IMPROVING OUTCOMES FOR CANCER IMMUNOTHERAPY

MODULATION OF TUMOR IMMUNOGENICITY TO IMPROVE OUTCOMES FOR CANCER IMMUNOTHERAPY

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TITLE: Modulation of Tumor Immunogenicity to Improve Outcomes for Cancer Immunotherapy

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Lay Abstract

Cancer is the leading cause of death in Canada and one of the leading causes of death worldwide. Conventional cancer therapies such as chemotherapy often include severe side effects that can decrease the quality of life of patients undergoing treatment. Immunotherapy is designed to harness the host immune response and enhance its ability to seek out and kill cancer cells. Immunotherapy has gained traction in the past decade due to its improved safety and efficacy over conventional cancer therapies. However, there is room for improvement as most patients fail to respond to immunotherapy. The work described in this dissertation involves the development of therapeutic combination platforms that are designed to improve upon immunotherapy outcomes. Murine tumor models were used to develop a better understanding of biological processes associated with therapeutic efficacy. These findings can be used for the development of therapeutic strategies that can further improve the efficacy of cancer immunotherapy.

Abstract

Cancer immunotherapy has demonstrated immense promise in the past decade. Immune checkpoint therapy has shown unprecedented responses in many cancers; however most patients fail to respond to checkpoint therapy. This highlights the need to develop a better understanding of factors in the tumor microenvironment that can influence therapeutic outcomes.

In this body of work, we have utilized oncolytic viruses (OVs) to enhance immunogenicity in the tumor and study the cellular mechanisms that enable a therapeutic response. We utilize a combination of OVs and low dose chemotherapy to further sensitize murine models of mismatch repair-deficient colorectal cancer to checkpoint therapy. Using a Clariom S transcriptome assay we found that the combination induced gene signatures associated with the recruitment and activation of myeloid subsets. When we assessed tumor infiltrates, we found that the combination induced the chemoattraction of several myeloid subsets, including type I conventional DCs (cDC1s) which are known for their role in antigen presentation. Using Batf3$^{-/-}$ mice, we demonstrated that the therapeutic efficacy of our combination platform was dependent on the presence of cDC1s.

In this dissertation, we also studied the role of OV-induced type I IFN (IFN-I) in enabling or suppressing antitumor immunity. We found that OVs induced the upregulation of PD-L1 in an IFN-I-dependent manner in cancer cells and circulating immune cells. Inhibition of IFN-I signaling using an anti-IFNAR monoclonal antibody partially prevented OV-induced upregulation of PD-L1. Furthermore, the combination of OV and

IFNAR blockade enhanced the effector functions of tumor-specific T cells and led to better tumor control compared to OV monotherapy.

Altogether, these findings demonstrate that OVs can be an effective agent for enhancing immunogenicity in the tumor and promoting the infiltration of inflammatory myeloid subsets. By combining OVs with checkpoint or IFNAR inhibitors, we prevent the onset of immunosuppression and enable a favorable therapeutic response.

Acknowledgments

First, I would like to thank Dr. Karen Mossman for her mentorship and guidance throughout my PhD. You have given me the opportunity to learn and develop as a researcher in a highly positive research environment. Your trainee-focused style of mentorship fosters the curiosity and ambition of your trainees, which is reflected by their outstanding track records across various fields and industries.

I would like to thank my supervisory committee members, Dr. Yonghong Wan and Dr. Jonathan Bramson for your guidance. You frequently encouraged me to collaborate with others within the institute and to use innovative approaches to address current gaps in the field.

I would like to thank all the members of the Mossman lab, who always maintained a friendly and enthusiastic environment. Lab colleagues can make or break a graduate student experience, and I was very fortunate to have joined a lab in which my colleagues interact with the utmost integrity and respect. I would like to thank Dr. Sam Workenhe, Dr. Arinjay Banergee and Dr. Alyssa Vito for their own style of mentorship. Not only have you helped me with long scientific discussions and career advice, but you have also led by example through your high levels of ambition, motivation, and productivity. I would also like to thank the Wan lab for their very frequent collaboration and for teaching me many immunology assays that I was unfamiliar with. In particular, I would like to thank Dr. Scott Walsh, who has guided me through both of my research projects and emphasized collaboration to answer critical gaps in the field. You have also led by example and have always maintained high levels of curiosity and motivation for research.

Finally, I would like to thank my family. Thank you to my parents, Ahmad and Iman, who have given me the opportunity (and encouraged me!) to pursue higher education. Your own ambitions and experiences have taught me to continue learning and applying myself. Thank you to my sister, Jenin, and my brother-in-law, Ala, for supporting me and listening to me complain about the many struggles of grad school. You have always managed to lift my spirits and I am very lucky to have you by my side.

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List of Abbreviations

ACT	Adoptive cell transfer
ANOVA	Analysis of variance
AP-1	Activator protein 1
APC	Antigen-presenting cell
BATF3	Basic Leucine Zipper ATF-Like Transcription Factor 3
CAD	Canadian dollar
CAF	Cancer associated fibroblast
CCL	Chemokine (C-C motif) ligand
cDC1	Conventional DC type 1
cDC2	Conventional DC type 2
CD-X	Cluster of differentiation - X (X being a number)
CM	Central memory
CRC	Colerectal cancer
CTLA-4	Cytotoxic T lymphocyte-associated molecule-4
DAI	DNA-dependent activator of IFN-regulatory factors
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DMEM	Dulbecco's modified Eagle's media
dMMR	Mismatch repair-deficient
DN	Double negative
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EM	Effector memory
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GFP	Green flourescent protein

HSV	Herpes simplex virus
i.d.	Intradermal
i.p.	Intraperitoneal
i.t.	Intratumor
i.v.	Intravenous
ICB	Immune checkpoint blockade
ICD	Immunogenic cell death
ICI	Immune checkpoint inhibitors
ICP0	Infected cell protein 0
ICS	Intracellular cytokine staining
IE	Immediate early
IFIT1	Interferon-Induced Protein With Tetratricopeptide Repeats 1
IFN	Interferon
IFNAR	IFN-α/β receptor
IFN-I	Type I interferon
IFNα	Interferon alpha
IFNβ	Interferon beta
IFNγ	Interferon gamma
IHC	Immunohistochemistry
IL-X	Interleukin X (X being a number)
IPS-1	IFN promoter stimulator 1
IRF1	Interferon regulatory factor 1
IRF3	IFN regulatory factor 3
IRF7	IFN regulatory factor 7
ISG	Interferon-stimulated gene
JAK1	Janus kinase 1
JNK	c-jun N-terminal kinase
KO	knockout

LCMV	Lymphocytic choriomeningitis virus
MAPK	Mitogen-activated protein kinases
MDA-5	Melanoma differentiation-associate gene 5
MDSC	Myeloid-derived suppressor cell
MFI	Mean flourescence intensity
MHC	Major histocompatibility complex
MHC I	MHC class I
MHC II	MHC class II
mito	Mitomycin-C
moDC	Monocytic dendritic cell
MOI	Multiplicity of infection
mRNA	Messenger RNA
MyD88	Myeloid differentiation primary response gene 88
NFκB	Nuclear factor kappa–light-chain-enhancer of activated B cells
NK	Natural killer
oHSV-1	Oncolytic HSV-1
OV	Oncolytic virus
oVSV	Oncolytic VSV
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononucelar cell
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed death-1
PD-L1	Programmed death-ligand 1
PFU	Plaque forming unit
Poly I:C	Polyinosinic:polycytidylic acid
PRR	Pathogen recognition receptor
RAG-2	Recombination activating gene 2

RIG-I	Retinoic acid-inducible protein I
RNA	Ribonucleic acid
RT-PCR	Real-time PCR
s.c.	Subcutaneous
STAT1	Signal transducer and activator of transcription 1
TAA	Tumor associated antigen
TAM	Tumor-associated macrophage
TCIR	T cell inhibitory receptors
TCR	T cell receptor
TGFβ	Transforming growth factor beta
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
TK	Thymidine kinase
TLR3	Toll-like receptor 3
TME	Tumor microenvironment
Treg	Regulatory T cell
T-Vec	Talimogene laherparepvec
TYK2	Tyrosine kinase 2
VacV	Vaccinia virus
VSV	Vesicular stomatitis virus
WT	Wild-type
YFP	Yellow flourescent protein

Declaration of Academic Achievement

The literature review in **Chapter 2** titled "Tumor heterogeneity: a great barrier in the age of cancer immunotherapy" was published on February 15th, 2021. The manuscript was conceived by me and cowritten with Dr. Alyssa Vito. The review describes mechanisms of acquired resistance that may prevent response to immunotherapy. In particular, the review investigates the role of tumor heterogeneity in driving acquired resistance and antigen loss. Additionally, we discuss methods being developed to characterize tumor heterogeneity and to overcome tumor heterogeneity to maintain durable responses to therapy.

The manuscript in **Chapter 3** titled "Combination of low-dose chemotherapy and oncolytic virotherapy sensitize MC38 tumors to immune checkpoint inhibitor therapy in a cDC1-dependant manner" was submitted to the *International Journal of Molecular Medicine* and is currently under review. I conceived the project along with Dr. Karen Mossman. Dr. Alyssa Vito assisted with several animal and flow cytometry experiments and with extracting RNA for the Clariom assay, Omar Salem assisted with some flow cytometry, Dr. Sam Workenhe assisted with the experimental design and execution of some animal experiment. Dr. Yonghong Wan and Dr. Karen Mossman provided scientific guidance, access to facilities, and funding. The Clariom S assay was performed by the Genetic and Molecular Epidemiology Laboratory at the David Braley Research Institute. The remainder of the experiments were designed and executed by me.

The manuscript in **Chapter 4** titled "IFNAR blockade synergizes with oncolytic virotherapy to prevent virus-mediated PD-L1 expression and promote antitumor T cell activity" was submitted to *molecular therapy – oncolytics* and is currently under review. I

conceived the project along with Dr. Scott Walsh and Dr. Karen Mossman. Dr. Scott Walsh also aided with the design and execution of some animal and flow cytometry experiments. Dr. Alyssa Vito aided with some animal experiments and with harvesting tumors for immunohistochemistry (IHC). Amir Reihani scanned the IHC slides and prepared the IHC images for publication. Kjetil Ask provided the facilities for IHC imaging. Dr. Yonghong Wan and Dr. Karen Mossman provided scientific guidance, access to facilities, and funding. IHC was performed at the McMaster Immunology Research Centre Histology Core. The remainder of the experiments were designed and executed by me.

The thesis was written by me and edited by Dr. Karen Mossman.

Chapter 1 : Introduction

1.1 Cancer

Cancer is currently the leading cause of death in Canada, and one quarter of Canadians are predicted to die from Cancer [1]. In addition, cancer poses an increasing risk of straining the health care system as half of Canadians are expected to develop cancer within their lifetime [1]. This burden will increase each year as the population continues to age [2]. Cancer is also accompanied by significant financial burden; the cost of cancer care in Canada was 7.5 billion CAD in 2012, which rose from 2.9 billion CAD in 2005 [3]. Expanding our understanding of cancer biology is crucial for the development of novel therapeutic platforms that can reduce the physical and financial burdens associated with cancer.

Cancer is an entire category of diseases that is characterized by the uncontrolled proliferation of cells that form a tumor. Given time, cancerous tumors can spread throughout the body via blood vessels and the lymphatic system to create secondary tumor growths known as metastases. Metastatic tumors can interfere with vital body functions and are the primary cause of death for cancer patients. Cancer can develop as a result of genetic mutations to genes that control cell growth and division. These mutations can be acquired in a several ways including exposure to carcinogens, radiation, or viral infections [4,5]. Furthermore, mutations in genes involved with cell division and DNA repair can impair these functions and create new mutations that further promote mutagenesis. This cascade of acquired mutations results in rapid evolution of cancerous cells as they continue to acquire

mutations that change many of their biological properties. Rapid evolution of cancer cells is often responsible of acquired resistance to many forms of cancer therapy, a topic that will be expanded on in **Chapter 2**. Despite this, there are a handful fundamental properties that are highly conserved amongst cancerous tumors.

1.2 Hallmarks of Cancer

There are several biological properties that collectively contribute to malignant growth. These properties were effectively summarized by Douglas Hanahan and Robert A Weinberg in the year 2000 and collectively termed the "hallmarks of cancer" [6], with additional properties added in 2011 [7]. The hallmark properties include limitless replicative potential, self-sufficiency in growth signals, insensitivity to growth inhibitory signals, evasion of apoptosis, sustained angiogenesis, immune evasion, and tissue invasion and metastasis (Figure 1.1). These properties can emerge as a result of tumorigenesis while simultaneously promoting tumor growth. On the other hand, these properties can also serve to differentiate cancer from healthy cells, and so are often exploited when developing cancer therapies. In this regard, understanding the hallmarks of cancer is crucial for the development of cancer therapies. Indeed, these hallmark properties serve as the foundation for the development of conventional cancer therapies such as chemotherapy, and novel therapies including immunotherapy and oncolytic virus therapy.

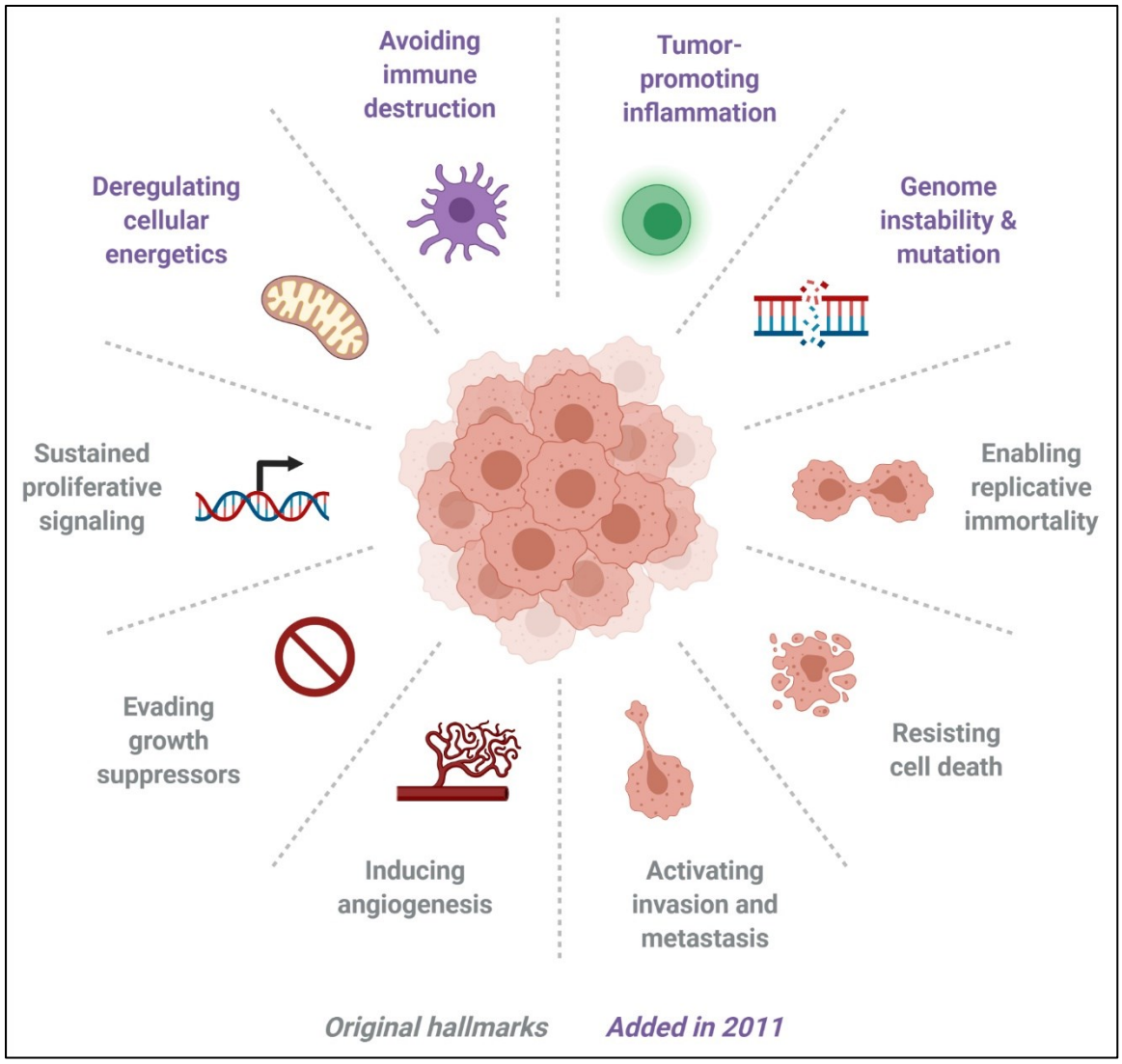

Figure 1.1. Hallmarks of cancer. Schematic was made on Biorender.com.

1.3 Chemotherapy

Chemotherapy coupled with surgical resection of the tumor mass is currently the most common treatment for cancer patients. Chemotherapeutic drugs are small molecules that exploit properties that are prevalent in cancer cells to selectively damage their cellular functions. Chemotherapy was developed as a

consequence of chemical weapons research during World War II. While chemical warfare was not used on the battlefield, an accidental spill of mustard gas on troops in Italy led to observations of bone marrow and lymph node depletions in exposed troops [8]. Upon observing the effects of mustard gas on rapidly dividing immune cells, researchers hypothesized that similar compounds might be effective against rapidly diving cancer cells. This led to the development of nitrogen mustards as the first chemotherapy and paved the way for the development of an entire class of chemotherapy known as alkylating agents. Alkylating agents capitalize on rapid DNA replication of cancer cells by cross-linking to the DNA during cell division causing cell death and apoptosis [9]. The success of alkylating agents led to the development of other classes of chemotherapy that were designed to capitalize on other properties prevalent in cancer. For example, antimetabolites exploit their enhanced metabolism [10], and microtubule inhibitors exploit their hyperactive cell cycle and division [11]. Moreover, different classes of chemotherapy were eventually combined to treat resistant tumors and prevent the onset of acquired resistance to a single class of chemotherapy [12]. Chemotherapy has been highly successful in treating many types of cancers, which is why it is the conventional choice for cancer therapy today. However, there is much room for improvement regarding efficacy and safety for patients. While chemotherapy exploits properties that are more prevalent in cancer cells, these properties are still crucial biological functions in normal cells. Furthermore, normal cells vary in their biological functions which can reduce specificity of chemotherapy even further. For example, neutropenia is a

common side effect of chemotherapy due to the toxicity of chemotherapy on rapidly dividing neutrophils [13]. The low specificity of chemotherapy for cancer cells can cause numerous and severe side effects, including nausea, vomiting, diarrhea, leukopenia and even sterility [14]. Therefore, it is imperative that novel therapeutic strategies are developed to improve upon antitumor efficacy while reducing the severity of side effects. The development of new classes of cancer therapy does not need to entirely replace chemotherapy. Instead, combinations of therapies can be developed to prevent the onset of acquired resistance while reducing the severity of side effects from a single class of cancer therapy.

1.4 Immunosurveillance and Immunoediting

Since the early 1900s, scientists have theorized that the host immune system could identify and control tumor growth [15]. This concept became known as immunosurveillance but was difficult to prove for several decades. In 1989, however, Boon *et. al.* demonstrated that $CD8^+$ T cell tolerance to self-peptides can be broken because of acquired mutations in cancer cells that render the peptides immunogenic [16]. These mutated peptides, termed neoantigens, occasionally have high affinity to major histocompatibility complex (MHC) class I molecules. Neoantigens with high affinity to MHC class I can break self-tolerance of $CD8^+$ T cells and are unique to the tumor, making them ideal targets for an antitumor immune response. The 1990s escorted a resurgence of enthusiasm for the concept of cancer immunosurveillance. In 1994 a study published in *Immunity* demonstrated that interferon gamma (IFNγ) can elicit protective immunity against transplantable tumors in

syngeneic murine models [17]. The development of new murine models facilitated the momentum of research in this area. For example, in 1992 a paper was published in *Cell* introducing recombination activating gene 2 (RAG-2)-deficient mice which fail to produce mature T or B lymphocytes [18]. Researchers found that RAG-2-deficient mice are more susceptible to both transplantable and spontaneous tumor formation compared to their wild-type (WT) counterparts [19]. Indeed, it quickly became established that the immune system can identify and eliminate cancer cells by recognizing tumor antigens and generating effector and memory cells that can survey the appearance of malignant tumors [15,20,21].

Despite the concept of immunosurveillance becoming more accepted, there was one major caveat to the theory. Given the ability of the immune system to monitor and control tumor growth, how do immunocompetent individuals develop cancer? A few studies began to answer this question by observing tumor formation under immunocompetent and immunocompromised conditions. For example, one study demonstrated that tumors formed in RAG2-deficient mice are often rejected when transplanted into WT mice, whereas tumors from WT mice readily grew in RAG2-deficient mice [19]. These data, combined with several other studies, suggest that tumor immunogenicity can be molded by their environment [22–24]. In a landmark review, Dunn and colleagues summarized findings that support this model of tumor plasticity and established the concept of immunoediting [25]. They describe a model in which the tumor will go through three stages of immunoediting termed the “Three Es” of cancer immunoediting (Figure 1.2). The first stage is

Elimination, which encompasses the model of immunosurveillance and the elimination of immunogenic cancer cells. This is followed by the Equilibrium stage, in which the immune response can create selective pressure towards less immunogenic cancer cell variants that are capable of immune evasion. The final stage, Escape, involves the rapid proliferation of the immunologically sculped tumor in an immunocompetent host. These concepts serve as a foundation for studying tumor-host interactions and had profound effects on the development of cancer therapies.

