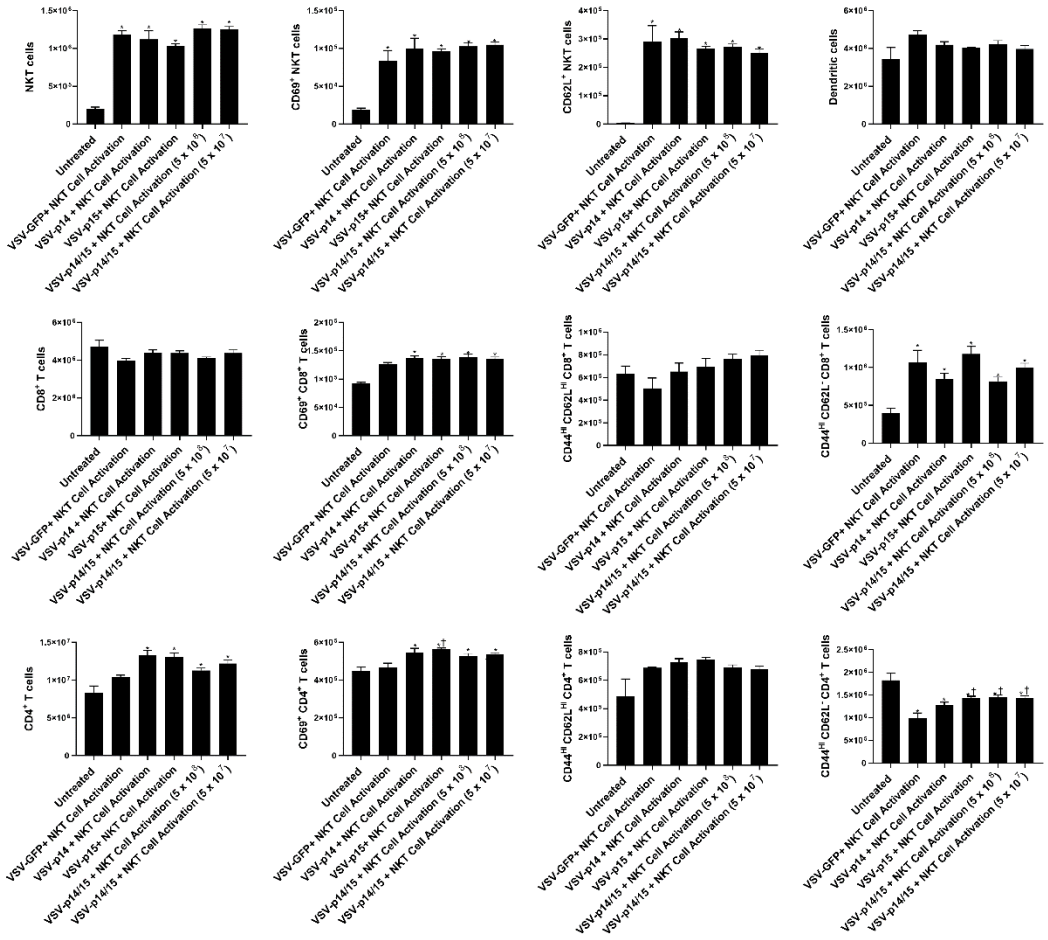


Figure 27: Rechallenged mice have increased splenic memory immune populations compared to naïve mice. Tumor-resected mice that survived to day 120 (Figure 25) were re-challenged in the contralateral mammary fat pad with 4T1 cells. Spleens of mice were isolated and dispersed into single cell suspensions.Flow cytometry was used to assess NKT cells (CD1d tetramer$^+$ TCRβ$^+$), CD8$^+$ T cells (TCRβ$^+$ CD8α$^+$), CD4$^+$ T cells (TCRβ$^+$ CD4$^+$), their activation (CD69$^+$), memory NKT cells (CD62L$^+$ CD1d tetramer$^+$ TCRβ$^+$) dendritic cells (MHC II$^+$ CD11c$^+$), effector memory CD4$^+$ T cells (CD44^{HI} CD62L^{neg} TCRβ$^+$ CD4$^+$), Effector memory CD8$^+$ T cells (CD44^{HI} CD62L^{neg} TCRβ$^+$ CD8$^+$), central memory CD4$^+$ T cells (CD44^{HI} CD62L^{HI} TCRβ$^+$ CD8$^+$), central memory CD8$^+$ T cells (CD44^{HI} CD62L^{HI} TCRβ$^+$ CD8$^+$). (n = 3-12 per group). *p< 0.05 compared to naïve control. †p<0.05 compared to VSV-GFP + Loaded DCs.

4.2.6 VSV-p14 or VSV-p15 in combination with NKT cell immunotherapy increases immune function towards 4T1 cells

Spleens from treated and untreated mice were isolated on day 25 to examine the immune response towards metastasis. $CD8^+$ T cells were sorted from untreated and treated spleens and co-cultured with Oregon green-labelled 4T1 cells for 18 hours. $CD8^+$ T cells from mice that received VSV-p14 or VSV-p15 treatment alone exhibited increase cytotoxicity against 4T1 cells (increased $7AAD^+$ Annexin V^+ 4T1 cells) (Figure 28A). Combination therapy of NKT cell immunotherapy with VSV-p14 or VSV-p15 significantly increased cytotoxicity compared to VSV-ΔM51 treatment alone, as well as VSV-GFP combined with NKT cell immunotherapy (Figure 28A). The increase in cytotoxicity was accompanied by increases in pro-inflammatory cytokines IFNγ (Figure 28B) and TNF (Figure 28C).

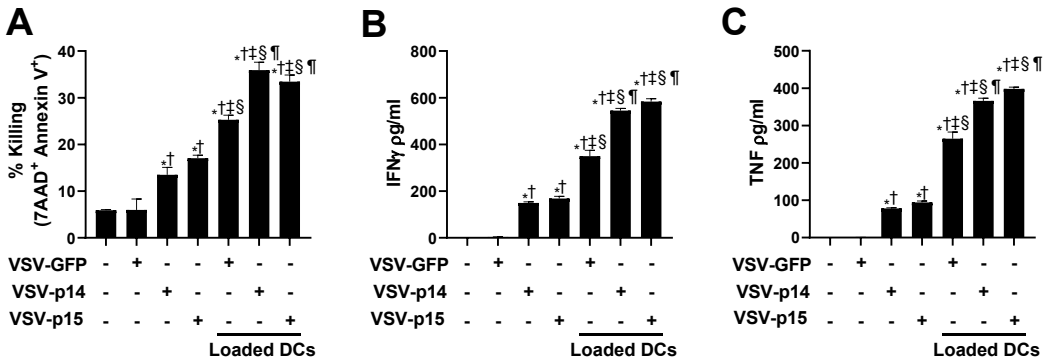


Figure 28: VSV-p14 or VSV-p15 in combination with NKT cell activation increases CD8+ T cell cytotoxicity and pro-inflammatory cytokine production. A) Magnetic bead sorted $CD8^+$ T cells were cocultured with Oregon green labelled 4T1 cells. 4T1 cells stained with Annexin V and 7AAD after 18 hour incubation at a 1:1 ratio with $CD8^+$ T cells isolated from untreated or treated mouse spleens. Supernatant was taken from an 18 hour incubation of 4T1 cells at a 1:1 ratio with $CD8^+$ T cells isolated from untreated or treated mouse spleens. Concentration of B) IFNγ and C) TNF was determined using Ready-Set-Go ELISAs. (n=3 per group). $^*p<0.05$ compared to untreated. $^\dagger p<0.05$ compared to VSV-GFP. $^\ddagger p<0.05$ compared to VSV-p10ARV. $^\S p<0.05$ compared to VSV-p14. $^\P p<0.05$ compared to VSV-GFP + Loaded DCs.

To further assess the immune response, we examined cell populations in the spleens of untreated and treated mice. Spleens of mice treated with VSV-p14 or VSV-p15 in combination with NKT cell activation therapy had a significantly increase in NKT and $CD8^+$ T cells compared to VSV-GFP alone or in combination with NKT cell activation (Figure 29). Furthermore, NKT and $CD8^+$ T cells from mice treated with VSV-p14 or VSV-p15 in combination with NKT cell activation had increased expression of PD-1 and CD69, demonstrating increased immune cell activation (Figure 29). Treatment with VSV-p14 or VSV-p15 alone increased CD69 expression on NK cells, with combination treatment increasing expression further. CD69 expression on NK cells did not increase in mice treated with VSV-GFP alone or in combination with NKT cell activation, indicating that the increase was due to FAST protein expression (Figure 29). While no treatment increased the number of DCs in the spleen, NKT cell activation in combination with VSV-p14 or VSV-p15 increased CD80 expression on splenic DCs, indicating increased antigen presentation (Figure 29). Taken all together, combination of VSV-p14 or VSV-p15 with NKT cell activation significantly increased immune activation and function, leading to complete clearance of lung metastasis.