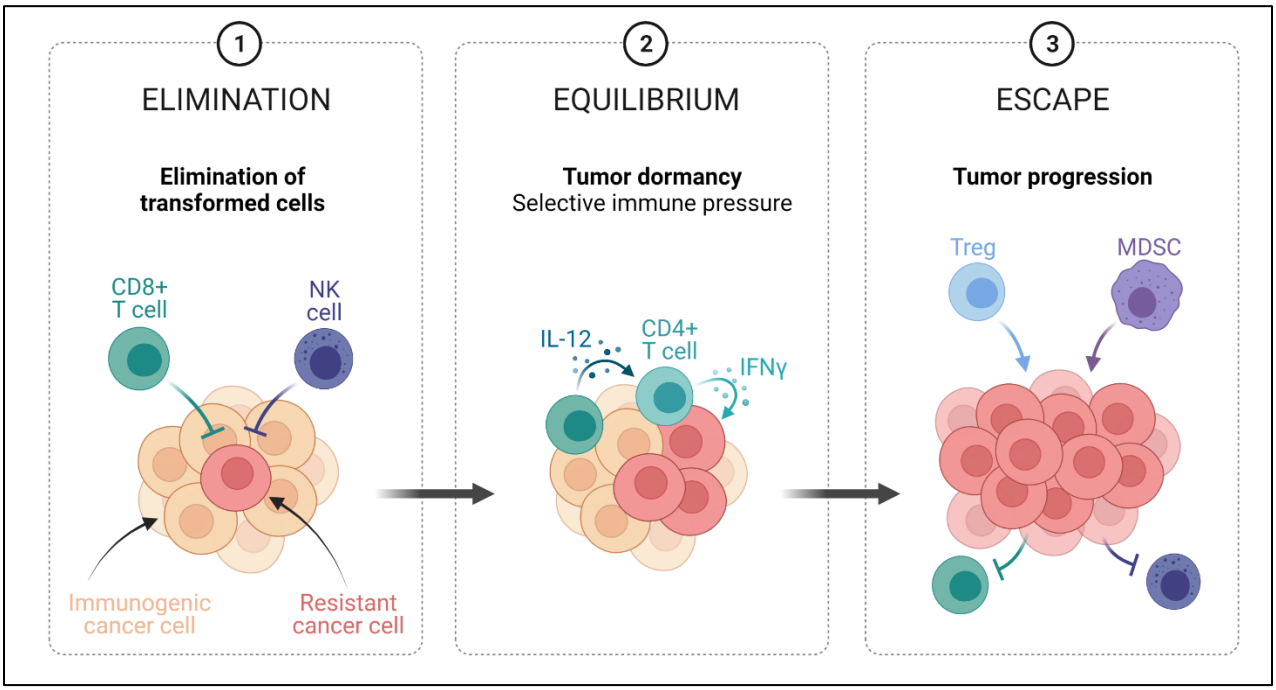

Figure 1.2. The three stages of cancer immunoediting. Schematic was made on Biorender.com.

1.5 Immunotherapy

Immunotherapy is a broad category of cancer therapies that is focused on enabling a robust immune response against the tumor. The immunogenicity of a

tumor can be sculpted by its environment enabling a variety of mechanisms for the evasion or suppression of the immune system [25]. Cancer immunotherapies include innovative strategies to increase the immunogenicity in the tumor. There is great enthusiasm for immunotherapy strategies because of their highly improved safety profile over conventional cancer therapies coupled with their promising therapeutic efficacy. However, there are many steps involved in generating and maintaining an effective antitumor response. In this regard, many different therapeutic strategies have been developed with their own range of applicability. Moreover, several strategies can often be combined to maximize therapeutic efficacy. Some strategies, such as cancer vaccines, are designed to initiate an endogenous immune response against the tumor [26]. In theory, this strategy can be personalized to promote a response against tumor-specific neoantigens. Adoptive T cell transfer therapy is another strategy that has shown great promise in the clinic, particularly for the treatment of hematological malignancies [26]. This strategy involves the stimulation or engineering of autologous or allogenic T cells that are then infused into patients. Interestingly, even conventional cancer therapies can be used as immunotherapies under the right circumstances. For example, both radiation therapy and low-dose chemotherapy have been shown to initiate an antitumor immune response by inducing immunogenic cell death (ICD) [27,28]. However, initiation of an antitumor response is not always sufficient for efficacy. Cancer cells can also suppress the activity of immune cells by expressing highly immunosuppressive ligands known as immune checkpoints. To this end, another strategy for immunotherapy

involves the development of immune checkpoint inhibitors (ICIs), which are monoclonal antibodies that block these ligands (this strategy is discussed in further detail in section 1.7). While these therapies have shown great potential in the past decade, there are many patients that do not exhibit durable survival outcomes [29]. Therefore, it is imperative that pre-clinical and clinical research is focused on identifying biological factors that enable durable responses in patients.

1.6 Tumor Antigen Presentation

$CD8^+$ T cells have long been the primary focus for cancer immunotherapy. While many other cell types can be involved in initiating an antitumor response, $CD8^+$ T cells seem to be the primary mediators of anticancer immunity. To elicit an antitumor $CD8^+$ T cell response, however, antigen presentation must occur at two distinct stages: 1) tumor antigens must be taken up by antigen presenting cells (APCs) and cross presented to prime naïve $CD8^+$ T cells. 2) tumor antigens must be presented on the surface of tumor cells to enable recognition by primed $CD8^+$ T cells. In both cases, antigens must be presented on MHC molecules for recognition by T cells. Antigens are recognized by $CD8^+$ or $CD4^+$ T cells when they are presented on MHC class I (MHC I) or MHC class II (MHC II), respectively. Normally, endogenous proteins are processed by proteosomes and presented on MHC I, while exogenous proteins are internalized before being processed and presented primarily on MHC II [30]. Tumor cells generally do not prime antigen specific $CD8^+$ T cells, which are often restricted to MHC I priming. Therefore, an alternate antigen presentation pathway is required for priming the MHC I-restricted $CD8^+$ T cells. Cross

presentation is process by which APCs can present exogenous antigens via MHC I [31]. In the context of cancer immunity, dendritic cells (DCs) are the most common APCs involved in priming $CD8^+$ T cells [32].

DCs can take up dying tumor cells and released tumor proteins to be processed and cross presented. Dying tumor cells also release danger-associated molecular patterns (DAMPs) that can promote maturation of DCs and initiate their migration to the draining lymph node, where cross presentation occurs [33]. DCs can be categorized into several subsets, however, the prominent DCs for initiating antitumor immunity are type 1 conventional DCs (cDC1s) [32,34]. cDC1s can be characterized by their expression of CD8α and/or CD103 and are highly dependent on the transcription factor Basic Leucine Zipper ATF-Like Transcription Factor 3 (BATF3) for their development [35]. BATF3 knockout mice, which are deficient for cDC1s, demonstrate dysfunctional cross presentation and are incapable of mounting an antitumor immune response [36,37]. Indeed, infiltration of cDC1s into the TME is associated with improved outcomes in patients and better response to immunotherapy [38–40]. cDC1s were also shown to be involved in reengaging central memory T cells and recruitment of adoptively transferred T cells to the TME [41,42]. Several chemokines can induce the recruitment of cDC1s into the tumor, including CCL4 and CCL5 [34,43]. On the other hand, tumors can modulate their antigenicity by releasing molecules that prevent the recruitment and maturation of DCs, including PGE_2, IL-6, TGFβ and IL-10 [44,45]. Therefore, some immunotherapy strategies employ adjuvants that can overcome the immunosuppressive

signals by inducing the secretion of molecules that promote DC recruitment and maturation [37,46,47].

1.7 Immune Checkpoint Inhibitors

ICIs are a class of immunotherapy that has been highly successful for treating immunosuppressive tumors. Immune checkpoints are co-inhibitory receptors that are expressed on activated T cells, B cells and myeloid cells. In the context of T cells, binding of immune checkpoints to their respective ligands inhibits T cell activation, proliferation, and effector functions [48]. The most widely known immune checkpoints are programmed cell death receptor-1 (PD-1) and cytotoxic T lymphocyte-associated molecule-4 (CTLA-4) which were studied by Tasuku Honjo and James Allison, respectively [49,50]. Both professors were awarded the 2018 Nobel Prize in Physiology or Medicine for their contribution. Under normal conditions, immune checkpoints serve to maintain self-tolerance, and ligands are expressed by APCs and vital organs such as the heart and lungs [51]. However, cancer cells can often exploit this mechanism by upregulating immunosuppressive ligands such as programmed death-ligand 1 (PD-L1, also known as CD274 and B7-H1). In doing so, antitumor T cells that identify their target are rendered dysfunctional and fail to carry out their effector functions (Figure 1.3) [48].

PD-1 is a transmembrane receptor with two primary ligands, PD-L1 and PD-L2. While PD-L2 has greater affinity for PD-1, PD-L1 is expressed on a greater variety of cells and more commonly expressed by cancer cells [52]. When PD-1 is engaged by its ligands, a signaling pathway is initiated that results in the

dephosphorylation of proteins downstream of the T cell receptor (TCR) and the co-stimulatory receptor, CD28 [53]. Inhibition of PD-1/PD-L1 signaling was shown to reinvigorate antitumor T cells and induce tumor regression in immunocompetent murine models [54,55]. As a result, monoclonal antibodies targeting PD-1 or PD-L1 were commercialized and approved for the treatment of multiple cancers, with remarkable clinical outcomes [56,57].

CTLA-4 is a transmembrane receptor that is highly homologous to CD28. CTLA-4 and CD28 share two primary ligands: CD80 and CD86 [58]. However, CTLA-4 interacts with both ligands with a higher affinity than CD28 [59,60]. Binding of CD28 to either of its ligands in conjunction with TCR activation is required for T cell activation, proliferation, and survival upon engaging antigens. CTLA-4 inhibits T cell activation by competing with CD28 for binding of CD80 and CD86, thereby preventing CD28-mediated co-stimulation [58]. Similar to PD-1, blocking CTLA-4 interactions promotes T cell activation and antitumor immunity, and induces tumor regression in murine cancer models [54,61]. Monoclonal antibodies against CTLA-4 have been trialed in a range of cancers with promising clinical outcomes [62–64]. Combinations of CTLA-4 and PD-1 blockade are under investigation for several types of cancer. While this combination has demonstrated synergistic effects in the clinic, the improved efficacy comes at the cost of greater toxicity [65–68]. Nevertheless, clinical results with ICIs shows great promise for the treatment of immunosuppressive tumors.

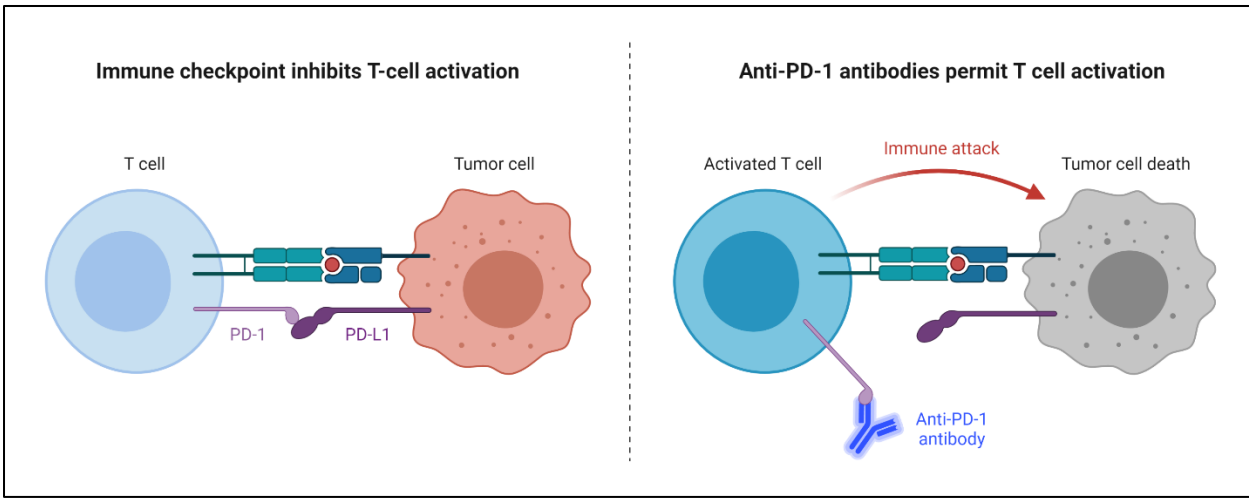

Figure 1.3. PD-1/PD-L1 axis as a target for monoclonal antibody therapy. Schematic was made on Biorender.com.

1.8 Tumor Microenvironment

One of the most important considerations for cancer pathology is the understanding that a tumor consists of an environment with large diversity of cell types, known as the tumor microenvironment (TME). The TME is an ecosystem that encompasses malignant cells, immune cells, blood vessels, and surrounding epithelial cells and fibroblasts. The interactions between transformed cells and other components of the TME can greatly influence tumor progression and patient prognosis. For example, abnormal vasculature in the tumor combined with increased oxygen consumption by cancer cells can create hypoxic conditions in the TME which is associated with poor prognosis, greater genomic instability, and the emergence of resistance clones [69]. Tumor-infiltrating immune cells also exert protumor and antitumor functions, which can influence tumor progression and response to therapy. For example, immune “hot” tumors are characterized inflammation and the tumor

infiltration T cells and NK cells that may exert antitumor cytotoxicity [70]. Hot tumors often include high expression of immunosuppressive ligands such as PD-L1 and are generally more responsive to ICI therapy [70]. On the other end of the spectrum, immune cold tumors are characterized by little infiltration of immune cells and are often unresponsive to ICI therapy [70]. Cold tumors require additional intervention to increase their immunogenicity and promote inflammation and tumor infiltration of antitumor immune cells [71]. Finally, immune excluded tumors are characterized by the infiltration of immunosuppressive cells such as regulatory T cells, Myeloid-derived suppressor cells, and M2 macrophages [70]. Indeed, it has become clear that consideration of the immune landscape in the TME is necessary for selecting the optimal therapeutic intervention.

1.9 Type I IFN Pathway

The innate antiviral response is the first line of defense against an invading pathogen. Innate antiviral signaling occurs through several signaling cascades simultaneously to initiate defenses at the molecular and cellular levels. Perhaps the most renowned members of antiviral signaling are interferons (IFNs). There are three distinct families of IFNs: type I, type II, and type III. Type I IFNs consist of the robustly studied IFNα and IFNβ, and the poorly defined IFNε, IFNτ, IFNκ, IFNω, IFNδ and IFNζ. The only member of the type II IFN family is IFNγ, which is mostly expressed by T cells and NK cells and is crucial for the initiation of adaptive immunity [72]. The type III IFN family contains IFNλ1, IFNλ2, IFNλ3 and IFNλ4, which have similar functions to type I IFNs but are more restricted since

their receptor is primarily expressed on epithelial cells [73]. In the context of anti-viral signaling, IFNα/β are the most widely studies since these cytokines and their receptors are ubiquitously expressed on most cell types. Production of IFNα/β is tightly regulated by three sets of transcription factors: IFN regulatory factors 3 and 7 (IRF3/7), activator protein 1 (AP1), and nuclear factor kappa–light-chain-enhancer of activated B cells (NFκB) (Figure 1.4) [74]. While each transcription factor complex can induce IFNα/β expression independently, all three are required for maximal induction of transcription [74]. The three sets of transcription factors assemble into a complex known as the enhanceosome, which binds to the promoter region and induces IFNα/β expression. Details concerning the regulation and downstream signaling of type I IFNs will be discussed in the following sections.

1.9.1 Pathogen Recognition Receptors

The host antiviral response is initiated when pathogen recognition receptors (PRRs) are engaged by pathogen-associated molecular patterns (PAMPs). There are several PRRs on the cell surface, in the cytoplasm, and in endosomal compartments that can recognize PAMPs associated with DNA and RNA viruses [75]. Toll-like receptors (TLRs) are a conserved family of transmembrane glycoproteins that reside on the cell membrane and/or endosomal compartments and can recognize a variety of PAMPs. For example, TLR4, which is expressed on the cell surface, can recognize viral glycoproteins [76,77]. On the other hand, TLR7 and TLR9 are located in endosomes where they can recognize viral RNA and DNA, respectively [75]. Upon pathogen recognition, all TLRs except for TLR3 initiate a signaling

cascade through myeloid differentiation primary response gene 88 (MyD88) [78]. The primary family of cytoplasmic PRRs responsible for recognition of RNA virus infections is the retinoic acid-inducible gene-I (RIG-I)-like receptor family. This family consists of RIG-I and melanoma differentiation-associated antigen 5 (MDA5), which recognize and bind to double stranded viral RNA [78]. Upon activation, both proteins undergo conformational changes that enable interaction with the mitochondrial transmembrane protein IFN promoter stimulator 1 (IPS-1) [75]. Similarly, cytosolic DNA sensors such as DNA-dependent activator of IFN-regulatory factors (DAI) can recognize and bind viral DNA leading to the initiation of downstream signaling cascades [75].

1.9.2 IFNα/β Production

Signaling cascades initiated by recognition of DAMPs generally converge on a few key transcription factors that are required for the transcription of IFNα/β genes. The IRF family of transcription factors include 9 members that share extensive homology in their N-terminal DNA-binding domain. IRF3 and IRF7 are amongst the most characterized of the family and are heavily involved in the production of IFNα/β. PRR signaling cascades result in the phosphorylation of IRF3, which induces its dimerization with other IRF3 or IRF7 molecules. The dimers interact with the cofactors cyclic-AMP-responsive-element-binding protein (CREB)-binding protein (CBP) or p300 in the nucleus [79–81]. This complex can then bind to a consensus DNA sequence resulting in the transcription of IFNα/β [82].

NFκB is another highly characterized transcription factor that is involved in the regulation of many cellular processes, including innate and adaptive immunity [83]. There are five known subunits of NFκB: p65 (RelA), p105/p50, RelB, c-Rel, and p100/p52. These subunits contain a Rel homology domain (RHD) which enables the homo- and heterodimerization of the subunits depending on the stimuli. Canonical NFκB signaling is mediated by the heterodimer of p65 and p50 and is the most commonly initiated upon PRR stimulation [84]. In quiescent cells, this dimer is sequestered in the cytoplasm by inhibitory proteins called inhibitors of NFκB (IκB) [83]. Upon initiation of PRR signaling cascades, IκB is phosphorylated by a family of kinases then ubiquitinated by a ubiquitin-conjugating enzyme and a ubiquitin-protein ligase[85,86]. This process flags IκB for degradation which, in turn, releases the NFκB dimers for nuclear translocation. In the nucleus, NFκB binds κB elements and initiates the transcription of many pro-inflammatory genes including IFNα/β [87].

Mitogen-activated protein kinases (MAPKs) are a large family of protein kinases that regulate many different cellular processes. In the context of viral infection, the MAPK known as c-jun N-terminal kinase (JNK) is also involved in the regulation of IFNα/β production [75]. Initiation of PRR signaling results in the phosphorylation of JNK, which in turn, translocates to the nucleus and phosphorylates a family of transcription factors known as AP1 [88]. Like NFκB, AP1 consists of homo- or heterodimers of several different subunits, including Jun, Fos, musculoaponeurotic fibrosarcoma (MAF), and activating transcription factor (ATF).

Activated AP1 dimers can then induce the transcription of several pro-inflammatory molecules including IFNα/β [89].

1.9.3 IFNα/β Signaling

Type I IFNs are secreted cytokines that can act in an autocrine and paracrine fashion to initiate antiviral defense mechanisms. IFNα and IFNβ can both bind to IFNα/β receptor (IFNAR) molecules expressed on the cell surface, which initiates a well characterized signaling cascade known as the JAK/STAT pathway. Binding of IFNα/β to IFNAR induces the autophosphorylation of the receptor-associated Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2) [90]. JAK1 and TYK2 then carry out phosphorylation of signal transducer and activator of transcription 1 and 2 (STAT1 and STAT2) [91]. Upon phosphorylation, STAT1/2 dimerize and associate with IRF9 to form the ISG factor 3 (ISGF3) complex [91]. ISGF3 then translocates to the nucleus where it binds the IFN-stimulated response elements (ISRE) promotor. This process initiates the transcription of hundreds of antiviral genes known as IFN-stimulated genes (ISGs) [91]. ISGs function through many different mechanisms to combat viral infection by inhibiting virus entry [92–94], virus replication [95,96], and virus egress [97].

1.9.4 Type I IFNs in Cancer Immunology

Aside from their role in combating virus infections, type I IFNs also influence the immune response against cancer. Type I IFN signaling is a crucial component in cancer immunosurveillance and the initiation of antitumor immunity [98]. Notably, type I IFN signaling is required for DC-mediated cross presentation of

tumor antigens and priming of tumor-specific T cells [99]. Furthermore, infiltration of T cells and NK cells into the tumor is often correlated with production of type I IFNs [98]. Type I IFNs have also been utilized as an anticancer therapy in preclinical and clinical studies [98,100–102]. While type I IFN is required for the initiation of antitumor, emerging studies suggest that they may also mediate forms of immunosuppression in the TME. Several reports have demonstrated type I IFN-dependent upregulation of immunosuppressive molecules such as PD-L1 and galectin 9 in the TME [103–106]. Furthermore, recent studies have associated sustained type I IFN signaling with resistance to several immunotherapies, including ICI therapy and CAR-T therapy [107–109]. Future work should focus on studying the kinetics of type I IFN signaling in the TME to initiate antitumor immunity while preventing IFN-mediated immunosuppression.

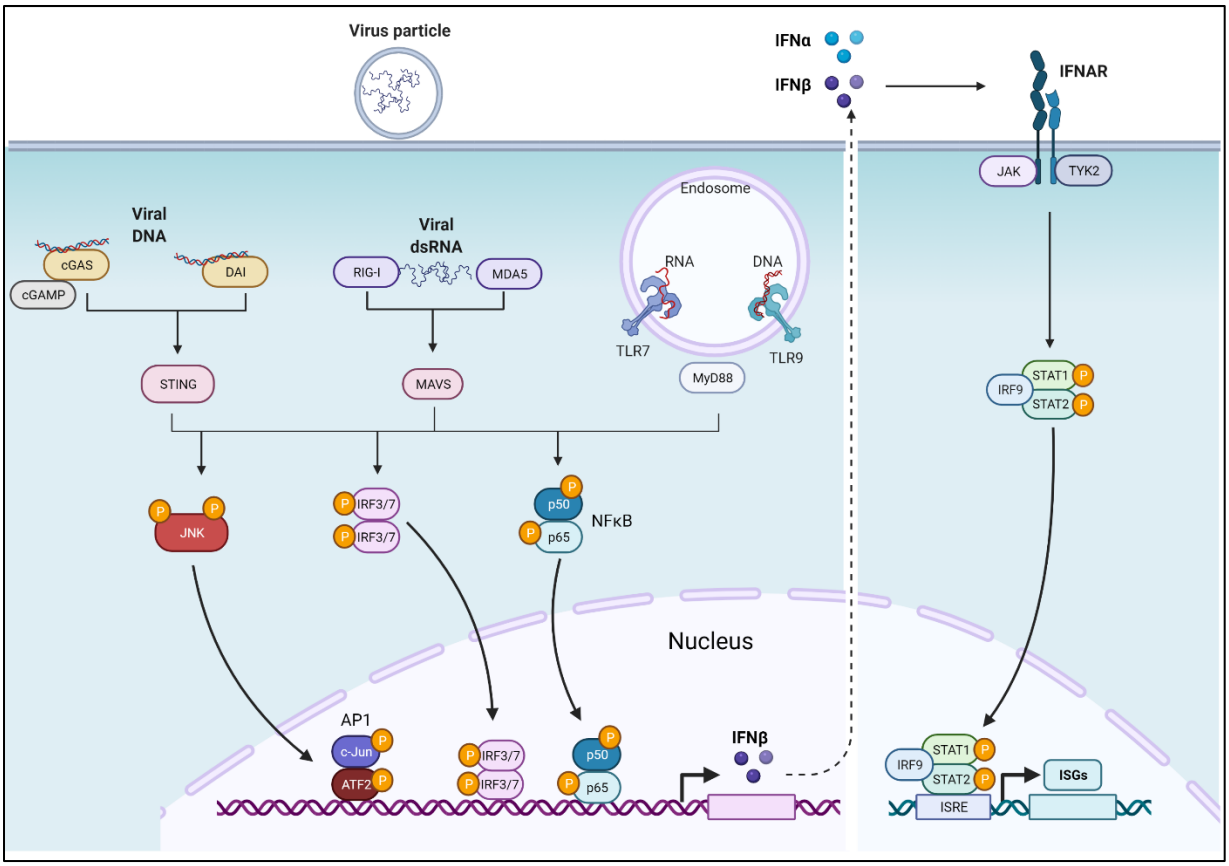

Figure 1.4. Type I IFN production and signaling. Schematic was made on Biorender.com.

1.10 Oncolytic Viruses

Oncolytic viruses (OVs) are a growing class of biotherapeutics that are engineered to preferentially infect and kill cancer cells. The basic concept of OVs emerged from the early observation that viruses have a preferential, though non-selective, tropism for cancer cells. These observations were first recorded in the early 20th century when some cancer patients entered a brief period of remission after contracting an infectious disease [110]. This phenomenon was occasionally observed throughout the 20th century and was even observed during the ongoing

COVID19 pandemic; a 61-year-old patient with Epstein–Barr virus (EBV)-positive Hodgkin lymphoma went into remission several months after recovering from SARS-CoV-2 infection [111]. The preferential tropism of viruses for cancer cells is attributed to many of the hallmark properties associated with cancer, including high metabolic rates, defective programmed cell death pathways, and immune evasion. Indeed, pathways involved with inflammation and immune activation often include pathways responsible for mounting cellular antiviral responses. Therefore, defects in inflammatory pathways that enable immune evasion can also result in defective antiviral signaling. Altogether, the properties that enable tumorigenesis provide a unique niche that supports virus replication. This phenomenon can be exploited by attenuating viruses so that they can effectively infect cancer cells without causing harm to normal cells with intact antiviral signaling.

OVs are multimodal agents that can eliminate cancer cells through several mechanisms. The most direct method of OV-mediated cytotoxicity is oncolysis of the infected cancer cells due to the natural life cycle of the virus. Additionally, OVs can reduce blood flow to the tumor mass by infecting tumor vasculature and causing thrombosis, leading to apoptosis and necrosis of cancer cells. Most importantly, however, the combination of infection and cell death results in the release of DAMPs that activate immune cells and attract them to the tumor bed, enabling a robust antitumor immune response [112–114]. Indeed, the ability of OVs to trigger an antitumor response is considered critical for therapeutic efficacy of OV therapy, with several studies demonstrating that depletion of $CD8^+$ T cells or tumor

engraftment in nude mice enables relapse of tumors following initial OV-mediated debulking [114,115]. Furthermore, it has been demonstrated that DCs are required for presentation of tumor antigens and the generation of an antitumor T-cell response during OV therapy [116,117]. Oncolysis of infected cancer cells can also result in the release of neoantigens that can be picked up by APCs leading to the recruitment of antigen-specific T cells [118]. Finally, OVs can be engineered to include transgenes that further diversify their utility. Transgenes incorporated into OVs often include immunostimulatory cytokines and chemokines, or tumor antigens that enable priming and/or boosting antitumor T cell response [119].

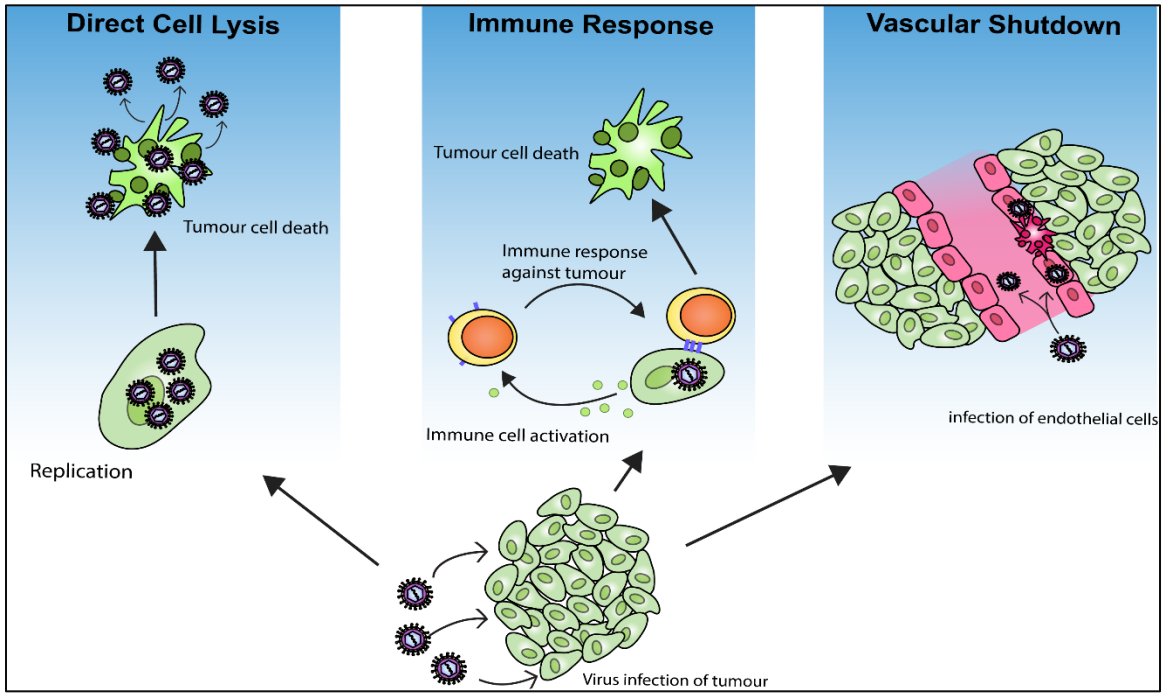

Figure 1.5. OV multimodal mechanism of action.

1.10.1 Oncolytic HSV

The herpesvirus family is a very large family of viruses that infect a wide variety of organisms. This family of viruses is characterized by enveloped particles containing a double-stranded DNA genome. Herpesviruses can be divided into the alpha, beta, and gamma subfamilies according to their biological characteristics and DNA sequences [120]. Herpes simplex viruses 1 (HSV-1) is part of the alpha herpesvirus subfamily that includes roughly 80 genes. HSV-1 can enter a cell through the herpes virus entry mediator (HVEM) and nectin-1 receptors, however other intracellular proteins are required for the virus genome to reach the nucleus where the viral genes are transcribed. The large genome of HSV-1 includes immediate early (IE), early, and late genes which can have a variety of roles in the life cycle of the virus.

In the early 90s, standardization of recombinant DNA technology enabled robust virus engineering. In 1991, Martuza *et. al.* found that a thymidine kinase (TK)-negative mutant of HSV-1 was able to prolong survival of mice with intracranial gliomas [121]. TK is an enzyme that catalyzes one of the steps in the synthesis of thymidine triphosphate. While TK is essential for HSV-1 to replicate efficiently in non-dividing cells that express low levels of cellular TK, the rapid division of cancer cells provides a steady supply of nucleotides that can complement the TK-negative virus. The many genes included in the HSV-1 genome enable a wide selection of genes that can be effectively attenuated to allow for preferential replication in cancer cells. For example, the IE gene ICP0 normally blocks IRF3

and IRF7-mediated IFN signaling [122,123]. Hummel and colleagues demonstrated that an ICP0-null variant of HSV-1 can selectively replicate in cancer cells *in vitro*. Furthermore, the oncolytic HSV-1 caused sustained regression of tumors in a murine model of breast adenocarcinoma [124]. The success of HSV-1 as an OV backbone is highlighted by the commercialization of Talimogene laherparepvec (T-Vec), which is the first OV to be clinically approved in the US and Europe. T-Vec is currently approved for use as a first-line therapy for the treatment of stage IIIb-IVM1c melanoma.

1.10.2 Oncolytic VSV

Vesicular stomatitis virus (VSV) is a single-stranded, negative-sense RNA virus of the family *Rhabdoviridae*. Although VSV normally infects insects, the virus has several mammalian hosts including cattle, swine, horses, and rodents [125]. VSV is a relatively small virus (11kb) that contains only five genes: nucleocapsid (N), phosphoprotein (P), matrix (M), glycoprotein (G) and large (L). The virus enters the host cell by binding to Low-Density Lipoprotein Receptors (LDLR) via endocytosis [126]. This process is dependent on VSV G, which is also essential for fusion and budding of the virus [127]. VSV replication occurs in the cytoplasm, where the VSV L protein consisting of an RNA polymerase initiates transcription of viral mRNA in conjunction with the P protein [128]. The N protein encapsulates viral RNA and protects it from degradation after VSV-L-mediated post-transcriptional modifications are applied [129,130]. Finally, the M protein is involved in the proper packaging of virions and dampening host antiviral signaling. For the latter,

the M protein has been shown to inhibit the nuclear export of cellular mRNA and their translation into functional proteins [131–133]. WT VSV is a highly IFN-sensitive virus that can act as an oncolytic virus due it's poor virulence in human cells. However, VSV's selectivity towards cancer cells can be further improved by attenuating the function of the M protein. For example, one variant of VSV was engineered with a deletion of methionine 51 in the M protein (VSVΔ51), which impairs the protein's ability to block antiviral signaling. This variant is even more sensitive to the intact antiviral signaling in normal cells but can still productively infect many cancer cells with defective antiviral pathways [134]. Variants of oncolytic VSV are currently being tested in phase I clinical trials as a monotherapy or as a combination therapy [119].

1.11 Resistance to Immunotherapy

Immunotherapy has demonstrated unprecedented response rates in cancer patients. However, many patients do not benefit from treatment, while other patients may relapse after a period of tumor regression. Resistance to immunotherapy can take the form of primary or acquired resistance, as discussed by Sharma et. al. [29]. Patients with primary resistance fail to respond to initial immunotherapy strategies. Primary resistance can come in the form of cell-intrinsic and cell-extrinsic mechanisms. Tumor cell-intrinsic mechanisms of resistance are mediated by expression of genes that can drive resistance. The most common example of this is upregulation of PD-L1, which is often expressed by high-risk cancers such as triple-negative breast cancer and colon carcinomas [135]. Other cell-intrinsic mechanisms

of resistance include prevention of immune infiltrates into the TME. One preclinical study demonstrated that tumors could reduce the levels of cDC1s in the TME through high activation of the WNT/β-catenin signaling pathway. WNT/β-catenin signaling indirectly inhibited the expression of CCL4, which resulted in exclusion of $CD8^+$ T cells infiltrates and resistance to ICI therapy [136]. Another example is the loss of PTEN expression in tumors, which increases the expression of immunosuppressive cytokines and impairs infiltration of T cells into the tumor [137]. Other examples of cell-intrinsic mechanisms of primary resistance include downregulation of MHC-I and alterations in antigen presentation machinery [29].

Cell-extrinsic mechanisms of primary resistance are mediated by components external to tumor cells. For example, PD-L1 can be expressed on a variety of cell types in the TME including cancer-associated fibroblasts (CAFs) and myeloid cells [138–140]. Another example is the frequent infiltration of regulatory T cells (Tregs) in human tumors [127,128], which can suppress effector T cell function through the secretion of immunosuppressive cytokines [129]. Similarly, myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs) can also suppress antitumor immunity. Infiltration of MDSCs into the TME is correlated with reduced efficacy of ICI therapy and adoptive T cell therapy in the clinical and preclinical studies [130,131].

Finally, acquired resistance can be potentiated by aberrant changes in the TME such as high mutational burden, hypoxic conditions, and abnormal vasculature. As a result, heterogenous subpopulations often exist within a single tumor

mass, and therapeutic intervention can exert selective pressure towards resistant variants. This concept is thoroughly expanded upon in **Chapter 2**.

1.12 Rationale and Hypothesis

ICI therapy has demonstrated very promising clinical outcomes in patients with several forms of cancer [48]. However, many patients with solid tumors fail to respond to initial ICI therapy, or relapse after a period of tumor regression [29]. Research has shown that highly inflamed tumors are more likely to respond to ICI therapy [132]. Furthermore, patients with phenotypically identical cancers may exhibit highly dichotomous responses to treatment [133]. **These observations highlight the need for developing strategies to increase inflammation in the TME to enable durable responses.** It is critical that molecular and cellular characteristics that enable durable responses are identified. To this end, we propose the use of OV platforms for improving tumor immunogenicity. **We hypothesize that OVs can enable response to ICI therapy by improving tumor immunogenicity though the induction of stimulatory cytokines and chemokines**. To address this hypothesis, we have developed the following aims:

1) **Establish therapeutic OV platforms that can enable response to ICI therapy in solid tumor models.**
2) **Identify immune infiltrates that drive therapeutic efficacy in solid tumor models.**
3) **Investigate the role of OV-mediated type I IFN induction on antitumor immunity.**

Our lab has previously developed oncolytic HSV-1 (oHSV-1) platforms in combination with low-dose chemotherapy for treatment of murine breast cancers

[134,135]. Specifically, the combination of low-dose mitomycin C (mito) with oHSV-1 enabled response to ICI therapy by inducing necroptosis in the tumor [135]. Here, we build upon these data in **Chapter 3** by testing the combination of mito + oHSV-1 in the immunosuppressive MC38 murine colorectal carcinoma model. Unlike the breast cancer models, mito + oHSV-1 was not successful in delaying tumor progression in MC38 tumor models. While MC38 tumors were partially responsive to ICI therapy, none of the mice demonstrated durable response to therapy. However, mito + oHSV-1 therapy was successful in sensitizing tumors further to ICI therapy, resulting in a complete response in more than 50% of mice. Using a Clariom S assay we identified chemokine signatures associated with chemoattraction of myeloid cells. We found that mito + oHSV-1 induced infiltration of cDC1s into the tumor, which was required for driving therapeutic efficacy. These data suggest that enhancing recruitment and activity of cDC1s in the TME is essential for enabling durable responses to ICI therapy.

In a collaboration with Dr. Yonghong Wan's lab, we investigated the role of type I IFN (IFN-I) signaling on antitumor immunity (**Chapter 4**). We used a non-immunogenic murine melanoma model, B16F10, to study the effects of oncolytic VSV (oVSV)-induced IFN-I signaling. We found that oVSV induced tumor expression of PD-L1 in an IFN-I-dependent manner. Furthermore, oVSV induced expression of PD-L1 on circulating T cells, B cells and monocytes, which was abrogated using anti-IFNAR-1 monoclonal antibody. While IFN-I is necessary for priming tumor-specific $CD8^+$ T cells [136], we found that combining oVSV with

anti-IFNAR-1 enhanced antitumor $CD8^+$ T cell effector functions. Additionally, IFNAR blockade improved therapeutic response to oVSV therapy in a B16F10-gp33 tumor model.

In the work presented in this thesis I have identified how OV-mediated inflammation can either enable or suppress antitumor immunity. Furthermore, I identified a crucial role of cDC1s for driving a durable response to ICI mediated by mito + oHSV-1. These results are in line with studies that suggest cDC1s are the primary APCs for priming antitumor $CD8^+$ T cells [37,38,43]. I have also identified immunosuppressive functions of IFN-I in the tumor, which is also supported by recent findings [137–139]. Future work should continue to characterize OV-mediated modulation of tumor immunogenicity to develop rational combination strategies that can maintain a durable response.

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Chapter 2 : Tumor Heterogeneity: A Great Barrier in the Age of Cancer Immunotherapy

Preface

This is an author-produced version of an article published in *Cancers*. This article can be accessed online at: https://www.mdpi.com/2072-6694/13/4/806. DOI: 10.3390/cancers13040806, PMID: 33671881. Authors: Nader El-Sayes, Alyssa Vito, Karen Mossman.

NES conceived and outlined the concepts discussed in the review and prepared the manuscript and figures. AV prepared the manuscript and figures. KM supervised the preparation and edited the manuscript.

This review discussed forms of acquired resistance to cancer immunotherapy. In particular, the focus of the review is to discuss how tumor heterogeneity can drive resistance to immunotherapy and prevent durable responses in the clinic. The review discusses how phenotypically identical cancers can greatly vary in immunogenicity from one patient to another. Furthermore, we discuss how tumors in a single patient can contain resistant subpopulations that can be selected for by therapeutic intervention. This work sets the foundation for the other chapters of this thesis: We must identify shifting factors in the TME that drive or prevent the onset of acquired resistance to immunotherapy. By understanding these shifting factors, therapeutic combinations can be developed and rationally selected to induce durable responses in the clinic.

Tumor heterogeneity: a great barrier in the age of cancer immunotherapy

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Simple Summary

Despite great advances in cancer therapy, tumor heterogeneity continues to be a great barrier for the successful treatment of cancer. It has long been established that tumor heterogeneity is prevalent in most cancer patients and is a major driver of acquired resistance to all forms of cancer therapy. In the case of immunotherapy, the response of the immune system against specific tumor antigens can drive selective pressure towards antigen-negative cells, which is a common cause of relapse in the clinic. In this review we summarize the mechanisms in which tumor heterogeneity can arise. Furthermore, we discuss recent advances in the field of oncology that can be used to better identify, study, and overcome tumor heterogeneity.

Abstract

Throughout the history of oncology research, tumor heterogeneity has been a major hurdle for the successful treatment of cancer. As a result of aberrant changes in the tumor microenvironment such as high mutational burden, hypoxic conditions and abnormal

vasculature, several malignant subpopulations often exist within a single tumor mass. Therapeutic intervention can also increase selective pressure towards subpopulations with acquired resistance. This phenomenon is often the cause of relapse in previously responsive patients, drastically changing the expected outcome of therapy. In the case of cancer immunotherapy, tumor heterogeneity is a substantial barrier as acquired resistance often takes the form of antigen escape and immuno-suppression. In an effort to combat intrinsic resistance mechanisms, therapies are often combined as a multi-pronged approach to target multiple pathways simultaneously. These multi-therapy regimens have long been a mainstay of clinical oncology with chemotherapy cocktails but are more recently being investigated in the emerging landscape of immunotherapy. Furthermore, as high throughput technology becomes more affordable and accessible, researchers continue to deepen their understanding of the factors that influence tumor heterogeneity and shape the TME over the course of treatment regimens. In this review, we will investigate the factors that give rise to tumor heterogeneity and the impact it has on the field of immunotherapy. We will discuss how tumor heterogeneity causes resistance to various treatments and review the strategies currently being employed to overcome this challenging clinical hurdle. Finally, we will outline areas of research that should be prioritized to gain a better understanding of tumor heterogeneity and develop appropriate solutions.