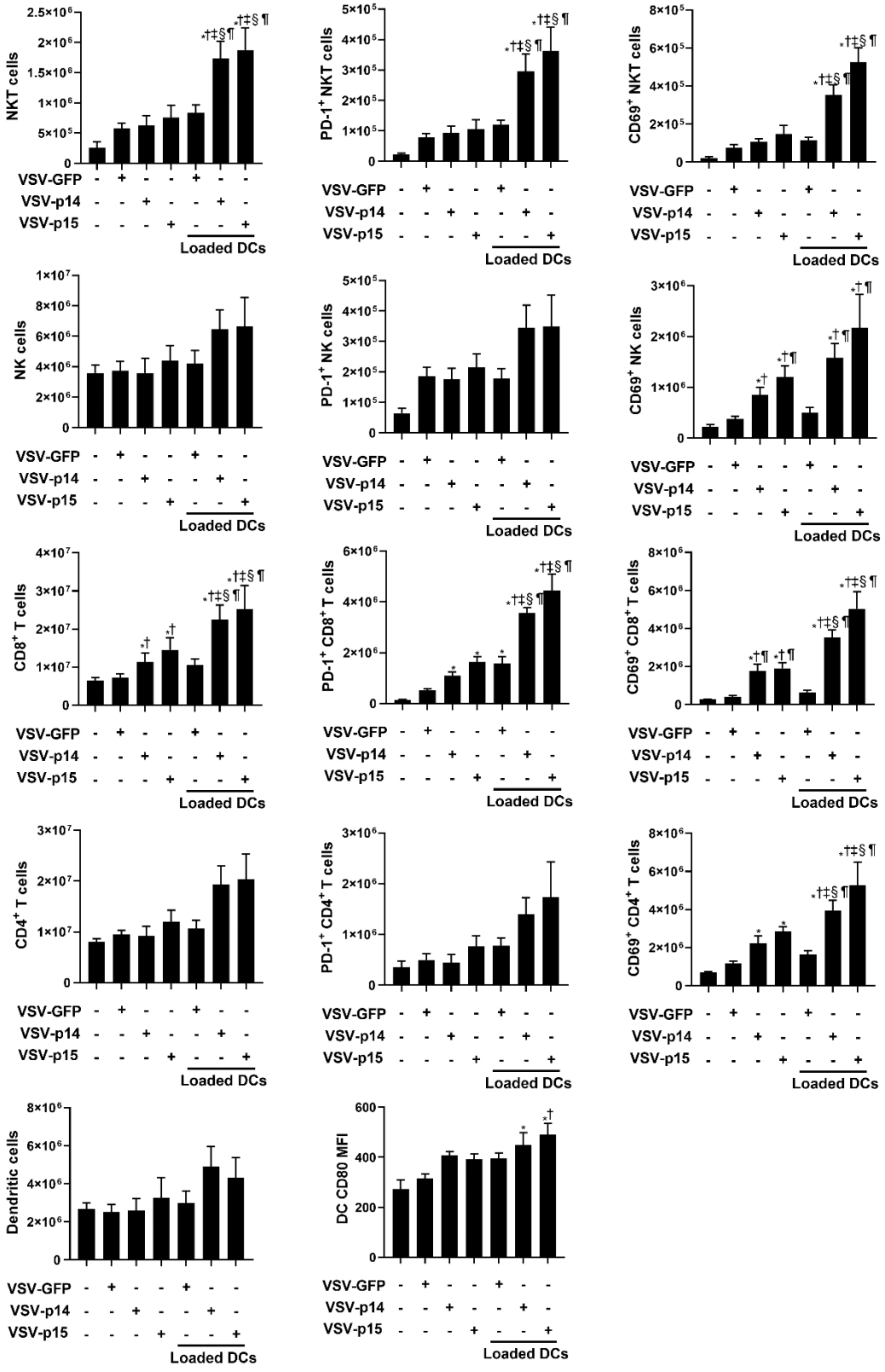

NKT cells
PD-1+ NKT cells
CD69+ NKT cells
NK cells
PD-1+ NK cells
CD69+ NK cells
CD8+ T cells
PD-1+ CD8+ T cells
CD69+ CD8+ T cells
CD4+ T cells
PD-1+ CD4+ T cells
CD69+ CD4+ T cells
Dendritic cells
DC CD80 MFI
VSV-GFP
VSV-p14
VSV-p15
Loaded DCs

Figure 29: VSV-p14 or VSV-p15 in combination with NKT cell activation clears lung metastasis and increases immune activation. Spleens of untreated and treated mice were isolated and dispersed into single cell suspensions. Flow cytometry was used to assess $CD8^+$ T cells ($TCR\beta^+$ $CD8\alpha^+$), $CD4^+$ T cells ($TCR\beta^+$ $CD4^+$), NK cells ($NK1.1^+$ $TCR\beta^-$) and NKT cells (CD1d $tetramer^+$ $TCR\beta^+$) dendritic cells (MHC II^+ $CD11c^+$) and their expression of CD69, PD-1, and CD80. (n=5-9). $^*p<0.05$ compared to untreated. $^\dagger p<0.05$ compared to VSV-GFP. $^\ddagger p<0.05$ compared to VSV-p10ARV. $^\S p<0.05$ compared to VSV-p14. $^\P p<0.05$ compared to VSV-GFP + Loaded DCs.

CHAPTER 5: DISCUSSION

5.1 NKT cell immunotherapy in combination with VSV-IL-15 and anti-PD-1 increases pancreatic cancer regression and tumor clearance

Current treatments for pancreatic cancer are largely ineffective and lead to many adverse events,[38,417] highlighting the need for new and more effective treatments. Here I report that NKT cell activation in combination with VSV-ΔM51 expressing IL-15 can be an effective treatment for pancreatic cancer. In both the subcutaneous and orthotopic models of pancreatic cancer, the combination of VSV-IL-15 and NKT cell increased overall survival and tumor regression (Figure 8 and 10). Interestingly, similar results were obtained in both models. The orthotopic model has been shown to better recapitulate the desmoplasia and immunosuppression seen in pancreatic cancer patients,[386,406] indicating that our treatment is still effective in a more immunosuppressive environment. This is an important factor in a highly immunosuppressive cancer, such as pancreatic cancer.[410] While our therapies induced tumor regression and increased anti-tumor immunity, the Panc02 tumors all eventually relapsed and progressed over time. This could be due to upregulation of immunosuppressive mechanisms that impair the immune response. The immunosuppressive checkpoint molecule PD-1 and its ligand PD-L1 are upregulated in response to pro-inflammatory cytokines, such as IFNγ and TNF,[69] which are generated in response to our NKT cell and combination therapies. The combination of NKT cell immunotherapy and VSV-IL-15 increased PD-1 expression on lymphocytes and PD-L1 expression on Panc02 tumors (Figure 14). The PD-1/PD-L1 axis regulates NKT cell anergy, and NKT cell activation can increase $CD8^+$ T cells' activity in anti-PD-1 resistant tumors, demonstrating an important immune interaction in the TME.[418,419] Therefore, we

examined the therapeutic benefit of adding PD-1 blockade to our combined VSV-IL-15 and NKT cell activation regimen. The addition of anti-PD-1 further increased tumor regression and overall survival in our subcutaneous model, by increasing immune activation and cytokine production, resulting in 20% of mice clearing their tumor burden (Figure 30).

Overall, the triple treatment of VSV-IL-15, NKT cell activation, and anti-PD-1 presents an effective and potentially safer approach to treat pancreatic cancer. Gemcitabine, FOLFIRFINOX, or a combination of gemcitabine and nab-paclitaxel, are the current standards of care for pancreatic cancer, but they are largely ineffective and come with severe adverse effects.[38,417] VSV-ΔM51 infection is associated with flu-like symptoms,[420] while NKT cell activation is mostly associated with mild adverse effects.[421] Anti-PD-1 therapy is mostly associated with grade 1-2 adverse effects, with severe adverse effects occurring in 10-20% of patients.[422,423] However, immunotherapies often have increased adverse effects when used in combination,[424] so a higher rate adverse effects can not be ruled out. Even though combined therapy was only able to clear tumors in 20% of mice, enhanced tumor regression in patients could make them better candidates for surgery, an important consideration as 80% of pancreatic cancer patients are ineligible for surgery.[425] This suggests that our treatment may be beneficial as a neoadjuvant therapy. Taken together, our combined immunotherapy approaches could provide an effective, safer alternative to the current standards of care.

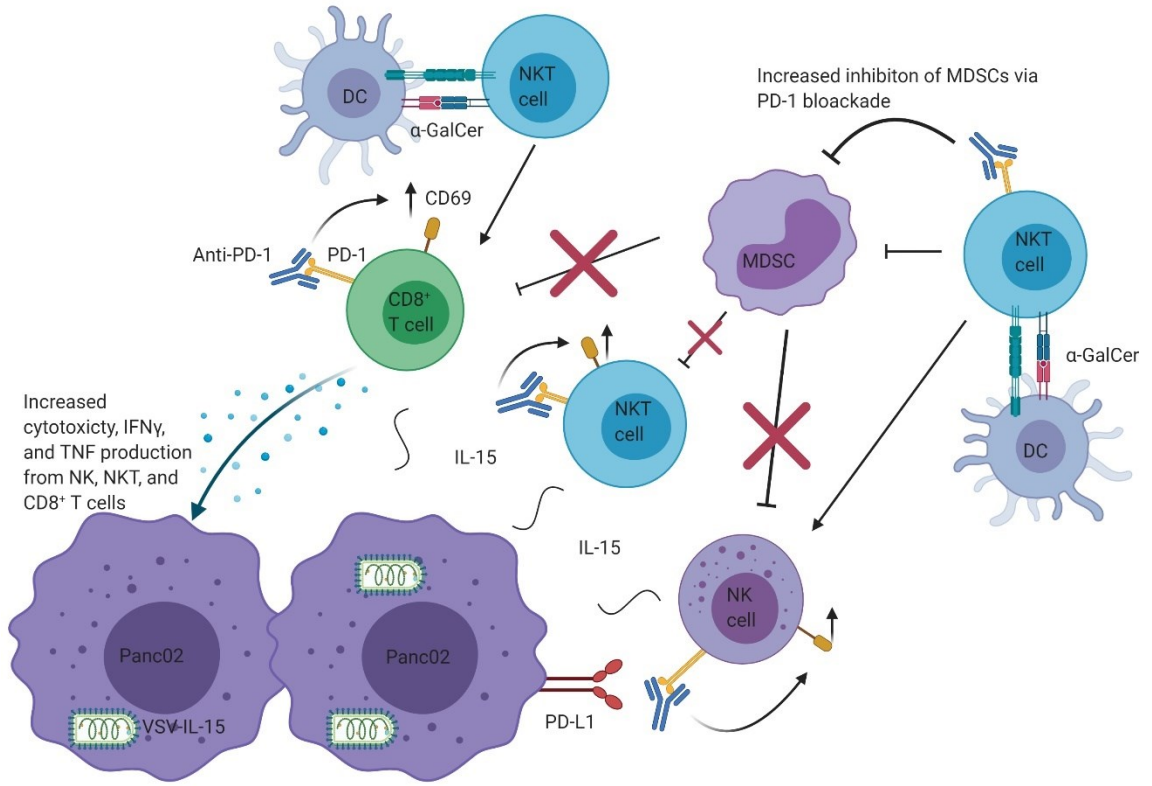


Figure 30: Triple therapy with VSV-IL-15, NKT cell activation, and anti-PD-1induces pancreatic tumor regression: potential mechanism. Potential mechanism of action by triple treatment of VSV-IL-15, NKT cell activation, and anti-PD-1 therapy. VSV-IL-15 infects Panc02 cells leading to viral replication, tumor lysis and release of IL-15. However, due to the high immunosuppression in pancreatic cancer, the IL-15 has limited effect on infiltrating lymphocytes. NKT cell activation inhibited MDSC immunosuppression and increased NK and $CD8^+$ T cell cytotoxicity and cytokine production. When VSV-IL-15 and NKT cell activation were used in combination, further increases in immune infiltration, cytotoxicity and cytokine production occurred. The addition of anti-PD-1 further increased NKT, NK, and $CD8^+$ T cell tumor infiltration, activation, cytotoxicity, and cytokine production, leading to increased tumor regression. The addition of anti-PD-1 also increased the ability of NKT cells to inhibit MDSCs, further countering immune suppression.

5.2 NKT cell immunotherapy in combination with VSV-IL-15 and anti-PD-1 increases tumor immune infiltration, activation, and cytokine production

Combination treatment with VSV-IL-15 and NKT cell immunotherapy led to increased immune cell infiltration and increased cytotoxic lymphocyte function, resulting in increased tumor regression and increased overall survival time in both subcutaneous and orthotopic models of pancreatic cancer (Figure 8 and 10). NK, NKT and $CD8^+$ T cells exhibited increased cytotoxicity and proinflammatory cytokine production in coculture with Panc02 cells. This increased cytotoxicity and cytokine production were dependent on NKT cell activation, as VSV-IL-15 therapy alone did not increase either cytotoxicity or cytokine production (Figure 12). VSV treatments did not enhance immune responses in wild-type or $J\alpha18^{-/-}$ mice, they did increase survival and reduce tumor weights (Figure 13A-B). Mice treated with VSV-GFP alone exhibited similar reduction in tumor weights and immune cell infiltration to mice treated with VSV-IL-15 alone (Figure 13B-C). This suggests that VSV is operating largely through oncolysis, and that IL-15 supports the NKT cell activation therapy. Furthermore, the increased cytotoxicity and cytokine production were lost in NKT cell-deficient mice, further reinforcing the central role of NKT cells in coordinating tumor control (Figure 13). The therapeutic benefit of combined VSV-IL-15 plus NKT cell activation could be augmented and prolonged by adding anti-PD-1 checkpoint therapy. The addition of PD-1 blockade increased NK, NKT and $CD8^+$ T cell activation, cytotoxicity and cytokine production, resulting in further tumor regression and complete tumor clearance in 20% of mice (Figure 14), which is

significant as Panc02 tumors are difficult to treat, and most therapies see moderate benefit with complete tumor regression being rare.[426–429]

Interestingly, the combination of NKT cell immunotherapy with VSV-p14 or VSV-p15 also increased cytotoxicity and cytokine production of $CD8^+$ T cells in the 4T1 breast cancer model (Figure 28), similar to the results found in the Panc02 model (Figure 12 and 19). This suggests that increased cytotoxicity and cytokine production induced by our combination therapy may be a common mechanism shared between cancer types and mouse genetic backgrounds.

5.3 NKT cell immunotherapy decreases MDSC immunosuppression in pancreatic cancer

Previously, our lab has shown NKT cell immunotherapy reduces MDSC immune suppression in a 4T1 breast cancer model.[201] Here, I show that NKT cell activation also reduces immune suppression in pancreatic cancer models. This is important since immunosuppression is a major factor in in pancreatic cancer disease progression.[410] While combining NKT cell therapy with VSV-ΔM51 did not increase the ability of NKT cells to suppress MDSCs, combination therapy with the addition of anti-PD-1 as a third therapeutic both enhanced and prolonged the response (Figure 20). Activation of NKT cells by α-GalCer induces anergy by upregulating expression of PD-1 on NKT cells.[418] Blocking the PD-1/PD-L1 axis can prevent the anergy induced by α-GalCer and maintain NKT cell anti-tumor function.[405,418] This is a potential mechanism driving the observation that anti-PD-1 increases the ability of NKT cells to suppress MDSCs; although, the exact mechanisms driving NKT cell-mediated inhibition of immunosuppression are still uncharacterized and need to be further elucidated. However, unpublished work in our lab

has indicated that NKT cells can both lyse MDSCs and convert them into APCs, similar to results found in an influenza A infection model.[200] Further work examining the effect of NKT cell immunotherapy on MDSC production of immunosuppressive mediators, such as TGFβ and nitric oxide is needed. Interestingly, mice that did not clear Panc02 tumors lost their anti-tumor immune response by day 55 (Figure 18). As PD-1 therapy was able to maintain anti-tumor immune responses, this suggests that the loss in anti-tumor activity by day 55 in mice without anti-PD-1 was primarily through immunosuppressive mechanisms. Whether anti-PD-1 can increase the ability of NKT cells to suppress MDSCs in other cancer models and mouse genetic backgrounds still needs to be tested.