2.1 Introduction

Major challenges with universal cancer therapy have historically been attributed to the large number of subtypes of the disease and the biological differences associated with cancers arising in different parts of the body. While this locational diversity remains a

challenge for unifying cancer treatment across various types, it has now become clear that even patients with phenotypically identical cancers often have dichotomous responses to treatment [1,2]. As we have continued to investigate the inner workings of a tumor, we have discovered that cancers are formed by many distinct cellular populations, rather than a homogenous cluster of identical cells. This tumor heterogeneity can take the form of cellular and genetic heterogeneity. Cellular heterogeneity is used to describe the diversity of cells that can be found in the tumor microenvironment (TME). The presence of cancer-associated fibroblasts, endothelial cells and immune cells plays an important role in tumor progression and response to therapy. In this review, however, we will focus on the genetic heterogeneity of cancer cells within one or several tumors, which is often a driver of acquired resistance in tumors and represents a major hurdle for proper diagnosis, prognostic prediction and therapeutic efficacy [3].

It is well established that tumor heterogeneity is largely driven by aberrant changes in the TME, such as high mutational burden, hypoxic conditions and abnormal vasculature [4]. Additionally, native TME factors aside, the administration of therapy can result in selective pressure towards subpopulations with acquired resistance mechanisms [3,5]. This phenomenon is often the cause of relapse in previously responsive patients, drastically shifting expected therapeutic outcomes. As oncology research has progressed and evolved, so too has our understanding of cancer biology and the immunological synapse involved in swaying clinical prognoses. But the question still remains, how do these environmental and therapeutic situations influencing the tumor create heterogeneity and distinctive cellular populations?

In 1976 Peter Nowell published a landmark perspective piece suggesting that cancer arises through an evolutionary process, similar in nature to Darwinian evolution and natural selection [6]. Nowell suggested that cancers arise through stepwise, somatic cellular mutations inevitably leading to multiple sub-clonal populations. This revelation changed the way that cancer was studied and further highlighted the need for multi-pronged therapeutic approaches and a deeper understanding of cancer at a genomic level. Indeed, as therapeutic approaches have evolved in recent decades, we have seen an insurgence of combined therapeutic modalities that can target cancer cells through differing mechanisms, with the goal of overcoming innate acquired resistance.

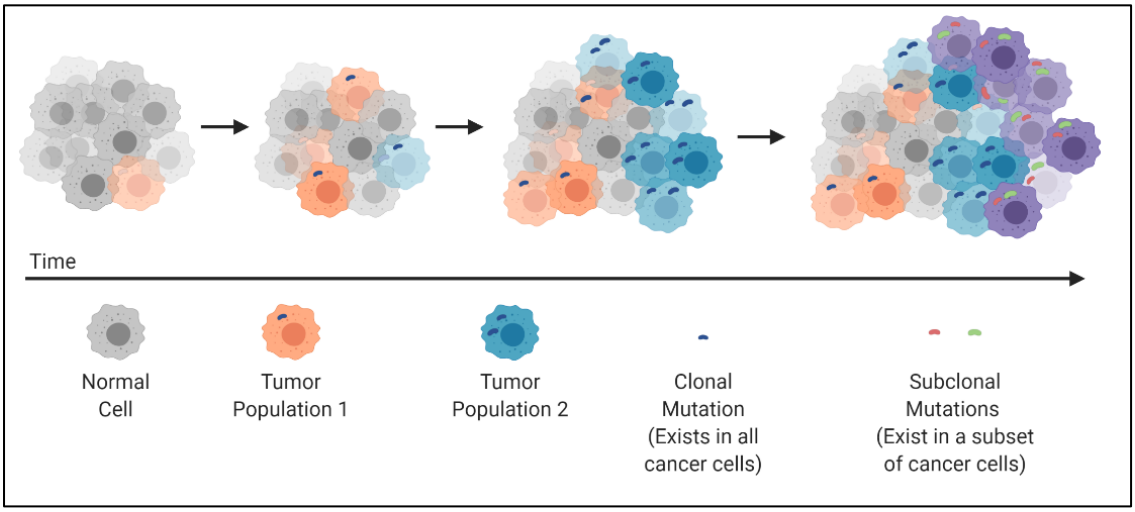

Figure 2.1. Clonal evolution and development of tumor heterogeneity.

In the last decade it has become evident that identifying the biological drivers of cancer will ultimately lead to personalized cancer treatment and improved clinical outcomes [7]. As such, oncologists now increasingly use molecular characterization of a sample from a primary or metastatic tumor lesion to guide their selection of treatments for an

individual patient. However, this approach can prove problematic as they rely on a limited sample of cancer tissue, often obtained from a needle biopsy or surgical excision, that is unlikely to accurately capture the complete genomic landscape of a patient's cancer. For example, clinical instances have occurred in which estrogen receptor (ER) expression in a primary breast cancer sample does not mirror what is later found in a distant metastatic lesion. In fact, in 7–25% of patients ER expression was different between the two sites [8–10]. Changes in ER status may have important clinical implications because patients with tumors that lack ER expression do not benefit from treatment with endocrine therapy such as tamoxifen or aromatase inhibitors [11]. This is just one example of how temporal heterogeneity can affect clinical outcomes. Furthermore, comprehensive characterization of multiple tumor specimens obtained from the same patient illustrates that remarkable intratumor heterogeneity can exist not only in the temporal sense as in the breast cancer example, but also between geographical regions in the same tumor.

Cancer genomics studies, including large-scale collaborative sequencing projects such as The Cancer Genome Atlas (TCGA) and the International Cancer Genome Consortium (ICGC), have catalogued genetic interpatient tumor heterogeneity for cancers of the same histological subtype. Non-genetic phenotypic and functional heterogeneity is also well recognized, as is heterogeneity within the TME. As high throughput technologies become more affordable and readily accessible, researchers continue to expand their understanding of the factors that influence tumor heterogeneity and shape the TME not only at the time of diagnosis, but also over the course of treatment regimens.

Here, we review the clinical implications of tumor heterogeneity for cancer diagnosis, prognostic predictions, treatment selection and resistance. We will investigate the factors that give rise to tumor heterogeneity and the impact it has on both established and emerging therapeutic regimens. We will discuss how tumor heterogeneity causes resistance to various treatments and review the strategies currently being employed to overcome this challenging clinical hurdle. Additionally, we will discuss how clinical trials that are restricted to molecular subtypes of cancer could incorporate studies of tumor heterogeneity so that we can better understand the clinical impact of heterogeneity on therapeutic efficacy and the emergence of acquired resistance.

2.2 Factors Contributing to Tumor Heterogeneity

Tumor heterogeneity can take different forms, each posing a unique challenge for successful treatment of disease and for overcoming the risk of acquired resistance. Patients with the same type of malignancy may experience vastly different clinical outcomes, both before and after treatment. This interpatient heterogeneity is often seen in the clinic and is largely attributed to differences in somatic mutations acquired in the tumor. Patients acquire mutations not only in different genes, but also in different domains within the same gene [12]. Heterogeneity is also prevalent within an individual patient and can take the form of intratumor, intermetastatic or intrametastatic heterogeneity. Intratumor heterogeneity consists of a single tumor mass which contains several distinct subpopulations of cells, each behaving differently with varied responses to therapeutic intervention [13]. Furthermore, intermetastatic heterogeneity can be observed between different tumor masses, as subpopulations of cells can migrate from the primary tumor and travel to distant sites

within the body to form metastatic lesions. In fact, heterogeneity between different malignant lesions is the most common clinical observation in patients with advanced metastatic disease [13–15]. Like intratumor heterogeneity, intrametastatic heterogeneity is defined by multiple cellular subpopulations within a single metastatic lesion. In the past decade there has been an increase in research focused on studying tumor heterogeneity and its role in developing acquired resistance to many different types of cancer therapies. While assessing the emergence of heterogenous cell populations throughout the course of tumorigenesis, there has been some debate as to whether heterogenous populations are pre-existing in the tumor or developed in response to therapeutic intervention. In reality, many factors may lead to heterogeneity during tumor progression, however some types of therapy also contribute to genomic diversity by increasing the tumor mutational burden.

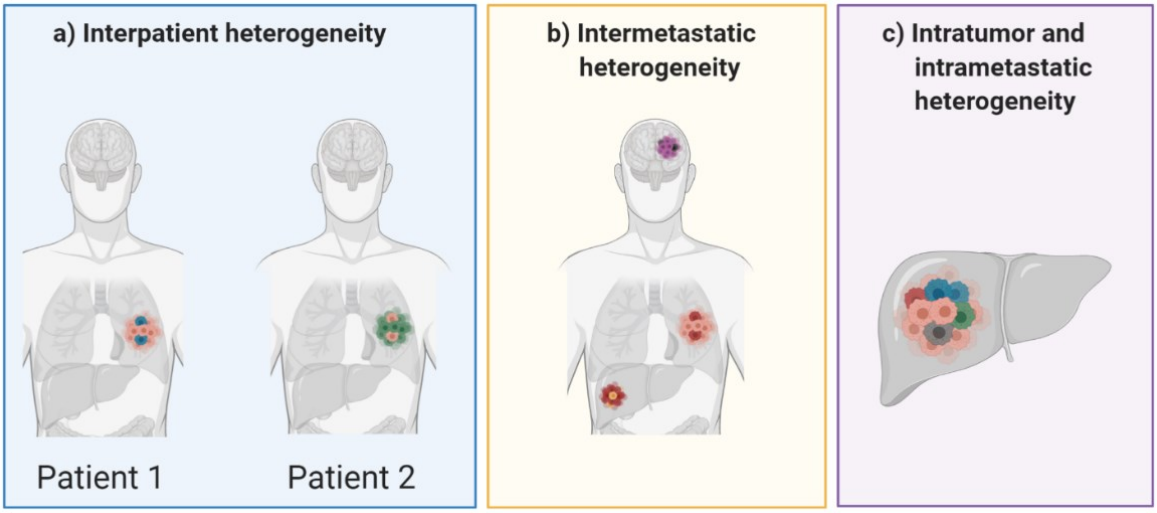

Figure 2.2. Forms of tumor heterogeneity that can occur between patients and in individual patients.

There have been many studies showing that heterogenous populations arise during the early stages of tumor progression, and that treatment leads to selective pressure towards resistant populations [16–20]. Among the many factors that drive tumor heterogeneity, genomic instability is most prominent in all malignancies. Many of the biological hallmarks associated with cancer development, such as limitless replicative potential, increase the mutational rate and genomic instability of malignant cells, which in turn give rise to other malignant traits [21]. This cascading effect often results in heterogeneity in the tumor as different cells acquire unique mutations that give rise to genetically distinct subpopulations [5,6,22]. For example, in normal cells the tumor suppressor p53 responds to DNA damage by triggering pathways involved in cell-cycle arrest, apoptosis, or DNA repair [23]. Mutations in p53 are some of the most frequent mutations found in human cancers and are largely associated with tumorigenesis and genomic instability [24,25]. Other hallmark traits associated with tumorigenesis may further contribute to genomic instability. For example, hypoxic conditions are often found in the TME due to the enhanced cellular kinetics and increased replicative potential that require greater oxygen uptake [4]. In one study, Lichun Ma and colleagues sampled the single-cell transcriptomic landscape of 19 patients with liver cancer [26]. They found that hypoxia-dependant VEGF expression was associated with higher transcriptomic diversity in the tumor. Furthermore, tumors with more heterogeneity contained T cells with lower cytotoxic activity and were associated with worse overall survival [5]. Indeed, hypoxia is well known to impede mismatch repair, cause genomic instability and promote the formation of subclones in the TME [4,27,28].

While tumor heterogeneity is unavoidable during tumor progression, some therapeutic interventions can further drive the formation of genetic and epigenetic diversity in the tumor. Many chemotherapies are DNA-damaging agents or agents that interfere with DNA replication/repair pathways [29,30]. This type of cellular damage results in the risk that persisting cancer cells may develop greater levels of genomic instability that can give rise to resistant populations [5]. Indeed, a study lead by McGranahan *et. al.* found an association between chemotherapy and neoantigen heterogeneity. Furthermore, neoantigen heterogeneity was associated with poor clinical outcomes in response to PD-1 and CTLA-4 blockade [31]. This concern also extends to radiation therapy, as ionizing radiation can also contribute to genomic instability and carcinogenesis [32].

2.3. Acquired Resistance and Antigen Escape

There is a plethora of innovative therapeutic approaches currently being developed for the treatment of cancer. Tumor heterogeneity acts as a major hurdle for treatment and a potentiator of acquired resistance regardless of the therapeutic approach or the type of cancer. Unlike primary resistance, acquired resistance can occur in patients that initially respond to therapy, resulting in a relapse after a period of tumor regression [33]. The mechanism of resistance caused by tumor heterogeneity is the same regardless of the treatment received. In a manner similar to that of natural selection, the composition of subpopulations in the tumor changes dynamically as a result of selective pressure exerted by therapeutic intervention and changes in the TME. Indeed, relapse of chemoresistant tumors is frequently observed in the clinic [5,34,35]. One such example was highlighted in a study led by Kim *et. al.* in which 20 patients with triple negative breast cancer (TNBC) were

observed during neoadjuvant chemotherapy (NAC). They identified 10 patients with persistent chemoresistant clones after treatment. Upon further analysis using single-cell DNA and RNA sequencing, the data indicated that the resistant clones were pre-existing and adaptively selected by NAC [20]. A similar study involved genetic and histological analyses of tumor biopsies from 37 patients with non-small cell lung cancer (NSCLCs) that developed resistance to treatment with EGFR inhibitors. Many of these tumors acquired various mechanisms of resistance, including mutations in the PIK3CA gene and epithelial to mesenchymal transition. Furthermore, some of the tumors lost their mechanisms of resistance in the absence of further EGFR inhibitor treatment and were later responsive to a secondary challenge with EGFR inhibitors [36].

Cancer immunotherapy has made extraordinary strides in the past decade, with the emergence of a variety of antibody, cell-based and virus-based therapies becoming first line treatments for some cancers. Unfortunately, populations with acquired resistance to immunotherapy can also arise because of tumor heterogeneity. The immune system can both hinder and promote tumor progression, a process known as cancer immunoediting. Cancer immunoediting can proceed through 3 distinct stages termed elimination, equilibrium and escape [37,38]. During the elimination phase, innate and adaptive immune cells work together to identify and eliminate malignant cells early during tumor progression. Some cancer cell variants may evade elimination and enter a phase of equilibrium, in which the immune system prevents further tumor progression but can inadvertently modify the immunogenicity of the cancer cells. Finally, the genetic instability of the cancer cells combined with the constant selective pressure of the immune system can give rise to new cancer

cell variants that escape immune control. In the case of immunotherapy, response against specific antigens exerts selective pressure towards antigen-negative subclones over time, a concept termed antigen escape [39]. An example of antigen escape was noted in two patients with stage IV melanoma treated by adoptive T-cell transfer. Expression of the T-cell-recognized neoantigen was lost in these patients, although it is unclear whether this form of antigen loss was caused by downregulation of the target antigen or the selection of antigen-negative variants [40]. In another study conducted by Sotillo and colleagues, samples were obtained from patients with B-cell acute lymphoblastic leukemias (B-ALL) that relapsed from CD19-specific CAR T-cell therapy. They found that relapsed cells contained alternatively spliced *CD19* mRNA with an omission of exon 2. The alternatively spliced form results in an N-terminally truncated form of CD19 that retains some functionality but avoids CAR T-cell-mediated killing [41].

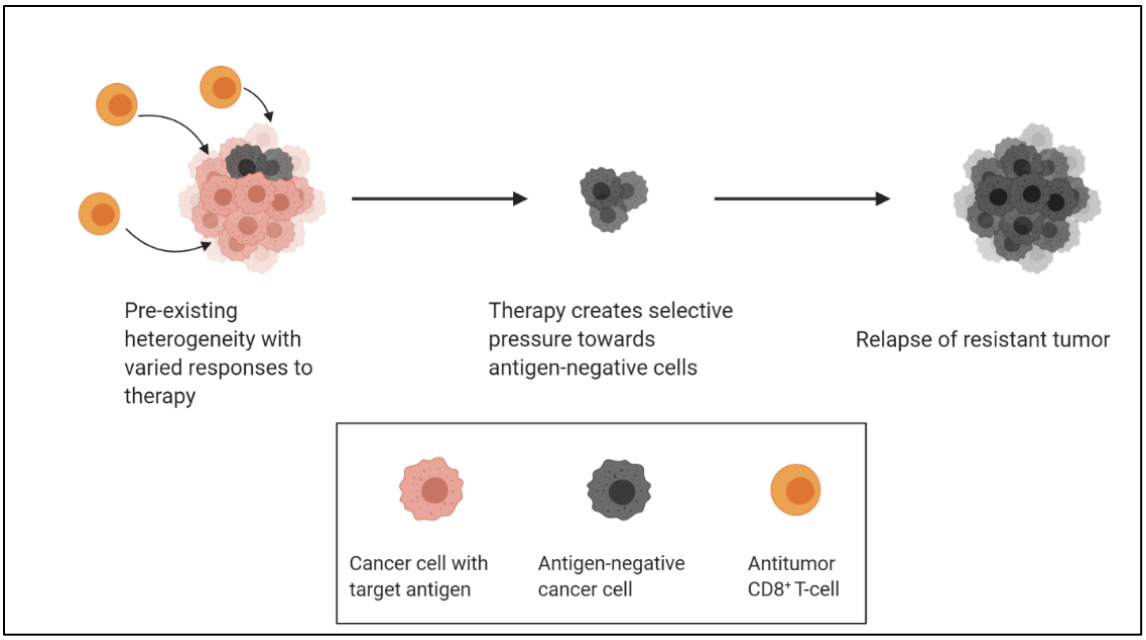

Figure 2.3. Selective pressure from antitumor T-cells drives resurgence of antigen-negative clones.

As previously discussed, antigen escape has become a well-documented hurdle for immunotherapy platforms that are designed to target specific antigens. At first glance this may indicate that spontaneous antitumor immunity is favorable over targeted responses and is less susceptible to acquired resistance mediated by antigen escape. Immunotherapy platforms such as immune checkpoint blockade (ICB) and *in situ* vaccines are examples of therapies that can potentiate spontaneous antitumor responses, however spontaneous $CD8^+$ T-cell immunity will likely be restricted to a few dominant neoantigens [42–45]. Therefore, spontaneous antitumor responses can also exert selective pressure towards antigen-negative clones. In one study, mice bearing B16 murine melanoma were vaccinated with plasmids encoding hsp70 and the HSVtk suicide gene. The authors found that suboptimal plasmid vaccination selected for aggressive tumor variants that lost the immunodominant

neoantigen while retaining the expression of other known melanoma antigens [46]. Similar conclusions can be made from another study that examined the evolving landscape of tumor neoantigens during the emergence of acquired resistance in patients with NSCLC after treatment with ICB therapy. They report that resistant tumors lost the expression of neoantigens that were recognized by the host antitumor T-cells [47]. Shifts from dominant to secondary neoantigens have been observed in some cases and will be discussed further in section 5, however shifts in antigen specificity are rare occurrences in the absence of deliberate intervention. The prevalence of antigen escape as a mechanism of acquired resistance to immunotherapy underscores the importance of further research to understand and manipulate the dynamic landscape of the TME.

2.4. Strategies for Identifying Tumor Heterogeneity

Since the first report of cancer genome sequencing appeared in 2006 [48], research involving genomic data has gained traction, with clinical implications rapidly revealing themselves. Initially, progress in obtaining such large datasets was hindered by the high cost and limited availability of whole tumor sequencing, preventing researchers and clinicians from readily taking advantage of the technology. However, over the years sequencing facilities have become more widespread and costs have gradually reduced, allowing for an explosion of cancer genomic data and publicly available datasets.

In 2006 the TCGA program began, a landmark cancer genomics program that characterized over 20,000 primary cancer and matched normal samples spanning 33 cancer types. This joint effort between the National Cancer Institute and the National Human Genome Research Institute brought together researchers from a wide range of disciplines and

multiple institutions. Over a span of 12 years, TCGA generated over 2.5 petabytes of genomic, epigenomic, transcriptomic and proteomic data. These datasets were released publicly and have since been used to improve diagnosis, treatment, and cancer prevention with over 11,000 publications utilizing the data from this massive collaborative effort.

The goal of sequencing cancer genomes is to develop a better understanding of factors that potentiate or hinder tumor progression at cellular and molecular levels. Sequencing data can be used for biomarker discovery to improve diagnoses, drug target discovery for therapeutic intervention and personalized medicine by matching patients with the treatment most likely to be efficacious against their disease. In the case of heterogeneity, the latter is of particular importance as sequencing efforts can help to identify heterogeneity within a tumor and also within multiple lesions from the same patient. However, most datasets obtained have one major limitation: clinical details of the sample donors are often incomplete or missing altogether. In fact, the first cohort of samples collected for TCGA were complimented with only the donor's gender, diagnosis and age at diagnosis. Key clinical data such as the administered therapy and clinical outcomes were often not included. The future of cancer genome sequencing aims to eliminate these restraints but will have to overcome strict patient privacy laws and the lack of a centralized hub for all data.