5.4 VSV-p14, VSV-p15, or VSV-p14/15 in combination with NKT cell immunotherapy clears breast cancer metastasis

Previously our lab has shown that VSV-ΔM51 and NKT cell immunotherapy can be effectively combined to increase survival in a metastatic 4T1 breast cancer model.[275] However, combination treatment only led to about 75% survival, demonstrating room for improvement. Here we demonstrate that NKT cell immunotherapy combined with oncolytic VSV-ΔM51 expressing the FAST proteins p14, p15 or p14/15 clears metastatic burden and leads to 100% survival by increasing immune infiltration and ICD (Figure 31). While VSV-p14 and p14/15 in combination with NKT cell immunotherapy led to 100% survival and complete lung metastasis clearance only when VSV-p14 and p14/15 were delivered at a dose of $5x10^8$, VSV-p15 was equally effective at $5x10^7$, a 10-fold dilution compared to VSV-p14 (Figure 25), suggesting that VSV-p15 may have greater efficacy. Overall, our current treatment of VSV-FAST combined with NKT cell immunotherapy presents an effective approach to treating breast cancer metastasis. While

immunotherapy and combination treatments have led to good survival rates in the 4T1 model, 100% survival is rare.[430–432] The current standard of care is largely ineffective and comes with many severe adverse events.[412,413] As stated before, VSV infection and NKT cell activation are associated with flu-like symptoms,[420] and minimal adverse effects, respectively.[421] Furthermore, the reduced therapeutic titre of VSV-p15 needed for treatment may further reduce adverse effects compared to combination treatment with VSV-GFP or VSV-p14. The decreased therapeutic viral titre of VSV-p15 may also reduce manufacturing and treatment costs, making the treatment more accessible.

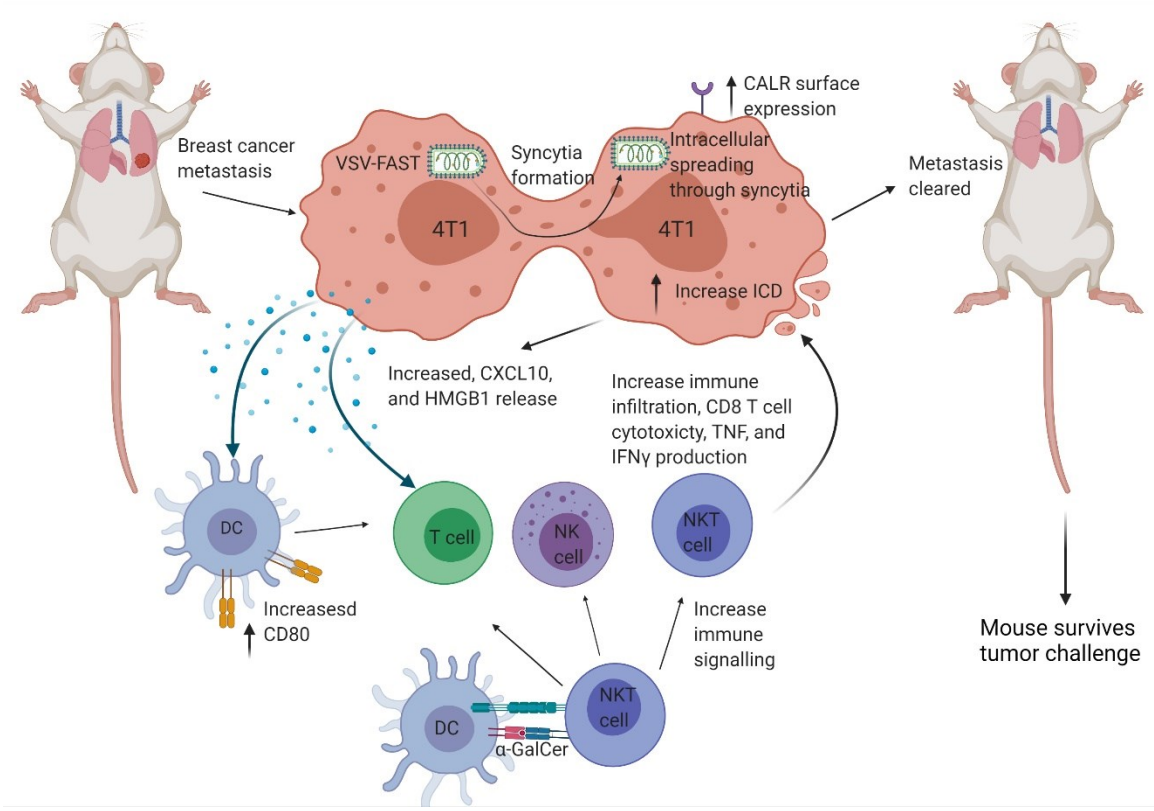


Figure 31: Combination therapy with VSV-FAST and NKT cell activation clears breast cancer metastasis. Potential mechanism of action of combination therapy with VSV-FAST and NKT cell activation. VSV-ΔM51 expressing a FAST protein infects 4T1 breast cancer metastasis leading to syncytia and viral spread. Syncytia formation due to FAST protein expression leads to tumor cells undergoing immunogenic cell death (ICD), marked by increased surface expression of calreticulin (CALR) and release of CXCL10 and high mobility group box protein 1 (HMGB1). The release of HMGB1 increases DC maturation and antigen presentation. CXCL10 increases immune cell trafficking to the site of metastasis. The addition of NKT cell activation further increases immune infiltration, cytotoxicity, and cytokine production leading to complete clearance of metastatic disease and survival of tumor challenge.

5.5 FAST proteins increase VSV-ΔM51 anti-tumor activity to different extents

FAST proteins are trafficked trough the endoplasmic reticulum-Golgi secretory pathway to the plasma membrane where they induce cell-to-cell fusion called syncytium.[346] VSV-p15 was more fusogenic and caused greater syncytia formation in 4T1 cells compared to VSV expressing other FAST proteins (Appendix Figure 1). Syncytium formation allows for the rapid, cell-cell transmission of the virus followed eventually by cell lysis.[354] Indeed, *in vitro* VSV-p15 was more effective at killing 4T1 cells compared to VSV-p14 and VSV-p10ARV (Appendix Figure 1). These results extended to an *in vivo* primary breast cancer model, where VSV-p15 increased killing of 4T1 cells and significantly increased overall survival time compared to VSV-p14, VSV-p10ARV, VSV-p10NBV, VSV-Δp14, and VSV-p14/15 (Figure 21). These *in vitro* results provide an explanation for VSV-p15's lower therapeutic titre.

5.6 Recombinant VSV-ΔM51 expressing p14, p15, p14/15 increases ICD

VSV has been shown to stimulate anti-tumor immunity by causing ICD of cancer cells.[416] FAST proteins disrupt calcium homeostasis, a major factor in ICD induction. [433,434] An important mediator of ICD is surface mobilization of CALR, which functions as an "eat me" signal and enhances uptake of tumor antigens into antigen presenting cells.[364] VSV-p14, VSV-p15, and VSV-p14/15 infection increased CALR surface expression more than VSV-GFP infection (Figure 22). However, VSV-p15 infection increased CALR even when VSV-p15 was delivered at a 10-fold lower dilution, indicating that VSV-p15 has a greater capacity to increase CALR than VSV-p14 or VSV-p14/15 (Figure 22). Other important mediators are extracellular release of HMGB1 and CXCL10. HMGB1 is an important mediator for migration and maturation of antigen presenting cells, including

DCs.[370] VSV-p15 significantly increased extracellular HMGB1 compared to VSV-p14 and VSV-GFP. This VSV-p14 and VSV-p15-induced HMGB1 secretion may provide a potential mechanism behind increased DC migration and activation, observed in both our primary and metastasis model (Figures 23, 24, and 29). CXCL10 is a potent chemoattractant for lymphocytes, including NKT cells, increasing immune cell tumor infiltration and increasing immunotherapy efficacy. [269,380] VSV-p15 significantly increased CXCL10 release compared to VSV-p14 and VSV-GFP (Figure 22). In summary, VSV-p15 infection increased ICD more than VSV-p14/15, VSV-p14, and VSV-GFP, even when used at a 10-fold dilution.

While VSV-ΔM51 expressing p14, p15, or p14/15 increased the ability of VSV-ΔM51 to induce ICD, we have not yet examined whether IL-15 can increase ICD caused by VSV-ΔM51 infection. While this information is lacking, many therapies that cause ICD increase the stimulator of interferon genes (STING) DNA recognition pathway and type I IFN signalling,[435] which in turn, increases IL-15 production.[436] In acute myeloid leukemia patients, chemotherapy induced ICD led to IL-15 trans-presentation to NK cells, resulting in greater innate immune activity towards the tumor.[369,437] Furthermore, treatment with IL-15 injections increased NK and $CD8^+$ T cell-mediated killing of tumor cells.[436] Therefore, while the addition of IL-15 itself might not increase initial induction of ICD, it may prolong or amplify VSV-ΔM51-induced ICD.

5.7 VSV-p14 or VSV-15 alone or in combination with NKT cell immunotherapy increases immune activation and anti-tumor activity

Oncolytic viruses work in part by stimulating anti-tumor immunity and increasing immune infiltration to make tumors hotter.[276] The increase in tumor infiltrating immune

cells makes immunotherapies more effective.[269,276] Treatment with VSV-p14 and VSV-p15 increased NKT, NK, $CD4^+$ and $CD8^+$ T cell infiltration of 4T1 tumors (Figure 23). Furthermore, these cells had higher expression of CD69 and greater IFNγ production indicating more immune activation and function, underscoring the ability of these treatments to further increase the efficacy of immunotherapies (Figure 23). Similar results were seen in the spleen (Figure 24). In the metastatic model, combination treatment increased NKT and $CD8^+$ T cell accumulation in the spleen (Figure 29). Furthermore, combination treatment increased CD69 expression on splenic, NKT, NK, $CD4^+$ and $CD8^+$ T cells, indicating strong immune activation (Figure 29). VSV-p14 or VSV-p15 alone in the primary tumor model, and combined with NKT cell immunotherapy in the metastatic model, increased the number of splenic and tumor infiltrating DCs as well as their expression of CD80, demonstrating that FAST proteins can increase the migration and activation of antigen presenting cells. This may be due to the ability of VSV-FAST constructs to induce increased production of HMGB1 (Figure 22), an important mediator for migration and maturation of antigen presenting cells, including DCs.[370]

5.8 NKT cell in combination with recombinant VSV-ΔM51 induces immune memory

Mice that survived initial Panc02 tumor challenge following triple therapy had decreased tumor growth following tumor re-challenge compared to naïve mice challenged with Panc02 cells, demonstrating that immune memory was formed and maintained (Figures 15 and 16). Similarly, 4T1 mice that survived initial tumor challenged decreased tumor growth following tumor re-challenge, compared to naïve mice challenged with 4T1 cells, further demonstrating that the combination of VSVΔ-M51 and NKT cell immunotherapy results in the formation of immune memory (Figures 26 and 27). Previous studies have

shown that NKT cell activation by α-GalCer induces $CD4^+$ and $CD8^+$ memory T cell formation.[438,439] Furthermore, α-GalCer presented by APCs induces a $CD62L^+$ NKT memory response.[228] $CD62L^+$ NKT have a longer persistence and anti-tumor activity compared to $CD62L^-$ NKT cells. Therefore, we expect that NKT cell activation drove memory responses in our cancer models.

In the Panc02 model, tumors had an increased number of effector memory $CD4^+$ and $CD8^+$ T cells (Figure 15). This immune memory may help limit pancreatic cancer recurrence. In humans, local recurrence after tumor resection occurs in the majority of patients in less than two years.[440] The immune memory formed after our combination treatment may extend the time until recurrence.

Tumors and spleens of 4T1 re-challenged mice had increased memory NKT, $CD4^+$, and $CD8^+$ T cells, demonstrating an immune memory response formed against the tumor (Figures 26 and 27). However, there was no significant difference in tumor growth between mice that had received VSV-GFP versus VSV-FAST treatment, indicating that previous treatment with VSV-ΔM51 expressing FAST proteins did not further protect mice upon rechallenge. As in pancreatic cancer, this immune memory may help limit breast cancer recurrence. TNBC has the highest recurrence rate of any breast cancer, occurring in 33.9 % of treated patients with in 2.6 years.[441] It is possible that the immune memory formed after our combination treatment may reduce the frequency of recurrence or extend the time until recurrence.

5.9 Future directions

The combination of recombinant VSV-ΔM51 and NKT cell-based immunotherapy has shown promise in murine models of TNBC and pancreatic cancer. However, further experiments (discussed below) are needed to progress this combination therapy towards clinical use. Furthermore, additional changes to the oncolytic virus or our NKT cell-based immunotherapy may yield increased therapeutic benefits.

5.9.1 Spontaneous cancer models

Murine tumor implant models do not recapitulate the development of cancer and often do not share all the characteristics as human cancers. Therefore, models that better simulate human cancers are needed. Spontaneous murine cancer models develop tumors driven by genetic mutations commonly found in human cancer. Therefore, spontaneous models better represent the natural process of tumor development and progression that better simulate the characteristics of human cancer.

The Lox-Stop-Lox (LSL)-*Kras*$^{G12D+}$*; Trp53*$^{fl/fl}$*; Pdx1-cre* (KPC) model develops pancreatic intraepitheliel neoplasias that progress into pancreatic cancer at an accelerated rate.[442] KPC mice have two main mutations that account for its accelerated development of pancreatic cancer. The first is a gain of function mutation in Kras, that causes it to lose the ability to hydrolyze GTP, leaving the protein constitutively active and leading to continuous growth signals.[443] The second is a deletion of *p53*, leading to loss of tumor suppression activity.[444] K-Ras and p53 mutations are common in human pancreatic cancer, with >95% of cases having mutated K-Ras function,[445] and >75% have mutated or loss of the p53 protein.[446] Both mutations in the KPC model are driven by Cre expression under the pancreas-specific promoter Pdx1.[447] The KPC model is

phenotypically similar to human pancreatic cancer, exhibiting similar stroma and chemotherapy resistance.[448,449]

Recently, I have bred KPC mice to test the combination therapies, however there were many challenges due to low incidence of pancreatic tumors and development of tumors in off target areas. Therefore, I switched to using tamoxifen inducible KPC mice (JAX stock#032429). These mice have the cre protein bound to the ligand binding domain of the estrogen receptor, keeping the cre protein in the cytoplasm and unable to access the loxP sites in the DNA.[450] Upon administration, tamoxifen binds the binding domain of the estrogen receptor, allowing the cre protein to access the nucleus and loxP sites.[450] Approximately 16 weeks after tamoxifen administration KPC mice formed pancreatic cancers (data not shown). These mice are currently being used for several experiments in the Johnston lab.

Experiments examining the therapeutic benefit and mechanisms of VSV-IL-15 combined with NKT cell-based immunotherapy and anti-PD-1 blockade should be repeated in this more clinically-relevant model of pancreatic cancer. Furthermore, our combination treatment can be tested against standard chemotherapy treatments, including gemcitabine, gemcitabine + albumin-bound paclitaxel, and FOLIFINIROX, to directly compare efficacy in a model with similar chemotherapy resistance to human pancreatic cancer.

The mouse memory tumor virus-polyoma middle tumor antigen (MMTV-PyMT) model is a spontaneous mouse model that simulates the progression of human TNBC.[451,452] MMTV-PyMT mice use a mammary tumor virus long terminal repeat promoter to drive expression of middle T oncogene in the mammary epithelia resulting in

tumor formation from the luminal cells in the mammary gland and metastasis to the lungs.[452] As MMTV-PyMT tumors progress, they lose expression of hormone receptors and overexpress cyclin D1, mimicking human breast cancer with a poor prognosis.[453] While the MMTV-PyMT model is the most common model, other models utilizing a Cre-lox system with Cre expression under MMTV, β-lactoglobulin, or whey acidic protein are available and provide options on different mouse backgrounds and can affect tumor and metastatic phenotypes.[454,455]

Experiments examining the therapeutic efficacy of VSV-p14 or VSV-p15 in combination with NKT cell immunotherapy should be repeated in PyMT mice to see if similar results are obtained in a more clinically-relevant model. While PyMT mice will be a useful model for examining the efficacy of our treatment on primary tumor growth, they will not be suitable for our tumor resection experiments, due to the fact that the model grows primary tumors in all mammary fat pads at different rates.[452] However, tumor cells can be isolated from PyMT mice and injected into wildtype mice, allowing us to examine our treatment's effect on breast cancer metastasis on the FVB/NJ mouse background.

5.9.2 Metastatic models of pancreatic cancer

A substantial challenge in treating human pancreatic cancer is the speed and rate at which the cancer metastasizes.[21,22] Therefore, treatments that can effectively target pancreatic metastasis are desperately needed. A big drawback of the subcutaneous and orthotopic Panc02 models is their lack of metastatic disease. Therefore, models where we can examine the therapeutic benefit of our treatment on pancreatic cancer metastasis are needed. Multiple options are available to study pancreatic metastatic disease. Panc02

cells can be delivered intrasplenically and grow in the liver, mimicking hepatic metastatic disease.[456] However, this does not simulate the natural growth and metastasis of human pancreatic cancer.