In the context of heterogeneity, the more recent development of single-cell RNA sequencing (scRNA-seq) is particularly exciting, as it enables the determination of not only the frequency of individual mutations, but also determination of co-occurring and mutually exclusive alterations. Indeed, scRNA-seq has been used to demonstrate the presence of

multiple cell populations within a tumor, each belonging to a distinct molecular group [49–51]. Furthermore, Kinker and colleagues have used scRNA-seq to investigate the ability of cultured cell lines to recapitulate the heterogeneity observed among malignant cells in human tumors [52]. They profiled 198 cancer cell lines from 22 cancer types and identified 12 expression programs as being heterogenous across multiple cancer cell lines. These programs, including cell cycle, senescence, stress and interferon responses, were further shown to recapitulate those recently identified as being heterogenous in expression within human tumors. This information allowed the researchers to identify specific cell lines as being the most relevant models of cellular heterogeneity, which they then used to study subpopulations of senescence-related cells, demonstrating their unique drug sensitivities, which were predictive of clinical response. This extensive and thorough body of work is a prime example of how scRNA-seq can be used to identify recurrent patterns of heterogeneity that are shared between tumors and the models we use for preclinical development.

Circulating tumor DNA (ctDNA), released from both normal and cancerous cells, has recently arisen as an exciting new biomarker in the field of oncology. ctDNA is characterized as DNA that contain genetic changes that can be used for identifying cancer. Many studies have reported impressive clinical data with cancer detection accuracy ranging from 50 – 70% [53–55]. Genomic characterization of ctDNA or circulating tumour cells may offer an opportunity to assess clonal dynamics throughout the course of a patient's illness and identify drivers of therapeutic resistance. In one study, Ma and colleagues showed that ctDNA can be used to assess tumor heterogeneity and predict treatment outcomes in metastatic breast cancer [56].

High throughput technologies such as scRNA-seq and innovative biomarker discovery strategies such as ctDNA are just some of the ways that the field of cancer research is rising to the challenge of identifying and tackling tumor heterogeneity. These technologies are becoming cheaper and more accessible, enabling a deeper understanding of the frequent changes in the TME and for the monitoring in both pre-clinical and clinical settings. These developments allow for more personalized solutions to the ever-changing landscape of tumors and can be used to predict the efficacy of therapeutic combination strategies, improving clinical outcomes and sparing patients from aggressive therapies that may provide no benefit to their tumor phenotype.

2.5. Emerging Strategies to Overcome Tumor Heterogeneity

The strong association between tumor heterogeneity and poor clinical prognosis has rekindled research for developing strategies to overcome tumor heterogeneity. The oldest and most common approach is to combine therapies with different mechanisms of action in a multi-pronged attempt to prevent the selection of resistant populations. Combination therapies have become commonplace for classical chemotherapy, with most therapeutic regimens involving the combination of different chemotherapeutic agents with or without surgical resection [57]. This strategy has extended to combining chemotherapy and immunotherapy with promising pre-clinical and clinical results. One meta-analysis of 12 phase-III clinical trials highlights the benefits of combining chemotherapy with atezolizumab and/or pembrolizumab in patients with NSCLC [58]. A similar example can be seen in a phase-III clinical trial in which the combination of Atezolizumab and nab-paclitaxel showed improved progression-free survival in patients with metastatic TNBC [59]. In a

pre-clinical study by Nguyen et. al., the authors utilized a combination strategy to eliminate antigen-negative tumors that arose after treatment with adoptive T cell therapy (ACT) followed by oncolytic virus vaccination. They found that the addition of a class I histone deacetylase inhibitor (HDACi) to the therapeutic regimen reprogrammed immunosuppressive tumor-infiltrating myeloid cells to eliminate antigen-negative tumor cells [60]. Indeed, many studies have demonstrated the efficacy of chemotherapy when combined with ICB [61,62], oncolytic viruses [63–65], and cell-based immunotherapies [66–68]. As discussed previously, some therapies may contribute to the genomic instability in cancer cells that can give rise to resistant subclones. Instead of attempting to prevent more genomic instability in the tumor, some combination therapies are designed to exploit this property for therapeutic benefit. Zhang and colleagues report that Cyclin-dependent kinase 7 (CDK7) can be used to potentiate genomic instability, which triggers an antitumor immune response. Addition of anti-PD-1 therapy enhanced the antitumor response and offered a significant survival benefit in a murine small cell lung cancer model [69].

Combination therapies for the treatment of cancer often outperform their respective monotherapies in progression-free survival and overall survival, however this does not guarantee the prevention of resistant subclones relapsing after initial treatment. In the context of immunotherapy, antigen escape is often a source of acquired resistance. One method to prevent antigen escape is to expand the antitumor response to generate a more diverse immune repertoire that would be better suited to target heterogenous tumors. Data from several studies suggest that multi-peptide vaccines can be designed to create a response against several tumor antigens simultaneously [70–72]. In one of these studies, the authors

designed a multi-antigen vaccine for renal cell cancer patients that increases the breadth of the immune response and resulted in better disease control compared to patients that responded to a single peptide [72]. Perhaps one of the greatest drawbacks to a multi-antigen vaccine strategy is the lack of known tumor antigens in many cancers. However, new generation high throughput technologies have enabled great strides for identifying tumor neoantigens [44]. In two preclinical studies, an in silico approach was used to identify immunogenic neoantigens which were then used to design multi-antigen vaccines [73,74]. Ideally, incorporating in silico and high throughput approaches could be used to design multi-antigen vaccines throughout the dynamic course of treatment and tumor progression.

Finally, enhancing the T-cell repertoire through antigen spread could allow for a broadened immune response regardless of the primary response. Antigen spread (also known as determinant spread and epitope spread) is the expansion of an immune response from a dominant antigen to secondary antigens. Expansion to secondary antigens could include different epitopes from the same antigen or from other antigens [75]. Antigen spread can occur during cell lysis, which releases potential secondary antigens that can be taken up and cross-presented by antigen presenting cells [75]. Interestingly, antigen spread has been observed in the clinic and is associated with improved prognosis [76]. In one clinical study, patients with stage III-IV melanoma were treated with autologous dendritic cells pulsed with an immunodominant epitope (MART-1 27-35). While MART-1-specific immunity did not correlate with clinical outcome, the only patient with a complete response developed immunity against other melanoma epitopes that were not included in the vaccine [77]. Antigen spread has been observed in several other pre-clinical and clinical studies

[78–81], however it is unclear if antigen spread can be deliberately induced through therapeutic intervention. One group demonstrated that radiation therapy can cause an expansion of the T-cell repertoire in a murine melanoma model, which allowed for improved survival when combined with ICB [82]. As previously mentioned, antigen spread likely occurs because of cell lysis in the TME, which suggests that immunogenic forms of cell death could be used to potentiate antigen spread, however more research is needed to support this theory.

Table 2.1 Therapeutic strategies to combat tumor heterogeneity. ICB = Immune checkpoint blockade, OV = oncolytic virus, ACT = adoptive T cell transfer, OVV = oncolytic virus vaccine, HDACi = class I histone deacetylase inhibitor, CDK7 = cyclin-dependent kinase 7, RCC = renal cell carcinoma.

Approach	Rationale	Examples
Combination Therapies	Using a multi-pronged approach to target multiple pathways simultaneously, preventing selection of resistant populations	1. Chemotherapy + ICB improves progression-free survival in patients [58,61,62,83] 2. Chemotherapy + OV improved therapeutic efficacy [63–65] 3. Chemotherapy + cell-based therapies [66–68]

		4. ACT + OVV + HDACi reprogrammed immunosuppressive myeloid cells, eliminating antigen-negative tumor cells in mice [60]
		5. CDK7 + ICB enhanced antitumor immunity and prolonged survival outcomes in mice [69]
Multi-Peptide Vaccines	Designed to create a response against several tumor antigens simultaneously	1. Multi-antigen vaccine in RCC patients increased the breadth of the immune response and resulted in better disease control [72]
		2. Immunogenic neoantigens were first identified and then used to design multi-antigen vaccines, improving therapeutic outcomes [73,74]

Antigen Spread	Intended to enhance the T-cell repertoire, allowing for expansion of the immune response from a dominant antigen to secondary antigens	1. Autologous DCs pulsed with an immunodominant epitope resulted in antigen spread in one patient, resulting in a complete response to treatment [77] 2. Radiation therapy can expand T-cell repertoire, allowing for improved survival when combined with ICB [82]

2.6. Conclusions and future perspectives

The phenomenon of tumor heterogeneity and clonal evolution in cancers has long been identified as a major driver of tumor development, metastasis and acquired resistance mechanisms. The availability of next-generation sequencing and advances in the field of bioinformatics have enabled clinicians to assess this previously elusive phenomenon in real-time clinical settings. However, as we uncover new ways of detecting and monitoring heterogeneity, we must also in parallel assess the ways that various forms of heterogeneity contribute to clinical outcomes across the full spectrum of cancer types and therapeutic modalities.

In order to fully understand the natural progression of tumor heterogeneity and the clinical implications associated with various forms of therapy, it is important that clinical trial design incorporates ways of assessing heterogeneity into newly developed studies. For high level reproducibility in clinical research and diagnostics, it will be necessary to

establish streamlined, standardized analytical methods. For example, liquid biopsies for plasma DNA analysis are currently recognized as the best method to study recurrent tumors that are refractory to therapy, and widespread clinical use of this technique could be highly beneficial to the field. Indeed, it has already been suggested that at least two types of methodological approaches should be considered to assess clinical heterogeneity [84]. Stanta and colleagues have reasonably suggested that surgically treated tumors should undergo a thorough analysis of tissues to drive the appropriately selected adjuvant therapy, and in recurrent cancer, follow-up should consider the inclusion of blood analysis of ctDNA. Another example is the use of radiomics, which is an emerging field of medical image analysis that utilizes radiological images to predict patient outcomes. It has been proposed that radiomics could be used to quantify tumor heterogeneity [85,86]. Since radiological images are frequently taken for diagnostic purposes, the ability to track tumor heterogeneity throughout tumor progression would be extremely valuable for furthering our understanding of tumor heterogeneity. Careful consideration of inclusion of such techniques into evolving clinical practice will further increase the wealth of data available to researchers and clinicians in an effort to identify correlative changes.

As the field of immunotherapy continues to progress and immunotherapies solidify their place as a true pillar of cancer therapy, the clinical hurdle of tumor heterogeneity so too moves to the forefront of oncologic research. As we study the immunological synapse associated with immunotherapy outcomes and in particular, patients who are refractory to treatment with immunotherapy, we need to simultaneously assess the dynamic shifts of the TME and heterogeneity levels. Incorporating innovative, high-level techniques, we can

move cancer treatment into the realm of personalized medicine and monitor patients and their response to therapy in real-time clinical settings. Understanding the evolutionary drivers for heterogeneity will be key in mapping out primary tumorigenesis, metastatic formation, and relapsed disease as we continue to work towards improving outcomes and quality of life for patients affected by cancer.

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Conflicts of Interest

The authors declare no conflict of interest.

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Chapter 3 : Combination of low-dose chemotherapy and oncolytic viro-therapy sensitize MC38 tumors to immune checkpoint inhibitor therapy in a cDC1-dependant manner.

Preface

This is an author-produced version of an article submitted for peer review in the *International Journal of Molecular Sciences*. Authors: Nader El-Sayes, Alyssa Vito, Omar Salem, Sam Workenhe, Yonghong Wan, Karen Mossman.

NES conceived and designed the project, acquired, and analyzed data, and wrote the manuscript. AV and OS acquired data. SW supervised the study and acquired data. YW supervised the study and reviewed the manuscript. KM supervised the study and revised the manuscript.

This manuscript explores the efficacy of combination therapies in murine models of mismatch repair deficient colorectal cancer (dMMR CRC). We demonstrate that the combination of low dose mitomycin C (mito) and oncolytic HSV (oHSV) can further sen-sitize dMMR CRC to immune checkpoint therapy. Furthermore, we show that mito + oHSV can induce the upregulation of gene signatures associated with the recruitment and activation of myeloid subsets. We found that mito + oHSV led to increased tumor infiltra-tion of several myeloid subsets, including type 1 conventional DCs (cDC1s). Finally, we demonstrate that cDC1s are required for enabling a response to immune checkpoint therapy in this model.

Title: Combination of low-dose chemotherapy and oncolytic virotherapy sensitize MC38 tumors to immune checkpoint inhibitor therapy in a cDC1-dependant manner.

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Abstract

Immune checkpoint therapy has shown great promise in cancers with high mutational burden, such as mismatch repair-deficiency colorectal carcinoma (dMMR CRC). However, most patients still fail to respond to immune checkpoint therapy. Using a mouse model of dMMR CRC, we demonstrated that tumors can be further sensitized to immune checkpoint therapy by using a combination of low-dose chemotherapy and oncolytic HSV-1. This combination induced the infiltration of $CD8^+$ and $CD4^+$ T cells into the tumor. Additionally, the combination induced the upregulation of gene signatures associated with the chemoattraction of myeloid subsets. When combined with immune checkpoint therapy, the combination promoted the infiltration of activated cDC1s into the tumor. Furthermore, we found our combination strategy is dependent on cDC1s, and therapeutic efficacy was abrogated in cDC1-deficient $Batf3^{-/-}$ mice. Thus, we demonstrate that the adjuvanticity of dMMR CRCs can be improved by combining low-dose chemotherapy and oncolytic HSV-1 in a cDC1-dependant manner.

3.1 Introduction

Colorectal cancer (CRC) accounts for roughly 10% of cancer-related deaths worldwide [1]. Even with early detection, 25-50% of patients with early-stage CRC develop metastatic disease [2]. Immune checkpoint inhibitors (ICIs) have shown tremendous promise in solid tumors with high mutational burdens, such as melanoma and lung cancer. To this end, mutational burden has been utilized as a biomarker for ICI therapy for multiple types of cancer [3,4]. CRC can be classified into two groups based on deficiencies in mismatch repair and microsatellite instability. Mismatch repair-deficient (dMMR) tumors have higher mutational burden, making them good candidates for ICI therapy. Indeed, ICI therapy has shown great promise in dMMR CRC with close to 40% of patients demonstrating an overall response to pembrolizumab [5]. While this level of response is promising, most patients with dMMR CRC do not respond to ICI therapy. Therefore, additional studies are required to identify factors in the tumor microenvironment (TME) that can enable a response to ICI therapy in dMMR CRCs.

While mutational burden and antigenicity are potential biomarkers for ICI therapy, several other factors can influence response to therapy. Namely, adjuvanticity is required to promote infiltration and activation of immune cells at the tumor site. Immunogenic cell death (ICD) can improve adjuvanticity through the release of DAMPs and other danger signals [6]. To increase adjuvanticity and sensitize tumors to ICI therapy, several combination strategies have been developed to induce ICD [7,8]. These include the combination of ICI therapy with clinically relevant chemotherapies and emerging therapies such as oncolytic virus (OV) therapy [9–11]. Our group has previously utilized combinations of

oncolytic HSV-1 (oHSV) and low-dose chemotherapies to sensitize breast adenocarcinomas to ICI therapy [12,13]. We further demonstrated that low dose mitomycin C (mito) combined with oHSV improves susceptibility of tumors to ICI therapy through induction of necroptosis [13]. While these combinations are effective in sensitizing tumors to ICI therapy, there is a fundamental lack of understanding of the required changes in the tumor microenvironment that improve adjuvanticity and drive therapeutic outcome.

In this study, we investigated the combination of mito + oHSV in MC38 tumors, a murine model of dMMR CRC with high microsatellite instability. We find that while mice harboring MC38 tumors moderately respond to ICI therapy, they do not maintain durable responses. Addition of mito + oHSV was successful in further sensitizing tumors to ICI therapy, resulting in durable responses in 55% of mice. The combination of mito + oHSV + ICI induces inflammation at the TME and promotes the recruitment of myeloid subsets. In particular, type 1 conventional DCs (cDC1s) showed high levels of tumor infiltration after treatment. Finally, we demonstrated that therapeutic efficacy driven by mito + oHSV + ICI is dependent on the presence of cDC1s. Altogether, these data show that therapeutic outcomes to ICI therapy can be further improved in dMMR CRC with the addition of combinations that improves tumor adjuvanticity. Furthermore, we demonstrate that enabling a response to ICI therapy in this model is dependent on the cDC1 subset. This is in line with other reports showing a crucial role of cDC1s in enabling a therapeutic response to ICI therapy [14,15].

3.2 Results

3.2.1 Combination of low-dose mitomycin C and oncolytic HSV-1 sensitize colon adeno-carcinoma tumors to ICI therapy

To assess the efficacy of ICIs (anti-PD-1 and anti-CTLA-4 monoclonal antibodies) in a murine model of dMMR CRC, C57BL/6 mice bearing MC38 tumors were treated with 250μg of anti-PD-1 and anti-CTLA-4 monoclonal antibodies every 3 days for a total of 8 doses while monitoring tumor growth (**Fig. 3.1A**). Tumor-bearing mice demonstrated a partial response to ICI therapy, with delayed tumor progression and prolonged survival (**Fig. 3.1B and 3.1C**). However, none of the mice demonstrated a durable response to ICI therapy. This finding is consistent with other reports that show a moderate response to ICI therapy in MC38 tumors [16–18]. To increase the immunogenicity of the MC38 tumors and sensitize the tumors further to ICI therapy, we treated mice with a combination of low dose mito and oHSV. MC38 tumor-bearing mice were treated with a therapeutic regimen consisting of mito, oHSV, and/or ICI (**Fig. 3.2A**). The combination of mito and oHSV showed no delay in tumor progression or survival benefit in the MC38 tumor model (**Fig. S1 and 3.2B**) . Similarly, the combination of mito + ICI and oHSV + ICI demonstrated no improvement over ICI therapy, however the full combination of mito + oHSV + ICI resulted in initial tumor regression in 100% of mice and a durable response in ~55% of mice (**Fig. 3.2B and 3.2C**). To assess the presence of persistent memory against MC38 tumors, mice that developed a complete response were rechallenged with MC38 CRC or E0771 breast carcinoma cells. 100% of the mice rejected MC38 rechallenge, whereas all the mice challenged with E0771 developed tumors within 15 days (**Fig. 3.2D**). These data suggest

that mito + oHSV therapy is not sufficient for therapeutic efficacy in MC38 tumors but can enable a durable tumor-specific response to ICI therapy.

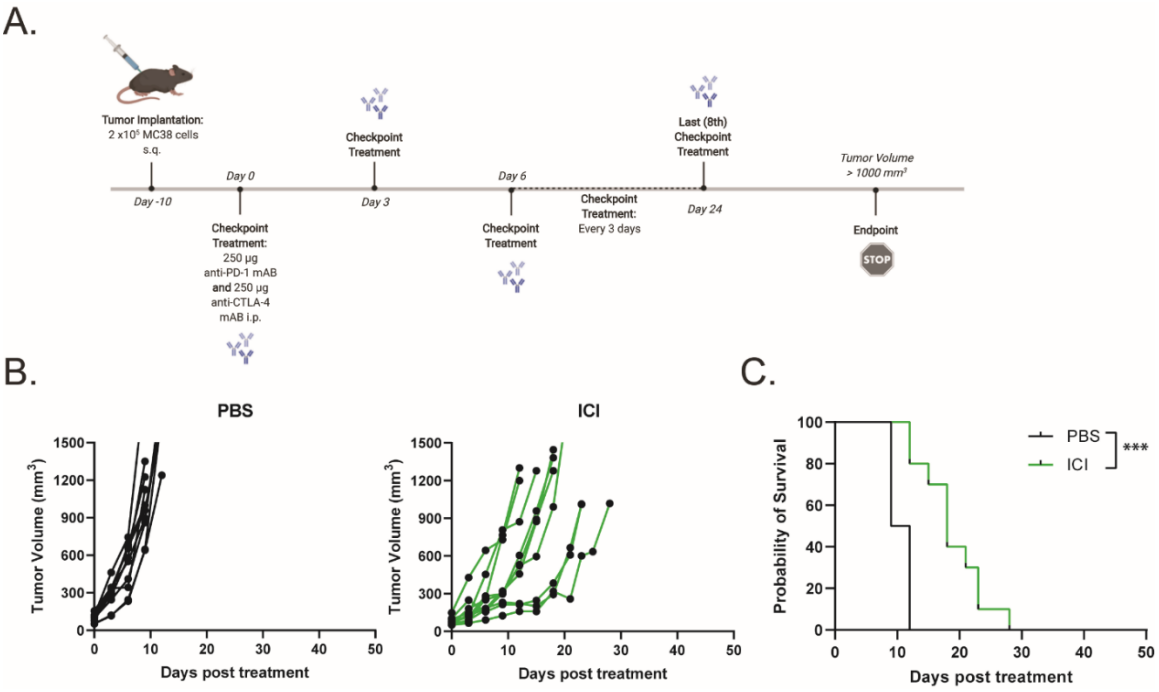

Figure 3.1. Immune checkpoint inhibitor therapy exhibits moderate response in MC38 tumors. **(A)** Schematic representation of the ICI treatment regimen. **(B)** Tumor growth kinetics and **(C)** Kaplan-Meier survival curves from MC38 tumor-bearing mice treated with saline or ICI.

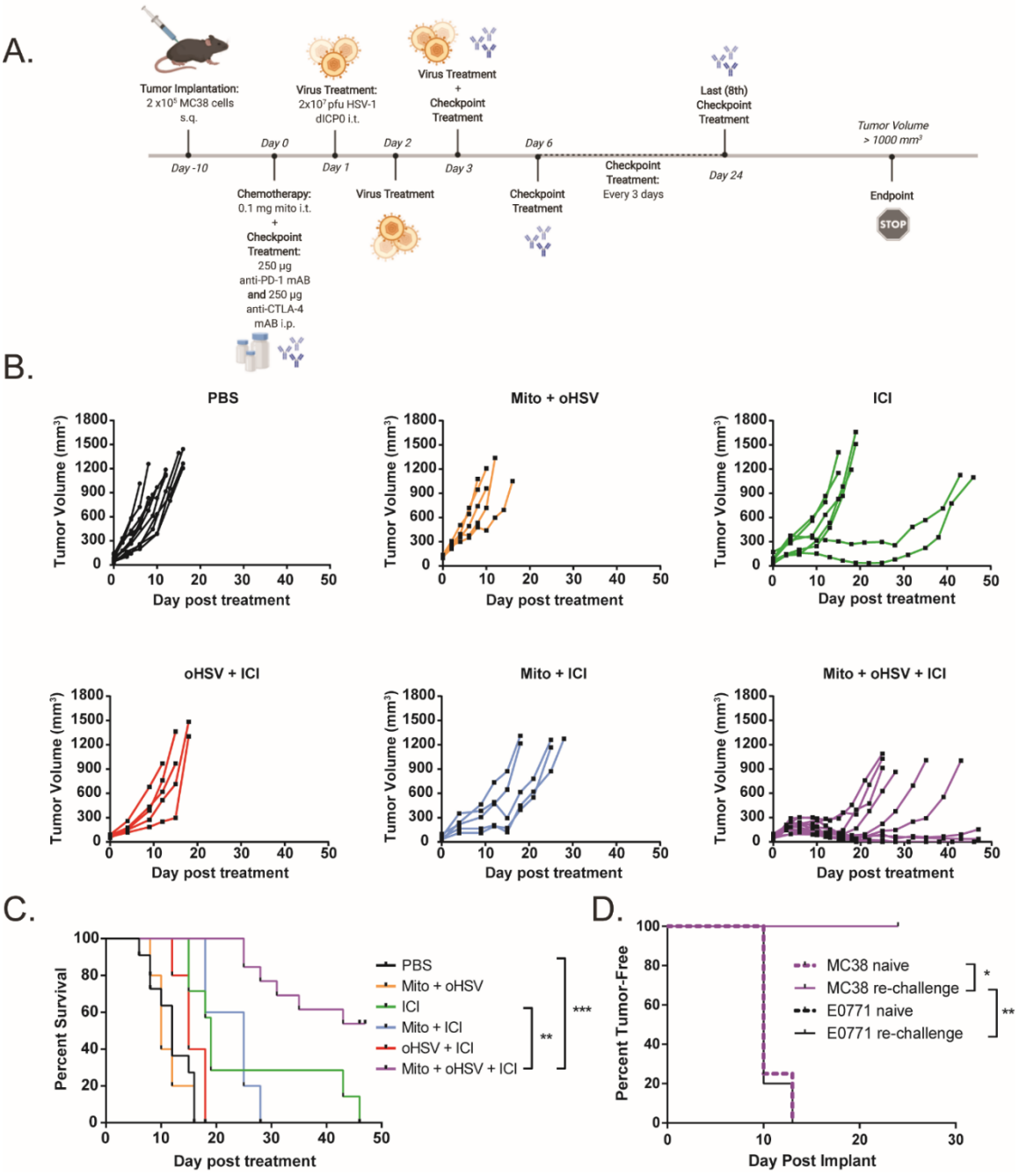

Figure 3.2. Combination of mitomycin and oHSV sensitize MC38 tumors to ICI therapy. **(A)** Schematic representation of the combination treatment regimen. **(B)** Tumor growth kinetics and **(C)** Kaplan-Meier survival curves from MC38 tumor-bearing mice treated with different combinations of mito, oHSV and/or ICI. **(D)** Mice that had a complete

response to mito + oHSV + ICI treatment were re-challenged with either MC38 or E0771 tumors and the percent of tumor-free mice was graphed as a Kaplan Meier curve.

3.2.2 Mito + oHSV + ICI induces tumor infiltration of T cells and is dependent on T cells for tumor control

We have previously shown that the combination of mito + oHSV can induce $CD8^+$ T cell tumor infiltration in breast adenocarcinoma [13]. Given that mito + oHSV fails to control tumor growth in the absence of ICIs in MC38 tumors, we investigated the level of T cell infiltration across treatment groups. Surprisingly, we found that mito + oHSV did not improve infiltration of T cells into the tumor. However, the addition of ICIs significantly improved infiltration of $CD8^+$ and $CD4^+$ T cells, despite the inability of ICI alone to induce T cell infiltration (**Fig. 3.3A and 3.3B)**. To further characterize the importance of T cells in mediating a therapeutic response to mito + oHSV + ICI, T cells were depleted using anti-CD8 and anti-CD4 monoclonal antibodies. Depletion of $CD8^+$ and $CD4^+$ T cells was confirmed by flow cytometry (**Fig. S2)**. We found that depletion of either $CD8^+$ or $CD4^+$ T cells abrogated tumor control and survival benefit mediated by mito + oHSV + ICI therapy (**Fig. 3.3C – F)**. This outcome is consistent with our previous findings in breast adenocarcinoma models [13].

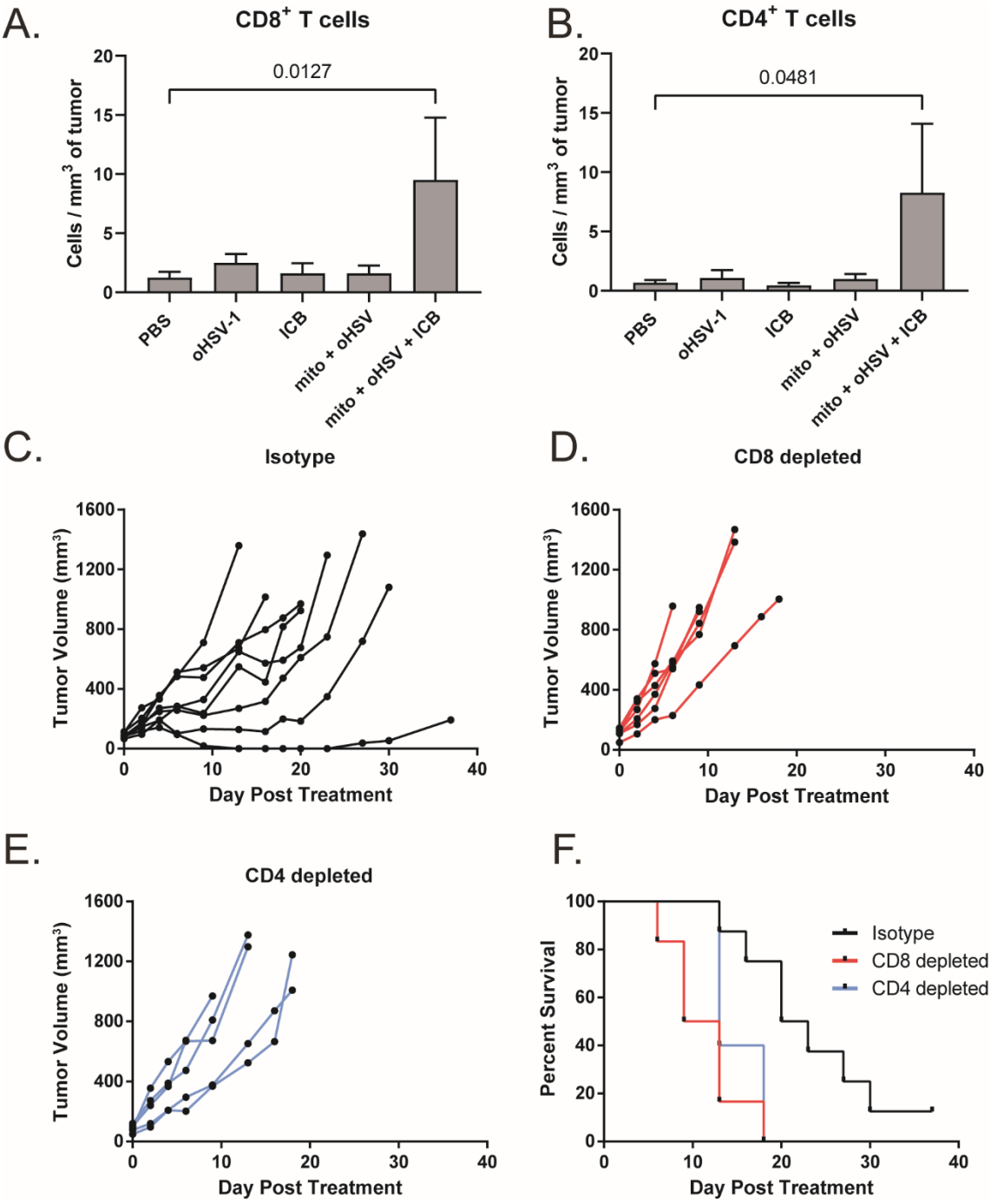

Figure 3.3. Efficacy of mito + oHSV + ICI combination is dependent on $CD8^+$ and $CD4^+$ T cells. **(A-B)** Mice bearing MC38 tumors were treated with mito, oHSV and/or ICI. Tumors were harvested 7 days after the final day of treatment and the infiltration of CD8 and

CD T cells was assessed by flow cytometry. **(C-E)** Tumor growth kinetics from tumor-bearing mice that were treated with mito + oHSV + ICI before administration of CD8 and CD4 depletion antibodies. **(F)** Kaplan-Meier survival curves from anti-CD8, anti-CD4 or isotype antibody treated mice.

3.2.3 Mito + oHSV combination induces transcriptome signature associated with myeloid cell recruitment and activation

The combination of mito + oHSV can enable a durable response to ICI therapy in MC38 tumors, however the combination is insufficient to generate even a moderate response in the absence of ICIs. To better characterize relevant changes in the tumor microenvironment that can enable a durable response to ICI therapy, we compared changes in the transcriptomes of treated mice. To this end, RNA was harvested from tumors that were treated with mito, oHSV, and/or ICI for analysis using a Clariom S assay. Principal component analysis shows that all groups involving treatment with mito clustered together, despite a lack of therapeutic efficacy with mito monotherapy or mito + ICI combination therapy (**Fig. 3.4A**). Pathway enrichment analysis identified several pathways that were upregulated in mito + oHSV + ICI groups compared to PBS controls (**Fig. 3.4B**). Of particular interest were pathways associated with chemokine signaling, inflammatory response, type II interferon signaling, and toll-like receptor signaling. Further in-depth analysis revealed upregulation of genes associated with the recruitment, maturation, and activation of myeloid subsets (**Table 1, Fig. 3.4C and 3.4D**). The same gene signature was upregulated in mito + oHSV groups relative to PBS controls (**Table 1, Fig. S3**). Furthermore, several of these signaling pathways and genes were upregulated in mito + oHSV +

ICI groups compared to ICI alone (**Table 1, Fig. 3.4E and 3.4F)**. In particular, mito + oHSV + ICI induced an upregulation of genes involved in DC recruitment, activation and antigen-presentation compared to ICI alone. These data suggest that the combination of mito + oHSV induce tumor infiltration and activation of DCs and other myeloid subsets, and that this phenomenon might be involved in sensitizing MC38 tumors to ICI therapy.

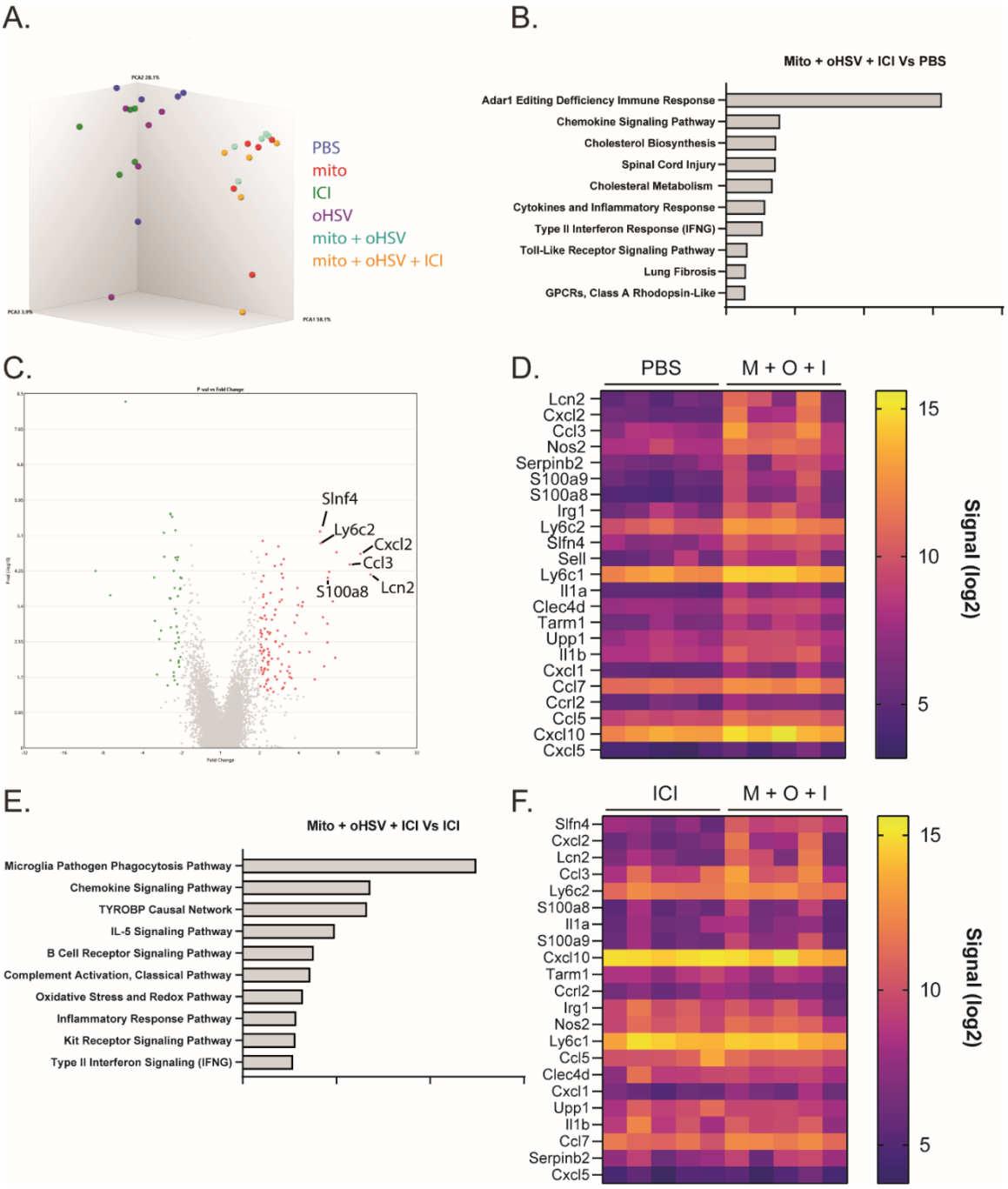

Figure 3.4. mito + oHSV + ICI induces RNA transcriptomes associated with recruitment and activation of myeloid subsets. Mice harboring MC38 tumors were treated with different combinations of mito, oHSV and/or ICI. RNA was harvested from the

tumors one day after the final treatment and sent for analysis by Clariom S assay. **(A)** 3-D cluster plot showing the RNA expression correlations between the different groups. **(B)** Pathway enrichment analysis showing the top ten signaling pathways differentially expressed between mito + oHSV + ICI compared to PBS control. **(C)** Volcano plot and **(D)** heat map showing genes differentially expressed between mito (M) + oHSV (O) + ICI (I) compared to PBS control. **(E)** Pathway enrichment analysis showing the top ten signaling pathways differentially expressed between mito + oHSV + ICI compared to ICI. **(F)** heat map showing genes differentially expressed between mito + oHSV + ICI compared to ICI.

Table 3.1. Differentially expressed genes associated with myeloid subset recruitment and activation. M = mito, O = oHSV, I = ICI.

Gene Symbol	M + O + I vs PBS	M + O vs PBS	M + O + I Vs I	Function
Lcn2	14.18	11.69	5.62	Expressed by DCs and contributes to CD8 T cell priming [19].
Cxcl2	11.77	23.53	4.99	Expressed by activated DCs [20]. Involved in chemoattraction of neutrophils [21].
Ccl3	9.78	16.41	3.09	Enhances recruitment of cDC1s and T cells to the tumor. Enhances priming and proliferation of antitumor T cells [22].
Nos2	7.73	4.83	1.76	Expressed by activated DCs [23]. Expressed by M1 macrophages [24].
Serpinb2	7.55	3.04	3.22	Expressed by conventional DCs and macrophages [25].
S100a9	6.76	5.87	3.73	Expressed by DCs, neutrophils, and macrophages [26]. Promotes inflammation through TLR4 and RAGE signaling [27].
S100a8	6.62	7.54	2.81	Expressed by DCs, neutrophils, and macrophages. Promotes inflammation through TLR4 and RAGE signaling.

Irg1	6.6	3.57	-1.57	Marker of myeloid cells [28].
Ly6c2	5.81	5.08	1.45	Marker of myeloid cells [29].
Slfn4	5.8	7.46	4.18	Involved in differentiation of myeloid cells [30].
Sell	5.24	5.23	2.58	Regulator of leukocyte adhesion [31].
Ly6c1	4.26	2.66	1.31	Marker of myeloid cells [29].
Il1a	4.21	4.15	1.79	Involved in DC activation and facilitates T cell priming [32].
Clec4d	3.85	3.54	1.19	Expressed by neutrophils and monocytes [33].
Tarm1	3.69	1.91	-1.36	Expressed by DCs, neutrophils, and macrophages. Enhances secretion of proinflammatory cytokines [34].
Upp1	2.99	2.08	-1.32	Associated with antigen-presenting myeloid cells [35].
Il1b	2.96	2.24	1.69	Involved in DC activation and facilitates T cell priming [32].
Cxcl1	2.92	1.84	1.77	Involved in neutrophil chemoattraction [36].
Ccl7	2.74	1.88	2.32	Involved in chemoattraction of immune cells [37].
Ccrl2	2.63	2.53	-1.06	Expressed by neutrophils [38].
Ccl5	2.47	3.11	1.42	Involved in chemoattraction of DCs [39].

Cxcl10	2.41	2.18	-1.47	Expressed by cDC1s and induces recruitment of T cells [40].
Cxcl5	2.01	1.38	1.07	Involved in neutrophil chemoattraction [41].

3.2.4 Mito + oHSV induces tumor infiltration of cDC1 subsets and is dependent on Batf3

To characterize myeloid subsets in the tumor, we treated tumor-bearing mice with combinations of mito, oHSV and/or ICI before harvesting tumors on day 4 of treatment. Tumor infiltrates were then characterized by multicolor flow cytometry. We found that several subsets of myeloid cells infiltrated the tumor after treatment with mito + oHSV (**Fig. 3.5A**). Interestingly, treatment with mito induced infiltration of monocytes ($CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$), while treatment with oHSV induced infiltration of neutrophils ($CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$) and DCs ($Ly6C^-$ $CD11c^+$ $MHCII^+$). In all three cases, however, the full combination of mito + oHSV + ICI induced the largest number of tumor infiltrates.

Given the role of DCs in antigen presentation, we further characterized the DC subsets infiltrating the tumor. Priming of antitumor T cells is dependent on cross presentation of tumor antigens by type 1 conventional DCs (cDC1s) [15,42,43]. Interestingly, treatment with mito induced infiltration of cDC1s ($CD8\alpha^+$ DCs) into the tumor, which was further increased with the addition of oHSV (**Fig. 3.5B and 3.5C**). However, treatment with mito + oHSV + ICI induced the greatest level of cDC1 infiltration into the tumor. Additionally, treatment with mito + oHSV + ICI induced the highest level of activated cDC1 infiltration characterized by their expression of CD40 (**Fig. 3.5D**). In contrast, type 2 conventional DC (cDC2) infiltration was improved with ICI therapy, but not with the full combination of

mito + oHSV + ICI (**Fig. S4)**. Infiltration of monocyte-derived DCs (moDCs) is also improved in all groups treated with mito or ICI (**Fig. S4**), however the role of moDCs in cancer immunotherapy is still under debate [44,45]. To establish the relevance of cDC1 tumor infiltration in enabling a therapeutic response to ICI therapy, we used Batf3$^{-/-}$ mice which are deficient for cDC1s [46]. We found that treatment of tumor-bearing Batf3$^{-/-}$ mice with mito + oHSV + ICI was ineffective in controlling tumor growth or prolonging survival (**Fig. 3.5E and 3.5F**). Altogether these data suggest that the recruitment and activation of cDC1s is required for mito + oHSV + ICI-mediated tumor control.