Another option is the use of Panc02-H7 cells. Panc02-H7 cells were developed by repeatedly isolating micro-metastases from orthotopically-injected Panc02 cells, resulting in a highly metastatic version of Panc02 cells.[388] When injected orthotopically, Panc02-H7 cells will aggressively metastasize to the liver, similar to human pancreatic cancer. However, Panc02-H7 metastatic models still don't recapitulate the natural progression of human pancreatic cancer and are still susceptible to chemotherapy that human pancreatic cancer is resistant to.[384,385,387]

Lastly, KPC mice represent another option. KPC mice exhibit tumor that metastasize to the liver, similar to human pancreatic cancer,[442] making this the most clinically relevant model. Furthermore, they share similar mutations and therapy resistance to human pancreatic cancer, making this option more clinically relevant than the Panc02-H7 model.[448,449] Giri et al.[457] recently developed a model using luminescent imaging to study liver metastasis after pancreatic tumor removal in KPC mice. Using this model would not only allow us to study the effects of our treatment on metastatic disease but would allow us to examine the potential therapeutic benefit of our treatment following surgery.

5.9.3 Combined VSV-FAST and NKT cell immunotherapy in pancreatic cancer and primary breast cancer

VSV-p14 and VSV-p15 reduced tumor progression in the primary 4T1 model while increasing ICD (Figure 22) and immune cell infiltration (Figure 23). While most VSV-

FAST constructs reduced tumor progression as monotherapies, all mice nevertheless succumbed to their disease. When used in combination with NKT cell immunotherapy, VSV-p14 and VSV-p15 were effective in the metastatic 4T1 model, resulting in complete metastatic clearance compared to only a reduced metastatic burden when virus was used alone (Figure 25). Therefore, VSV-p14 or VSV-p15 in combination with NKT cell immunotherapy may increase therapeutic benefit in the primary breast cancer model. Furthermore, this will allow us to examine differences in tumor immune infiltration and ICD markers between virus treatment alone and virus treatment in combination with NKT cell immunotherapy, giving us better insight into the mechanisms behind our combination therapy.

Due to the success of VSV-FAST constructs in the 4T1 model, examining their therapeutic potential in pancreatic cancer is warranted. Examining the therapeutic benefit of the VSV-FAST viruses alone or in combination with NKT cell immunotherapy could elucidate therapeutic mechanisms that are unique between pancreatic and breast cancer. Furthermore, the effect of the mouse background (i.e BALB/c vs. C57BL/6) on therapeutic benefit and mechanisms could be examined.

We demonstrated that anti-PD-1 increased the therapeutic efficacy of VSV-IL-15 in combination with NKT cell immunotherapy (Figure 14). Treatment with VSV-FAST did increase PD-1 expression (Figure 23 and 24), a major indicator of the therapeutic efficacy of anti-PD-1.[254] Therefore, anti-PD-1 therapy may increase the therapeutic benefit of VSV-FAST and NKT cell combination therapy in both the pancreatic and primary breast cancer model.

5.9.4 Examining the effects of IL-15 and FAST proteins on VSV-ΔM51 induced panoptosis

In this dissertation, treatments with VSV-p14 or VSV-p15 were shown to increase markers of ICD (CALR, HMGB1, and CXCL10), apoptosis (Annexin V) and necrosis (7AAD) (Figures 23 and 28). Taken together, this demonstrates that VSV-ΔM51 can induce many types of cell death. Recent work has detailed "panoptosis", which refers to the activation of multiple cell death pathways by infection or treatment, leading to inflammatory cell death.[458–461] Panoptosis is controlled via the panoptosome, a complex comprised of pyroptotic, apoptotic, and necroptotic proteins, which induces inflammatory cell death.[458–460] Multiple OVs, including wildtype VSV, have been shown to drive panoptosome formation and panoptosis.[460,461] Importantly, panoptosis has been shown to kill cancer cells and inhibit spontaneous tumor growth, suggesting its importance in cancer treatment.[462,463] Combination treatment with recombinant VSV-ΔM51 and NKT cell immunotherapy in both the pancreatic and breast cancer models increased immune cell production of TNF and IFNγ, which drive panoptosis (Figures 12 and 28).[463,464] Therefore, examining important effector proteins of panoptosis (RIPK1, Caspase 1,3, and 8) in the therapeutic benefit of our treatment is warranted.[459] Furthermore, simultaneous inhibition of proteins important for panoptosome formation (ZBP1, Aim2, and TAK1) during our treatment regime could demonstrate the importance of panoptosis in treatment efficacy.[458,459] Combination treatments with VSV-ΔM51 expressing IL-15, p14, or p15 further increased immune cell production of TNF and IFNγ compared to VSV-GFP (Figures 12 and 28). Furthermore, VSV-p14, VSV-p15, and VSV-p14/15 increased ICD, which leads to downstream TNF and IFNγ production, potentially driving

panoptosis.[463,464] Therefore, we should examine whether IL-15 and the FAST proteins mentioned above can further increase VSV-ΔM51 induced panoptosis.

5.9.5 Alternative recombinant VSV-ΔM51

While VSV-IL-15, VSV-p14, VSV-p15, and VSV-p14/15 yielded significant results in our pancreatic and breast cancer models, VSV-ΔM51 expressing other genes of interest should also be investigated for their potential benefits. Recently, our lab has created a VSV-ΔM51 mutant that expresses IL-12, an important cytokine for NK, NKT, and T cell activation.[465] IL-12 released by APCs induces and enhances cell-mediated anti-tumor immunity by multiple mechanisms: (i) IL-12 increases Th1 differentiation;[466] (ii) increases activation of NK, NKT, and $CD8^+$ T cells and their ability to kill tumor cells;[467–469] (iii) increases chemokine expression to attract NK, NKT, and T cells;[470] (iv) re-programs immunosuppressive cells, such as TAMs and MDSCs, decreasing tumor-associated immunosuppression[471,472] and; (v) increases antigen presentation molecules on cancer cells, allowing for better recognition by the immune system.[473] While systemic IL-12 immunotherapy causes dose-limiting toxicities, local delivery of IL-12 has shown to be effective, with limited adverse effects.[474] Therefore, local expression of IL-12 by VSV-ΔM51 may be an effective way to increase subsequent NKT cell immunotherapy, while limiting dose-limiting toxicities.

Fusogenic *Aquareoviruses* and *Orthoreviruses* encode six different FAST proteins with limited sequence similarity (p10, p13, p14, p15, p16, p22).[345] The FAST proteins cause different levels of syncytia and cell death *in vitro* (Appendix Figure 1). Furthermore, in our 4T1 breast cancer models, we found p10, p14 and p15 were able to increase the efficacy of VSV-ΔM51 virotherapy to different extents. Therefore, we

should examine the ability of the remaining FAST proteins, p13, p16, and p22, to increase the efficacy of VSV-ΔM51 virotherapy and subsequent NKT cell immunotherapy.

Alternatively, combining several VSV-ΔM51 expressing different genes of interest during the same treatment may yield a synergistic effect and better therapeutic outcomes. Combining multiple VSV-ΔM51's expressing immunomodulatory genes (i.e. VSV-IL-12 and VSV-IL-15) or combining an VSV-ΔM51 expressing an immunomodulatory gene with a VSV-ΔM51 expressing a FAST protein may lead to increased tumor regression and immune infiltration.

Lastly, VSV-ΔM51 can be engineered to have two genes of interest inserted into the genome for expression at the tumor site. The recombinant VSV-ΔM51 constructs used in this thesis had their gene of interest inserted in between the G and L protein.[303,304] VSV-ΔM51 can also have a gene of interest inserted in between the M and G protein using similar methods.[475] Multiple VSV-ΔM51 currently in clinical trials (NCT03865212 and NCT03647163) have two gene inserts, demonstrating the overall efficacy of this approach. However, multiple inserts could reduce VSV-ΔM51 viral replication and egress. VSV-ΔM51 expressing IFNβ and a sodium iodine transporter had reduced viral titres compared to VSV-GFP,[475] Despite this, therapeutic titres could still be reached for clinical trials.[476] Therefore, VSV-ΔM51 expressing multiple genes of interests, either IL-15, IL-12, or FAST protein should be tested in the pancreatic and breast cancer models.