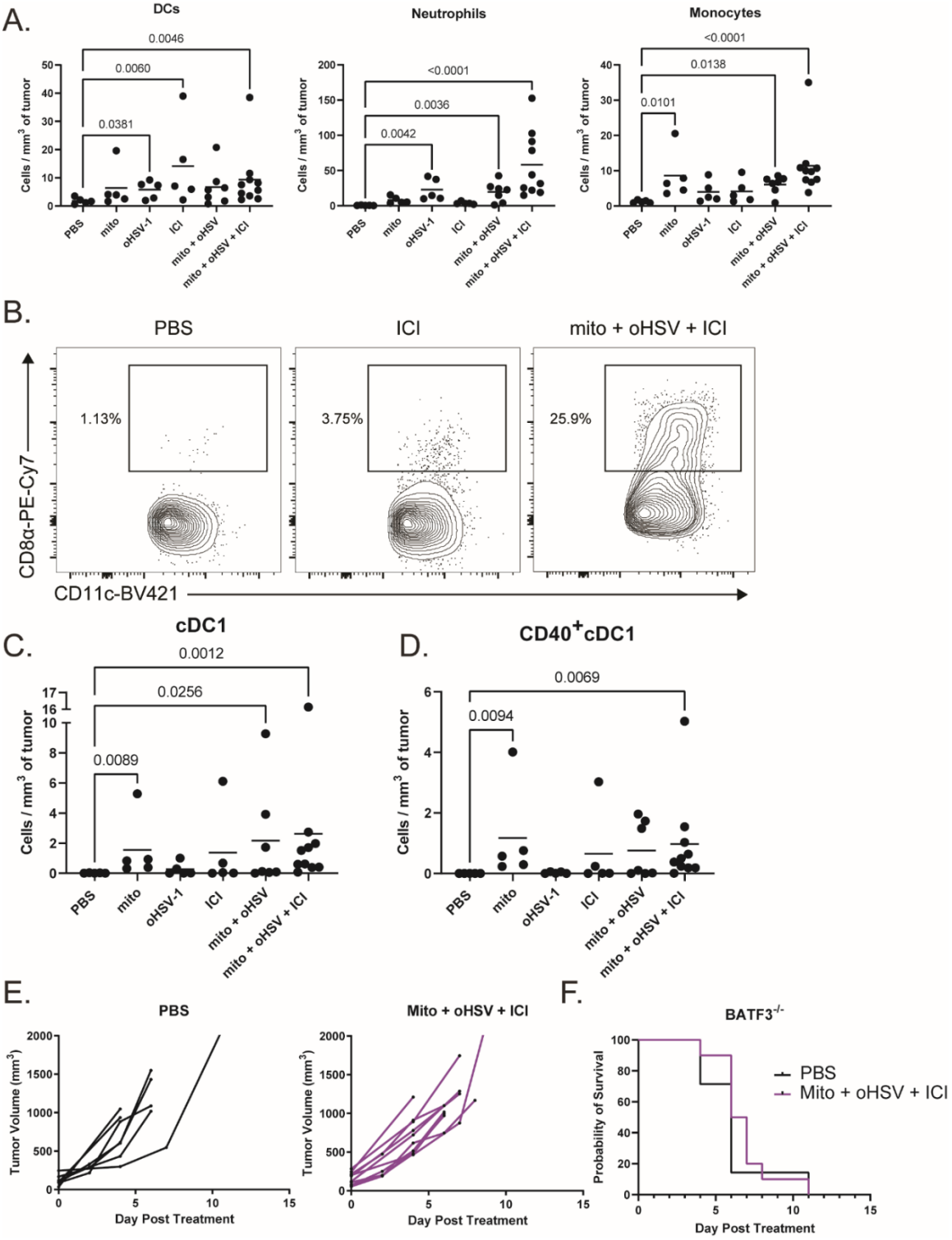
A.
DCs
Neutrophils
Monocytes
Cells / mm3 of tumor
0.0046
0.0060
0.0381
<0.0001
0.0036
0.0042
<0.0001
0.0138
0.0101
PBS
mito
oHSV-1
ICI
mito + oHSV
mito + oHSV + ICI
B.
PBS
ICI
mito + oHSV + ICI
1.13%
3.75%
25.9%
CD8α-PE-Cy7
CD11c-BV421
C.
cDC1
0.0012
0.0256
0.0089
D.
CD40+cDC1
0.0069
0.0094
E.
PBS
Mito + oHSV + ICI
Tumor Volume (mm3)
Day Post Treatment
F.
BATF3-/-
Probability of Survival
PBS
Mito + oHSV + ICI

Figure 3.5. mito + oHSV + ICI promotes tumor infiltration of cDC1s. **(A)** Mice bearing MC38 tumors were treated with different combinations of mito, oHSV and/or ICI. Tumors were harvested 4 days after start of treatment and the frequency of infiltrating immune cells was analyzed by flow cytometry. DCs ($CD11c^+$ $MHCII^+$), neutrophils ($CD11b^+$ $Ly6C^{mid}$ $Ly6G^+$) and monocytes ($CD11b^+$ $Ly6C^{hi}$ $Ly6G^-$) were graphed. **(B)** Representative flow plots of $CD8^+$ DCs (cDC1s). **(C)** graphs of tumor-infiltrating cDC1s and **(D)** $CD40^+$ cDC1s **(E)** $BATF3^{-/-}$ mice harboring MC38 tumors were treated with PBS or mito + oHSV + ICI. Tumor volumes and **(F)** Kaplan-Meier survival curves were graphed.

3.3 Discussion

In this study, we demonstrate that response to ICI therapy can be improved by utilizing a combination of mito + oHSV in a murine model of dMMR CRC. In the absence of ICIs, mito + oHSV fails to control growth or prologue survival of tumor-bearing mice. Although we have previously demonstrated improved infiltration of T cells in murine models of breast adenocarcinoma after treatment with mito + oHSV [13], this combination was ineffective in improving T cell infiltration in CRC tumors in the absence of ICI therapy. This outcome is likely a result of the immunosuppressive nature of MC38 tumors, which have been reported to maintain high levels of PD-L1 expression that contributes to immune evasion [47]. However, addition of ICIs resulted in a significant increase in T cell infiltrates. These data suggest that the combination of mito + oHSV can further improve response to ICI therapy. Indeed, several clinical trials are underway in dMMR CRCs combining chemotherapy with ICIs [2].

Despite having no therapeutic efficacy in MC38 tumors, mito + oHSV induced the upregulation of genes associated with recruitment of myeloid subsets. Furthermore, the combination induced infiltration of DCs, neutrophils and monocytes into the tumor. This observation is in line with our previous work in which we found chemokine signatures associated with myeloid cells in breast adenocarcinoma after treatment with mito + oHSV [13]. Of particular interest was the improved infiltration of cDC1s into MC38 tumors, which are required for priming endogenous tumor-specific T cells and enabling response to ICI therapy [46]. We found that response to mito + oHSV + ICI was abrogated in Batf3$^{-/-}$ deficient for cDC1s. Indeed, other reports have shown that strategies to promote infiltration and activation of cDC1s can sensitize tumors to immunotherapy. For example, one study led by Salmon *et. al.*demonstrated that the combination of FLT3L and poly I:C can improve the expansion and activation of cDC1s, leading to improved priming of antitumor T cells and a better response to ICI therapy [14]. Similarly, FLT3L and poly-ICLC enhanced cDC1-mediated cross priming and synergized with anti-CD137 and anti-PD-1 therapy in MC38 tumors [15]. While mito + oHSV induced infiltration of cDC1s in the absence of ICI therapy, the addition of ICIs resulted in consistently elevated infiltration of activated cDC1s. Interestingly, one report has demonstrated that CD40 expression on DCs is inhibited in MC38 tumors [48]. Furthermore, PD-1 expression on DCs was shown to dampen their activation [49]. These findings suggest that the addition of ICIs can improve the activation of cDC1s, while mito + oHSV improves cDC1 infiltration into the tumor. Indeed, the full combination of mito + oHSV + ICI is the only group that demonstrated significantly improved infiltration of $CD8^+$ and $CD4^+$ T cells. While $CD4^+$ T cells are MHC-II restricted,

cDC1-mediated priming of $CD4^+$ T cells is required for optimal antitumor activity [42]. These reports are in line with our results showing that depletion of $CD4^+$ T cells abrogates tumor control by mito + oHSV + ICI. Future studies should assess the ability of mito + oHSV to improve MHC-I and MHC-II-mediated antigen presentation by cDC1s.

While this study focused on the infiltration of cDC1s, mito + oHSV induced the infiltration of several other myeloid subsets, including neutrophils. The role of tumor-associated neutrophils (TANs) in immunotherapy has been highly controversial. N1 TANs can exert antitumor activity through direct and indirect cytotoxicity, while N2 TANs are widely associated with immunosuppression and metastasis. It is currently unclear whether the induction of TAN infiltration by mito + oHSV + ICI is beneficial, detrimental, or irrelevant for therapeutic efficacy. We also found that ICI therapy can induce the infiltration of cDC2s into the tumor, which is decreased by the addition of mito + oHSV. Future work should assess the potential of mito + oHSV to promote the differentiation of pre-DCs into cDC1s rather than cDC2s.

ICI therapy has demonstrated most success in solid tumors with high mutational burden, such as melanoma, lung cancer, and dMMR CRC [3–5]. However, the majority of patients still fail to respond to ICI therapy, highlighting the need for improvement. Recent preclinical reports suggest that increasing tumor adjuvanticity through ICD-inducing therapies can enable better responses to ICI therapy [6,7]. We show that the combination of low-dose mitomycin C and oncolytic HSV-1 can enable response to ICI therapy in a cDC1-dependant manner in dMMR CRC. We believe that combination therapies that can induce

ICD have the potential for improving tumor adjuvanticity, which can improve therapeutic outcomes in cancers with sufficient antigenicity.

3.4 Material and Methods

Cell Lines

MC38 cells (ATCC) were maintained in Dulbecco's modified Eagle's media (DMEM) supplemented with 5% fetal bovine serum (FBS, ATCC 30-2020) 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco).

Virus propagation

HSV-1Δ810 (oHSV) is an oncolytic attenuated variant of the HSV-1 with a deletion in the ICP0 region. The virus was propagated, purified, and quantified on U2OS cells as described previously [50].

In vivo experiments

Mice were maintained at the McMaster University Central Animal Facility and all the procedures were performed in full compliance with the Canadian Council on Animal Care and approved by the Animal Research Ethics Board of McMaster University. MC38 tumors: 2 x 10^5 cells were implanted subcutaneously into the left flank of 6–8-week-old female C57/Bl6 mice (Charles River Laboratories, Wilmington, MA). On the first day of treatment 0.1 mg of Mitomycin C (Sigma-Aldrich), 250µg of αPD-1 and αCTLA-4 (InVivoMab) antibodies were administered by i.t. and i.p. injections respectively. For the following 3 days, 2 x 10^7 pfu of oHSV was administered by i.t. injection (total of 3 doses). Experimental groups receiving αPD-1/CTLA-4 followed a dosing schedule of 250µg treatments every 3

days for a total of 8 doses. Tumor volumes were monitored and measured every 2-3 days until they reached their endpoint volume (1000 mm^3).

Immune analysis and flow cytometry

Tumors were harvested on days 4 and 7 of treatment before being processed. The tumors were diced into fine pieces then subject to digestion using Liberase (Sigma-Aldrich) as described in the manufacturer's protocol. The digested tumors were then passed through a 100µm cell strainer. Red blood cells were lysed with ACK buffer, and the remaining cells were transferred to a round-bottom 96-well plate. The cell suspensions were stained with fixable viability stain 510 (BD Biosciences) for 30 min at room temperature then treated with anti-CD16/CD32 (Fc block; BD Biosciences) for 15 min at 4 degrees. Cell surface staining was done for 30 min at 4 degrees. Intracellular staining was done using cytofix/cytoperm Fixation/Permeabilization kit (BD Biosciences). Data acquisition was done on the LSRFortessa (BD), and data were analyzed using FlowJo.

Clariom S Assay

Tumors were harvested one day after the final treatment and homogenized in trizol. RNA was extracted using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's protocol. RNA was reverse transcribed, and cDNA was purified via magnetic beads and fragmented using UDG. Fragmented cDNA was then hybridized to the Affymetrix Clariom S mouse arrays (Thermo Fisher Scientific) and the stained arrays were scanned to generate intensity data. Raw data was analyzed using the Thermo Fisher Transcriptome Analysis Console software.

Statistical Analysis

Results are presented as means ± standard deviation. Log-rank (Mantel-Cox) tests were used to analyze the statistical significance between treatment groups for Kaplan–Meier survival graphs. Ordinary one-way ANOVA was used to determine the statistical significance between means of treated groups according to the normality of their distributions. In all cases the null hypothesis was rejected when p values < 0.05. All statistical analysis was performed using GraphPad Prism 9.

3.5 Supplementary

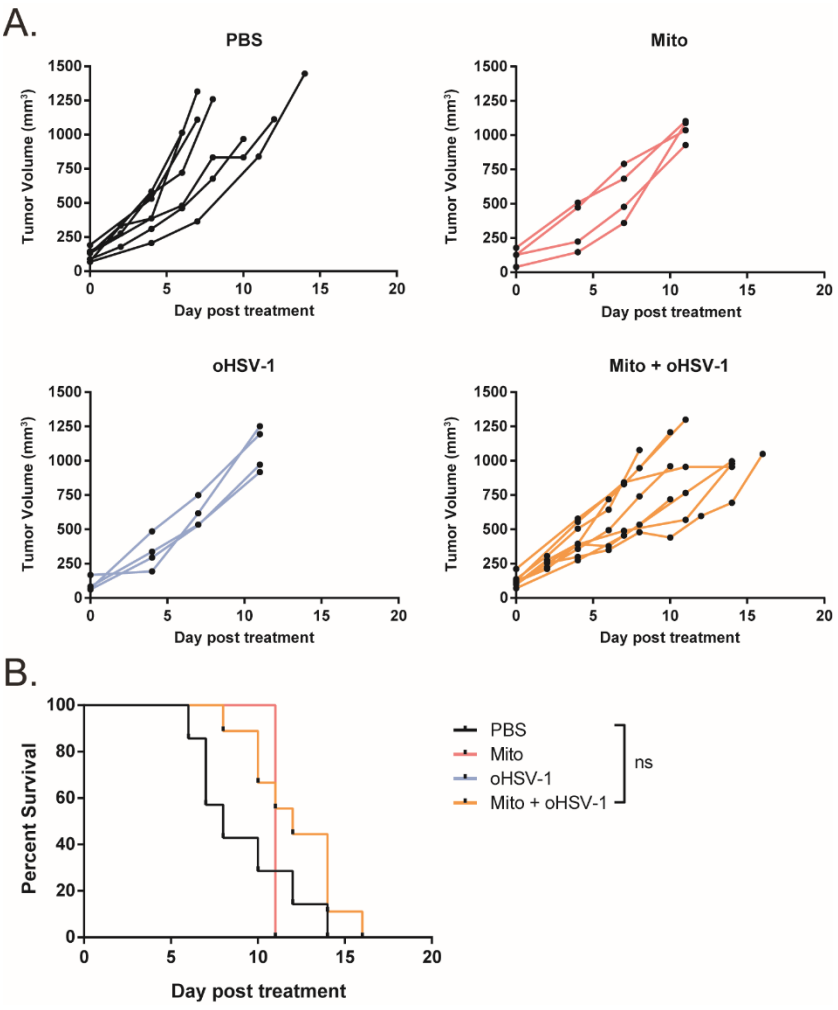

Figure S1. Combination of mito and oHSV fails to control MC38 tumors. **(A)** Tumor growth kinetics and **(B)** Kaplan-Meier survival curves from mice bearing MC38 tumors treated with different combinations of mito and oHSV monotherapy and combination therapy.

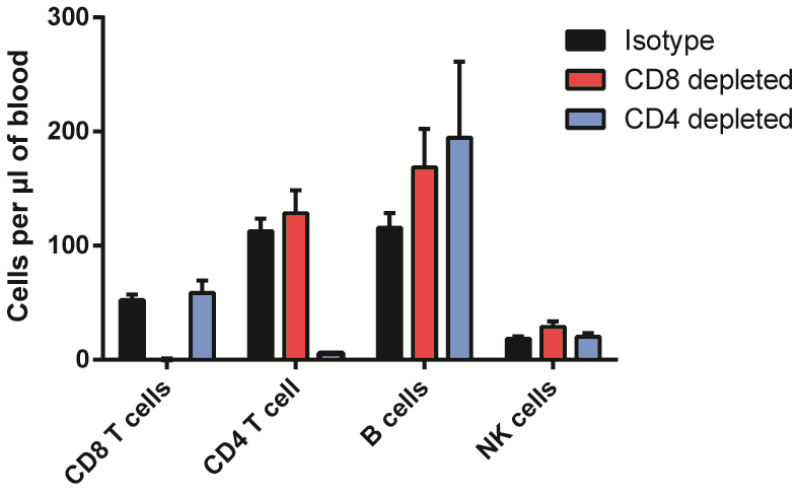

Figure S2. Depletion of T cells with monoclonal antibodies**.** Anti-CD8 or anti-CD4 monoclonal antibodies were administered to tumor-bearing mice by i.p. Number of CD8 T cells, CD4 T cells, B cells and NK cells in circulation were assessed by flow cytometry.

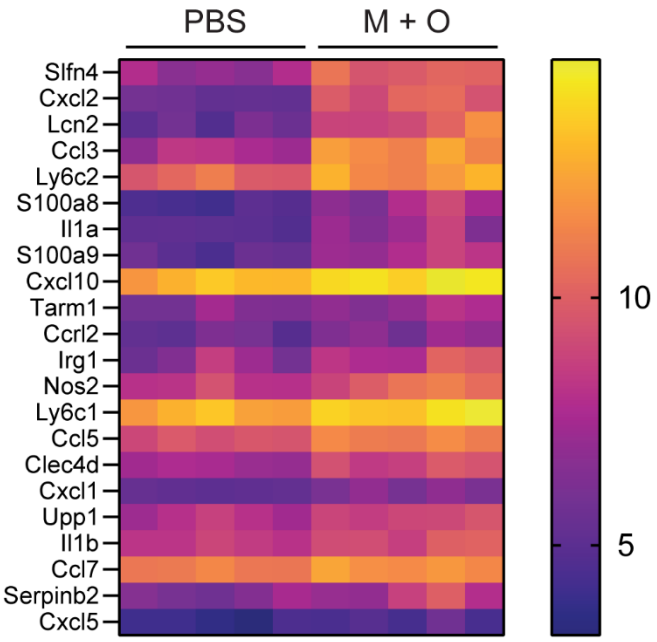

Figure S3. mito + oHSV induces RNA transcriptomes associated with recruitment and activation of myeloid subsets. Mice harboring MC38 tumors were treated with different combinations of mito, oHSV and/or ICI. RNA was harvested from the tumors one day after the final treatment and sent for analysis by Clariom S assay. The heat map shows genes differentially expressed between mito + oHSV compared to PBS control.

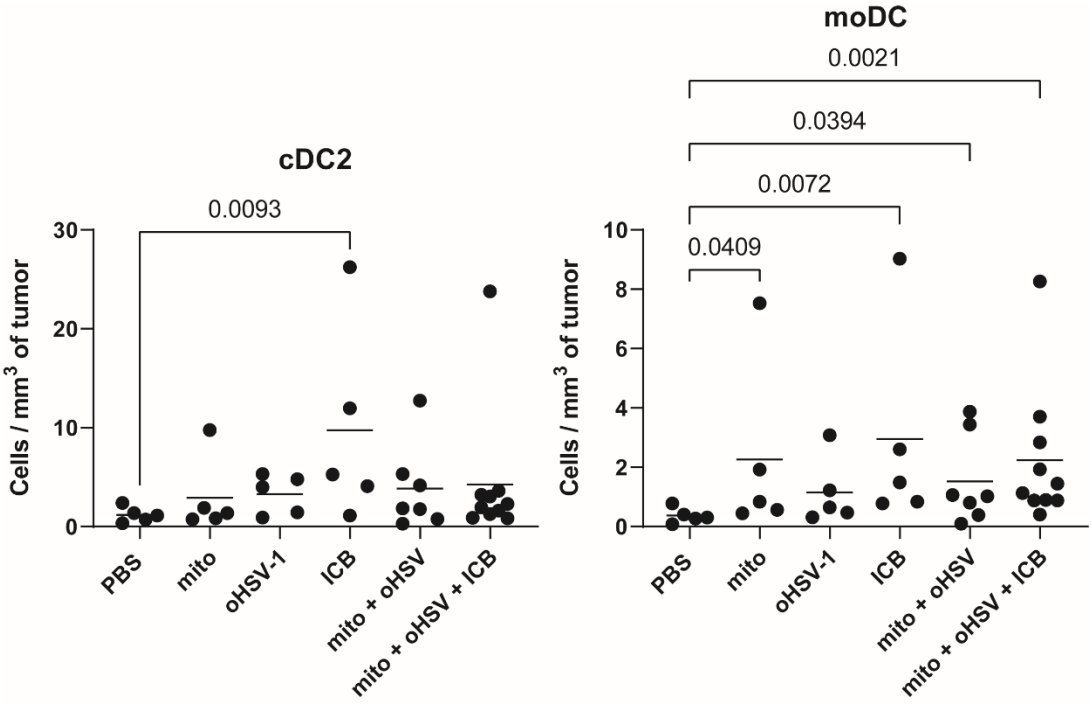

Figure S4. Tumor infiltration of cDC2s and moDCs. Mice bearing MC38 tumors were treated with different combinations of mito, oHSV and/or ICI. Tumors were harvested 4 days after start of treatment and the frequency of infiltrating immune cells was analyzed by flow cytometry. cDC2s ($CD11c^+$ $MHCII^+$ $CD11b^+$ $Ly6C^-$) and moDCs ($CD11c^+$ $MHCII^+$ $CD11b^+$ $Ly6C^+$) were graphed.