5.9.6 NKT cell immunotherapy in combination with other oncolytic viruses

While recombinant VSV-ΔM51 increased NKT cell immunotherapy efficacy in models of breast and pancreatic cancer, other oncolytic viruses have not been tested and may

have differing abilities to increase the efficacy of NKT cell immunotherapy. For example, the combination of reovirus with NKT cell immunotherapy increased overall survival compared to single treatments in an ovarian cancer model but not in a 4T1 breast cancer model,[275] demonstrating differing abilities of OVs to increase the efficacy of NKT cell immunotherapy. Other prominent OVs, including oncolytic herpes simplex virus-1, vaccinia virus, and adenovirus have been shown to increase the efficacy of other immunotherapies, such as ICIs,[477–479] and may increase the efficacy of NKT cell immunotherapy. As discussed, VSV-ΔM51 infection increases markers of ICD and immune cell infiltration, resulting in increased efficacy of NKT cell immunotherapy (Figures 22 and 23).[275] Other oncolytic viruses have also been shown to increase ICD and immune cell infiltration[273,479] and therefore may increase NKT cell immunotherapy in a similar manner to VSV-ΔM51. One limitation of VSV-ΔM51 is the limited size of genetic material that can be added to the VSV-ΔM51 genome.[480] Larger genomic inserts, such as larger or even multiple genes, can be added to the genomes of double-stranded DNA OVs without affecting their oncolytic activity,[480] potentially allowing for better immunostimulatory effects or stimulation of multiple pathways.

Previously, our lab has shown that different OVs have differing abilities to increase the efficacy of NKT cell immunotherapy in a manner dependent on the cancer type.[275] To overcome this limitation, one option is to engineer OVs to increase their efficacy. Directed evolution involves the engineering and selection of a new serotype of OV with increased efficacy towards a certain type of cancer while maintaining its low efficacy in normal cells. Enadenotucirev is a direct evolved adenovirus with increased selectivity for colon carcinoma cells and efficacy compared to its parent serotypes.[481] In

preclinical models, enadenotucirev increases ICD and immune cell infiltration, which, as mentioned above, could potentially increase the efficacy of subsequent NKT cell immunotherapy.[482,483] Clinical trials using enadenotucirev showed strong oncolytic activity in the tumor accompanied by increased $CD8^+$ T cell infiltration, demonstrating potential to increase anti-tumor immune responses in humans.[484] Furthermore, enadenotucirev has shown the ability to increase subsequent immunotherapy treatments, and is currently in clinical trials with PD-1 inhibitors (NCT02636036).[485] Therefore, enadenotucirev may increase NKT cell immunotherapy as well. While enadenotucirev has seen the most success out of any directed evolution OVs, other OVs have directed evolved serotypes, including wildtype VSV.[486,487] Therefore, direct evolving an OV may overcome its limitations in certain models.

5.9.7 Alternative glycolipids for NKT cell stimulation

All the work in this dissertation has used α-GalCer to stimulate NKT cells. However, there is a growing list of alternative glycolipids that can stimulate a strong Th1 response from NKT cells. Chemical modifications to α-GalCer have produced multiple analogs that produce a stronger Th1 cytokine response from NKT cells. α-C-GalCer replaces the O-glycoside linkage found in α-GalCer with a C-glycoside linkage, resulting in increased IFNγ and IL-12 production.[205,206] Importantly, IL-4 production from NKT cells stimulated by α-C-GalCer was decreased, demonstrating increased skewing towards a Th1 phenotype.[205,206,488] During preclinical trials in a metastatic melanoma model, α-C-GalCer showed increased therapeutic benefit compared to α-GalCer, mediated by increased IFNγ production by NKT cells and DC coreceptor expression.[205,489] While α-C-GalCer exhibited therapeutic potential in preclinical models, α-C-GalCer does not

stimulate human NKT cells well.[490,491] However, chemical modification of α-C-GalCer may have overcame this limitation.[491] Taken together, this demonstrates a proof of principle, that alternative glycolipids have differing abilities to stimulate NKT cells and may increase the therapeutic potential of our combination treatment.

Another alternative glycolipid is 7DW8-5, which structurally resembles α-GalCer, but is distinguished by a shorter acyl chain which ends in a fluorinated benzene ring.[207] Compared to α-GalCer, 7DW8-5 has a stronger binding affinity to CD1d and the TCR of NKT cells, which leads to greater production of IFNγ and IL-2 downstream.[207] While research examining the therapeutic potential of 7DW8-5 in cancer models is lacking, stimulation of NKT cells with 7DW8-5 increases Th1 and $CD8^+$ T cell responses in multiple vaccine models.[492,493] 7DW8-5 did increase NKT cell-mediated cytotoxicity and overall survival compared to α-GalCer in a humanized breast cancer model.[494] However, it only demonstrated this increased efficacy compared to α-GalCer when the cell line was made to express CD1d.[494] Further examination of the 7DW8-5-induced immune response in cancer models is needed.

Recently, a new glycolipid antigen termed RK has been synthesized.[495] RK-loaded-DCs increased IFNγ production by both human and mouse NKT cells and promoted long-term T cell memory.[495] Strikingly, mice treated with RK-loaded-DCs had complete eliminated melanoma metastases, whereas mice treated with α-GalCer-loaded-DCs had minimal therapeutic benefit, demonstrating increased therapeutic efficacy of RK, at least in this model.[495] In future, 7DW8-5 and RK, as well as other alternative glycolipids, should be tested as part of our combination treatment in both the 4T1 and Panc02 models to assess whether they display any increased therapeutic benefits.

5.10 Clinical translation

I have shown that combining recombinant VSV and NKT cell immunotherapy is an effective treatment in pancreatic and breast cancer mouse models. Furthermore, I showed that the addition of anti-PD-1 can increase the efficacy of the combination treatment in the pancreatic subcutaneous model (Figure 14). Further experiments are needed to move the combination treatments towards clinical trials. Testing our combination treatments in more clinically relevant models that better recapitulate human pancreatic and breast cancer is needed. As previously mentioned, combination therapies should be tested in spontaneous cancer models to indicate whether the combination treatments are effective in a model more similar to human patients.[448,449] Furthermore, spontaneous models have similar chemotherapy resistance to their human counterparts, allowing us to compare the efficacy of the combination therapies to the chemotherapies a patient would receive.[448,449,453] Another option is humanized mouse models. $CD34^+$ human stem cells can be injected into immunodeficient mice, creating mice with human immune systems.[496] Human pancreatic or breast cancer cells or patient derived cancer cells could then be injected into the mice to form tumors.[497,498] Treating humanized mice with the combination therapy will allow us to examine whether our combination treatments are effective when used with human immune cells.

It is well established that patients whose tumors express high levels of CD1d or have high infiltration of NKT cells, benefit more from NKT cell immunotherapy.[184,499] Therefore, I believe that patients with these qualities would also benefit the most from my combination therapies. Furthermore, we have previously shown that VSV-ΔM51 can increase CD1d expression on 4T1 cells *in vitro*.[275] While this still needs to be examined

in vivo and in pancreatic cancer, this demonstrates that treatment with VSV-ΔM51 before NKT cell immunotherapy may increase the overall efficacy of the combination treatment in patients. Furthermore, OVs infection can increase immune infiltration of the tumor, turning a "cold" tumor "hot" and overcoming a major barrier to immunotherapy.[269] Lastly, both OV infection and NKT cell immunotherapy can increase PD-1 on immune cells, and PD-L1 on tumor cells. Higher expression of PD-1 and PD-L1 is a major determiner of anti-PD-1 efficacy.[254] Similarity, in our pancreatic cancer model I found that anti-PD-1 alone was ineffective, and only increased overall survival when used after our combination treatment (Figure 14). Therefore, the order of recombinant VSV, then NKT cell immunotherapy, followed by anti-PD-1, all delivered intravenously, should lead to the most therapeutic benefit for patients.

5.11 Limitations

While the data presented in this thesis show that the combination treatments have good efficacy, there are some limitations to the data presented. In both models we see increased immune infiltration of the tumor. However, whether the tumor infiltrating immune cells are anti-tumor or anti-viral has not been tested. IFNγ production and degranulation of immune cells from the tumor and spleen following VSV-ΔM51 peptide stimulation should be examined.[500] Furthermore, neutralization assays using serum from untreated and treated mice would allows us to examine the humoral response towards VSV-ΔM51.[501]

In pancreatic cancer, I showed that NKT cell activation overcame MDSC ($Ly6G^+$ $CD11b^+$) immunosuppression (Figure 20). Previous work showed similar results in a

breast cancer model.[201] However, Ly6G$^+$ CD11b$^+$ cells could also be neutrophils as there no validated markers to differentiate granulocytic-MDSCs and neutrophils in mice. Multiple markers to separate the two population have been suggested, including CD84 and FATP2,[502,503] however they may be model dependent.[504] Further work using additional cell markers, will have to examine whether the cells are solely granulocytic MDSCs. Furthermore, examining MDSC production of immunosuppressive mediators, such as TGFβ and IL-10 by ELISAs will give us a better understanding of how NKT cell immunotherapy affects MDSCs.

While 4T1 tumors have similar receptor expression, chemotherapy resistance, and metastatic signature as human TNBC,[390,391] Panc02 cells do not do a good job of recapitulating human pancreatic cancer. Panc02 tumors are highly immunosuppressive and have limited CD8$^+$ T cell infiltration, similar to human pancreatic patients.[384,385] However, they have a higher mutational burden, making them more responsive to many immunotherapies, including immune checkpoint therapy.[505] Furthermore, they have limited desmoplasia formation and are more susceptible to chemotherapy.[505] Panc02 cells are not highly metastatic, whereas human pancreatic cancer aggressively metastasizes to the liver and 80% of patients have metastatic disease at the time of diagnosis.[505,506] Lastly, Panc02 cells lack an activating KRAS mutation, which occurs early in PanIN development and is present in 95% of pancreatic cancer patients.[505] Taken together, it is important that treatment is tested in more physiological relevant models.

5.12 Concluding remarks

NKT cells have an important role in immunosurveillance and anti-tumor immunity. Activation of NKT cells using α-GalCer loaded DCs can increase anti-tumor immunity

and overall survival in preclinical models. Here, I looked to improve upon NKT cell immunotherapy by using combination therapy with recombinant VSV-ΔM51 expressing either IL-15 or FAST proteins in models of pancreatic and breast cancer, respectively.

Combination therapy with VSV-IL-15 and NKT cell immunotherapy increased overall survival in both subcutaneous and orthotopic models of pancreatic cancer. Tumors had increased infiltration of $CD8^+$ and $CD4^+$ T cells, NK cells, and NKT cells. Furthermore, the $CD8^+$ T cells, NK cells, and NKT cells displayed increased cytotoxicity towards Panc02 cells, accompanied by increased Th1 cytokine production. The addition of anti-PD-1 to our VSV-IL-15 plus NKT cell-based combination immunotherapy further increased overall survival, immune activation, cytotoxicity, and cytokine production, ultimately resulting in 20% of mice experiencing complete tumor regression in our subcutaneous model. Activation of NKT cells via α-GalCer loaded DCs decreased MDSC immunosuppression, which can be further enhanced by subsequent anti-PD-1 therapy. Taken together, this demonstrates that VSV-IL-15 in combination with NKT cell immunotherapy and anti-PD-1 checkpoint blockade can be an effective triple therapy for pancreatic cancer.

VSV-ΔM51 expressing the FAST proteins p14, p15, or p14/p15 increased tumor regression and overall survival in a primary 4T1 breast cancer model compared to VSV-GFP and untreated mice. These therapeutic effects were accompanied by increased tumor infiltration and markers of ICD. NKT cell immunotherapy combined with VSV-ΔM51 expressing p14, p15, or p14/15 protein resulted in 100% survival in a 4T1 metastatic model. Mice treated with VSV-p14 or VSV-p15 combined with NKT cell-based immunotherapy had complete lung metastasis clearance accompanied by increased $CD8^+$

T cell cytotoxicity and Th1 cytokine production. Furthermore, mice that survived initial tumor challenge had decreased tumor growth after tumor rechallenge. Rechallenge tumors had increased immune infiltration and memory populations, demonstrating the formation of anti-tumor immune memory. In summary, the combination of VSV-p14 or VSV-p15 with NKT cell immunotherapy increases anti-tumor immunity and clears metastatic disease.

Overall, the combination of recombinant VSV-ΔM51 and NKT cell immunotherapy can provide therapeutic outcomes in multiple types of cancer and on different mouse genetic backgrounds. Furthermore, subsequent anti-PD-1 therapy can further increase the therapeutic efficacy of our treatment. Testing our therapy in more clinically relevant models and against standard treatment will provide the next steps in moving our treatment towards clinical application.

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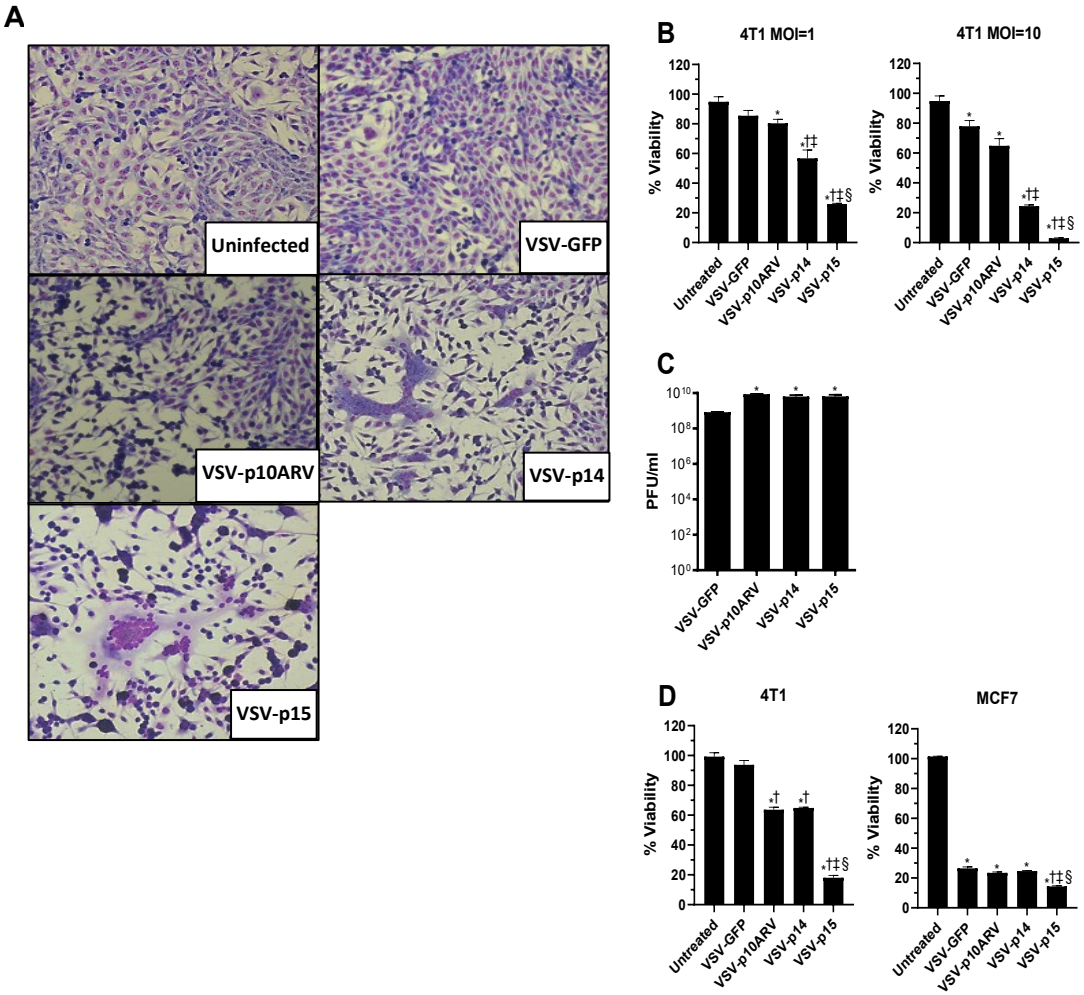
APPENDIX



Appendix Figure 1: VSV-FAST increases 4T1 cell fusion and cell death *in vitro.* (A) 4T1 cells were uninfected or infected at an MOI of ~1 with VSV-GFP, VSV-p10ARV, VSV-p14, or VSV-p15. Images of syncytia were taken 12.5 hpi. (B) 4T1 cells were infected at an MOI of 1 or 10 for 20 hours with either VSV-GFP or a VSV expressing a FAST protein. Cells were incubated with 0.5mg/ml of MTT reagent for 2 hours before adding DMSO and reading cell viability at 540nm and 690nm (n=3 per group). $^{*}p<0.05$ compared to untreated. $^{\dagger}p<0.05$ compared to VSV-GFP. $^{\ddagger}p<0.05$ compared to VSV-p10ARV. $^{\S}p<0.05$ compared to VSV-p14. C) 4T1 cells were infected at an MOI of ~5 for 24 hours. Supernatants were taken and titres were determined by plaque assay. (n=3 per group). $^{*}p<0.05$ compared to VSV-GFP (D) 4T1 and MCF7 spheroids were infected with VSV-GFP or VSV expressing a FAST protein for 20 hours. Cell viability was read at 405nm. (n=3 per group) $^{*}p<0.05$ compared to untreated. $^{\dagger}p<0.05$ compared to VSV-GFP. $^{\ddagger}p<0.05$ compared to VSV-p10ARV. $^{\S}p<0.05$ compared to VSV-p14. Data generated by Nichole McMullen and Roberto De Antueno