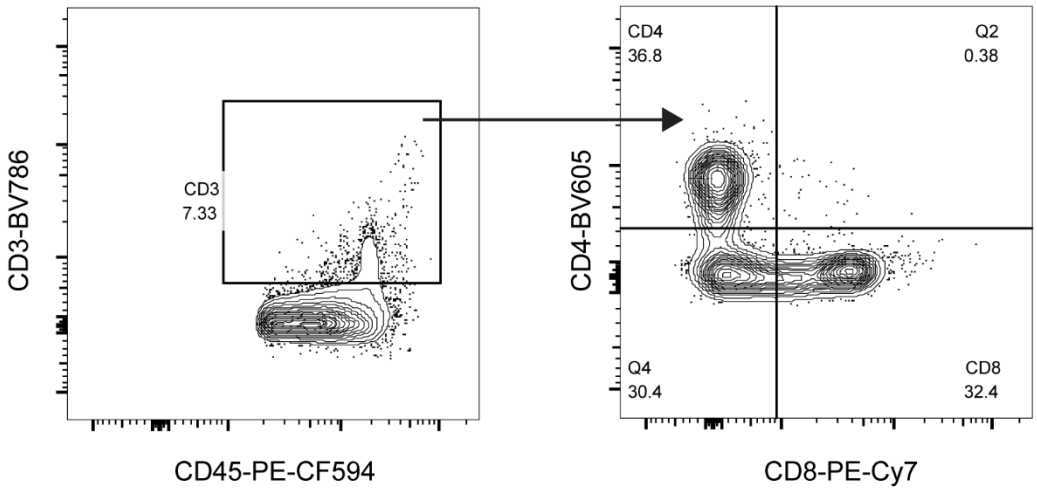

Figure S5. T cell gating strategy. Mice bearing MC38 tumors were treated with different combinations of mito, oHSV and/or ICI. Tumors were harvested 7 days after start of treatment and the frequency of infiltrating immune cells was analyzed by flow cytometry. Cells were gated on viable $CD45^{+}$.

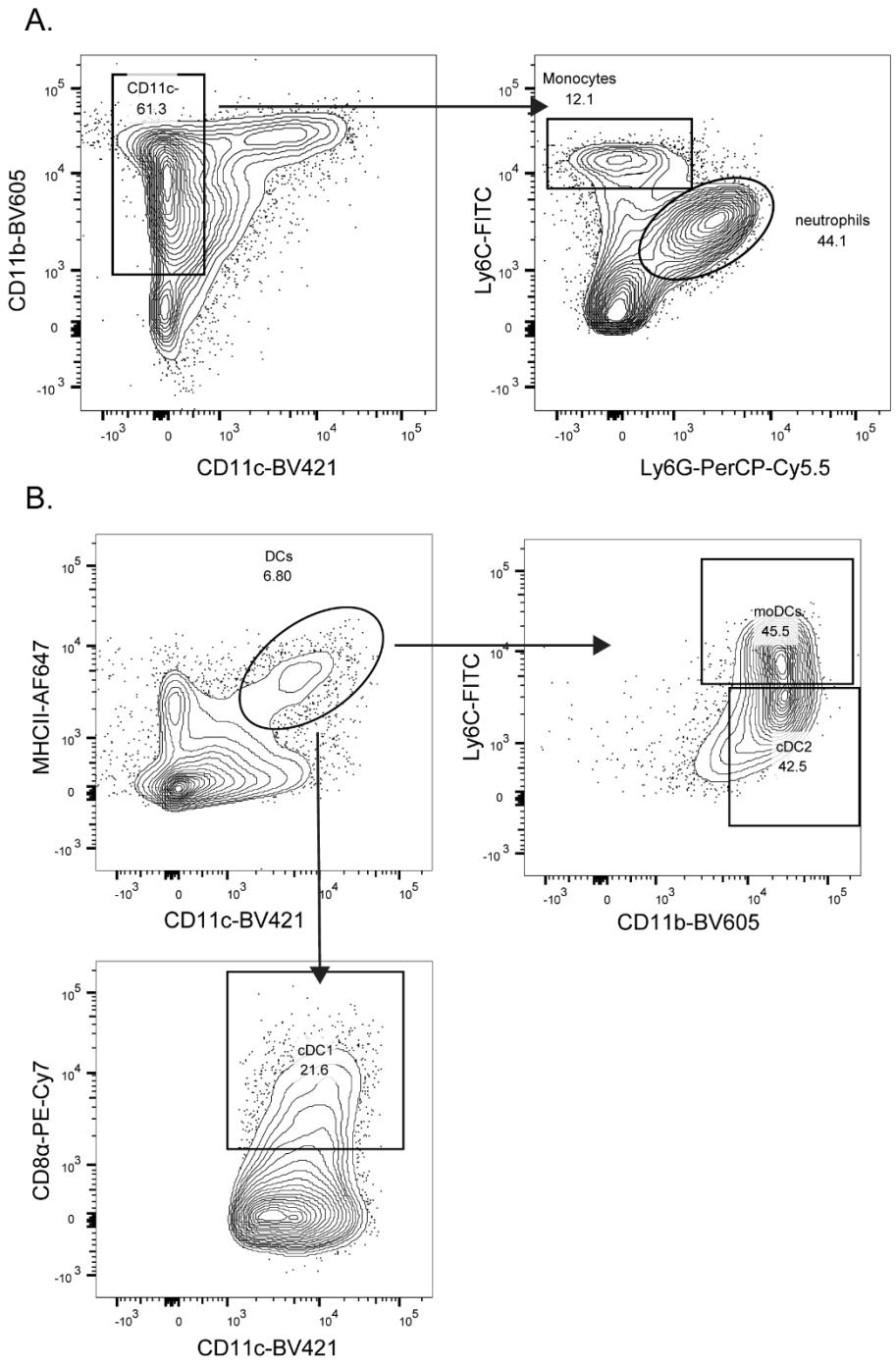

Figure S6. Myeloid gating strategy. Mice bearing MC38 tumors were treated with different combinations of mito, oHSV and/or ICI. Tumors were harvested 4 days after start of

treatment and the frequency of infiltrating immune cells was analyzed by flow cytometry. Cells were gated on viable $CD45^+$. **(A)** Neutrophils and monocytes. **(B)** DCs.

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Chapter 4 : IFNAR blockade synergizes with oncolytic virotherapy to prevent virus-mediated PD-L1 expression and promote antitumor T cell activity.

Preface

This is an author-produced version of an article submitted for peer review in *Molecular Therapy - Oncolytics*. Authors: Nader El-Sayes, Scott Walsh, Alyssa Vito, Amir Reihani, Kjetil Ask, Yonghong Wan, Karen Mossman.

NES conceived and designed the project, acquired, and analyzed data, and wrote the manuscript. SW designed the project and acquired and analyzed data. AV acquired data. AR acquired data. KA reviewed the manuscript. YW supervised the study and reviewed the manuscript. KM supervised the study and revised the manuscript.

This manuscript explores the role of type I IFN (IFN-I) signaling on antitumor immunity. We used a non-immunogenic murine melanoma model, B16F10, to study the effects of oncolytic VSV (oVSV)-induced IFN-I signaling. We found that oVSV induced tumor expression of PD-L1 in an IFN-I-dependent manner. Furthermore, oVSV induced expression of PD-L1 on circulating T cells, B cells and monocytes, which was abrogated using anti-IFNAR-1 monoclonal antibody. While IFN-I is necessary for priming tumor-specific $CD8^+$ T cells [135], we found that combining oVSV with anti-IFNAR-1 enhanced antitumor $CD8^+$ T cell effector functions. Additionally, IFNAR blockade improved therapeutic response to oVSV therapy in a B16F10-gp33 tumor model.

Title: IFNAR blockade synergizes with oncolytic virotherapy to prevent virus-mediated PD-L1 expression and promote antitumor T cell activity.

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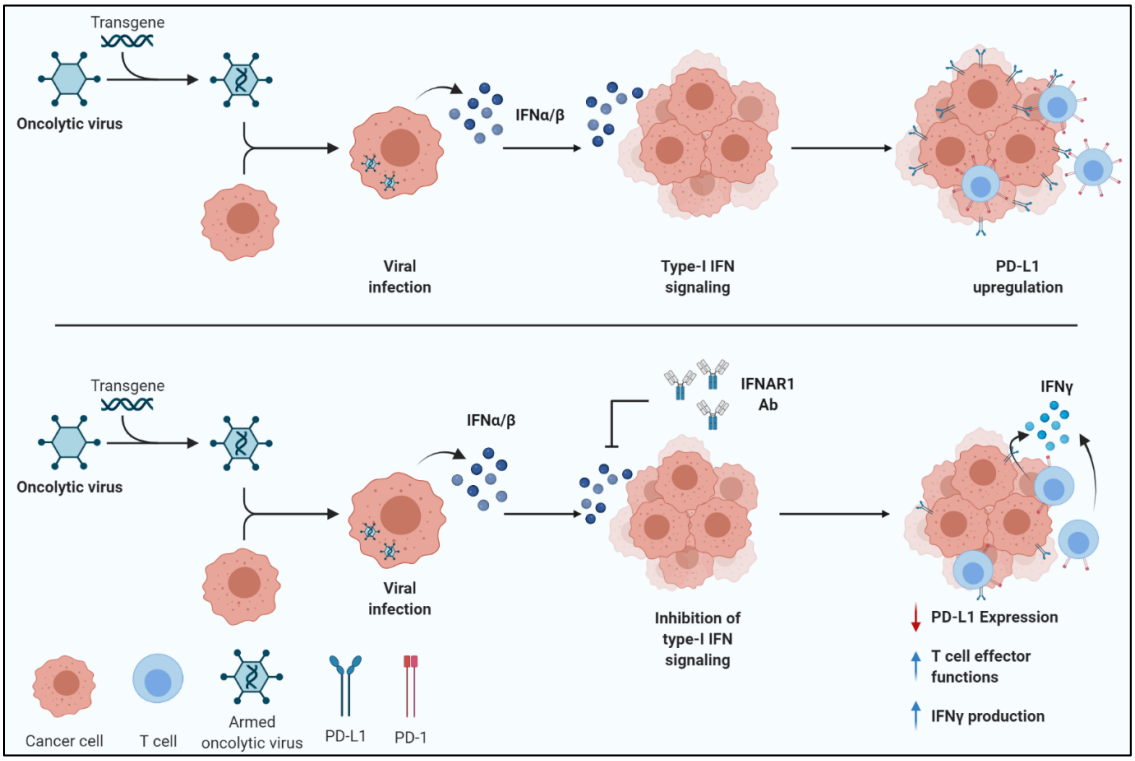
Graphical Abstract



Abstract

Oncolytic virotherapies have shown excellent promise in a variety of cancers by initiating endogenous antitumor immunity. However, the effects of oncolytic virus-mediated type I interferon (IFN-I) production on antitumor immunity remains unclear. Recent reports have highlighted immunosuppressive functions of IFN-I in the context of checkpoint inhibitor and cell-based therapies. In this study, we demonstrate that oncolytic virus-induced IFN-I promotes the expression of PD-L1 in tumor cells and leukocytes. Inhibition of IFN-I signaling using a monoclonal IFNAR antibody partially abrogated IFN-I-induced PD-L1 expression and promoted tumor-specific T cell effector functions when combined with oncolytic virotherapy. Furthermore, IFNAR blockade improved therapeutic response to oncolytic virotherapy in a manner comparable to PD-L1 blockade. Our study highlights a critical immunosuppressive role of IFN-I on antitumor immunity and employs a combination strategy that improves the response to oncolytic virotherapy.

4.1 Introduction

Oncolytic viruses (OVs) are a growing class of biotherapeutics that have demonstrated remarkable potential in the treatment of solid tumors. OVs are multimodal agents that induce cancer cell death through a variety of mechanisms. While the most direct mechanism of OV-mediated cytotoxicity is oncolysis of cancer cells, OVs can also generate a robust antitumor immune response by inducing localized inflammation in the tumor microenvironment (TME). OVs have been shown to sensitize otherwise immune "cold" tumors to immune checkpoint inhibitors (ICI) therapy, a strategy that has seen some success in both pre-clinical and clinical studies [1–3]. Furthermore, OVs are attractive candidates for

use as vectors for tumor associated antigens (TAAs) and chemoattractants due to their selective replication at the tumor site, with active replication causing many changes in the TME. Most notably, the presence of a virus will highly upregulate the production of type I interferons (IFN-I). The role of IFN-I in cancer immunotherapy has become highly controversial, as they have been shown to have both pro-tumor and antitumor properties [4]. IFN-I can enhance immunogenicity of the tumor by upregulating the surface expression of major histocompatibility complex-I (MHC-I) and TAAs [5–7]. However, IFN-I signaling can also potentiate resistance through upregulation of T cell inhibitory receptors and their respective ligands, including programmed death-ligand 1 (PD-L1) and galectin-9 [8–12]. There is also accumulating evidence that IFN-I can potentiate resistance to ICI therapy via PD-L1-independent mechanisms, and that blocking IFN-I signaling can improve the function of exhausted T cell subsets [13,14]. Furthermore, IFN-I potentiates autoimmune side effects of antigen-targeted adoptive cell therapy, and modulation of IFN-I signaling ameliorates side effects without compromising antitumor efficacy [15]. Finally, the antiviral functions of IFN-I can be detrimental to OV therapy by preventing infection of tumor cells and subsequent expression of encoded tumor antigens. Several groups have seen improved outcomes in pre-clinical models by using small molecule inhibitors of type I IFN signaling combined with OV therapy [16–18].

In this study, we show that OVs can induce upregulation of PD-L1 in tumors in an IFN-I-dependent manner. Differences in PD-L1 upregulation induced by two OVs are directly correlated with the level of IFN-I induction by the respective virus. Additionally, IFN-α/β receptor (IFNAR) knockout cells demonstrate vastly reduced OV-mediated

expression of PD-L1, which was also observed *in vivo*. Finally, IFNAR blockade prevented OV-mediated upregulation of PD-L1 in circulating leukocytes, and vastly improved anti-tumor $CD8^+$ T cell activity resulting in improved therapeutic efficacy. Our strategy of combining OVs with IFNAR blockade can prevent the onset of IFN-I-mediated immunosuppression in the TME.

4.2 Results

4.2.1 Virus-mediated PD-L1 upregulation corresponds with IFN-I production

To begin characterizing the role of OVs in regulating the expression of PD-L1, we decided to compare two commonly used OVs. B16F10 cells were infected with VSVΔ51-GFP (VSV) or Vaccinia Virus-YFP (VacV) at a multiplicity of infection (MOI) of 1. Relative expression of PD-L1 mRNA was assessed via RT-PCR 24 hours post infection. GFP or YFP expression was used to confirm initiation of replication (Figure S4.1). While both OVs induce PD-L1 mRNA expression, VSV induces PD-L1 expression at a much higher magnitude (Figure 4.1A). VSV also upregulated PD-L1 in MC38 cells (Figure S4.2), which express higher basal levels of PD-L1 and are responsive to PD-L1 blockade therapy [19]. To see how PD-L1 data compare with other virus-induced genes, we assessed the mRNA expression of the interferon-stimulated gene IFIT1. Similar to PD-L1, IFIT1 mRNA was highly expressed in cells infected with VSV (Figure 4.1B). As IFIT1 expression can be independent of IFN-I signaling [20], we assessed virus-induced expression of IFN-I . Here we also found that VSV induces much higher expression of IFNα and IFNβ mRNA (Figure 4.1C and 4.1D) and protein (Figure 4.1E and 4.1F) compared to VacV.

While IFNγ is a known and potent inducer of PD-L1 expression in the TME [21–23], several emerging studies suggest that IFN-I also regulates PD-L1 expression in the tumor [10–12]. Since we see a correlation between virus-induced PD-L1 expression and IFN-I production, we tested the effects of IFN-I on PD-L1 expression in B16F10 and MC38 cells. Treatment with either IFNα or IFNβ induced expression of PD-L1 mRNA in both cell lines (Figure 4.2A and 4.2B). Furthermore, IFNα/β receptor (IFNAR) knockout cells showed no increase in PD-L1 expression when treated with either IFNα or IFNβ (Figure 4.2A and 4.2B). These data match the IFN-induced expression of IFIT1 (Figure S4.3A and S4.3B). Finally, these results were validated at the protein level by measuring the mean fluorescence intensity (MFI) of PD-L1 surface expression via flow cytometry (Figure 4.2C – 4.2F).

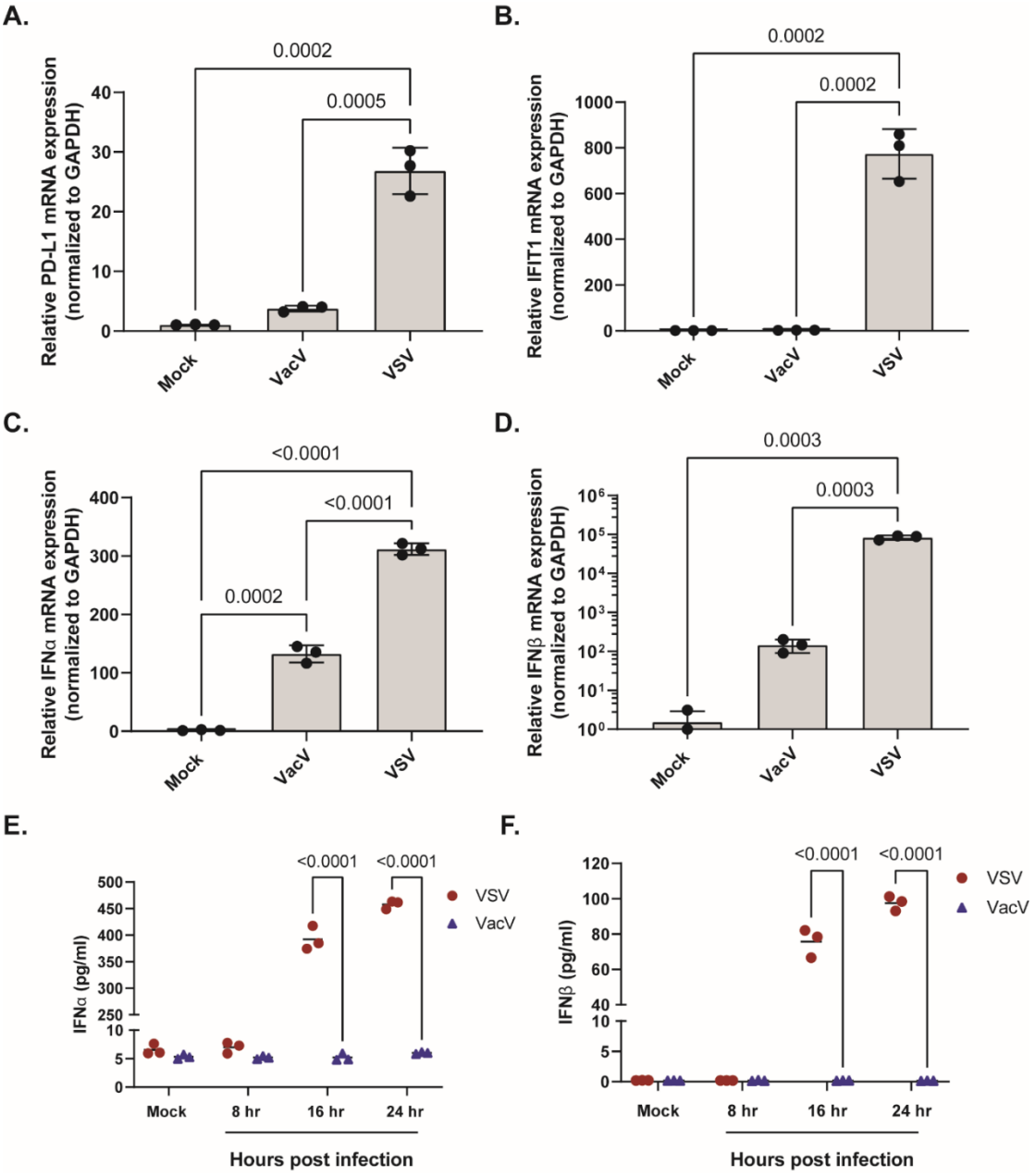


Figure 4.1. Virus-induced expression of PD-L1 correlates with type I IFN production. B16F10 cells were infected with VSVΔ51-GFP (VSV) or vaccinia virus-YFP (VacV) at an MOI of 1. RNA was harvested 24 hours post infection and RT-PCR was used to assess

mRNA expression of **(A)** PD-L1, **(B)** IFIT1, **(C)** IFNα and **(D)** IFNβ. Supernatants were also collected and used to measure the concentration of **(E)** IFNα and **(F)** IFNβ secreted into the supernatant.

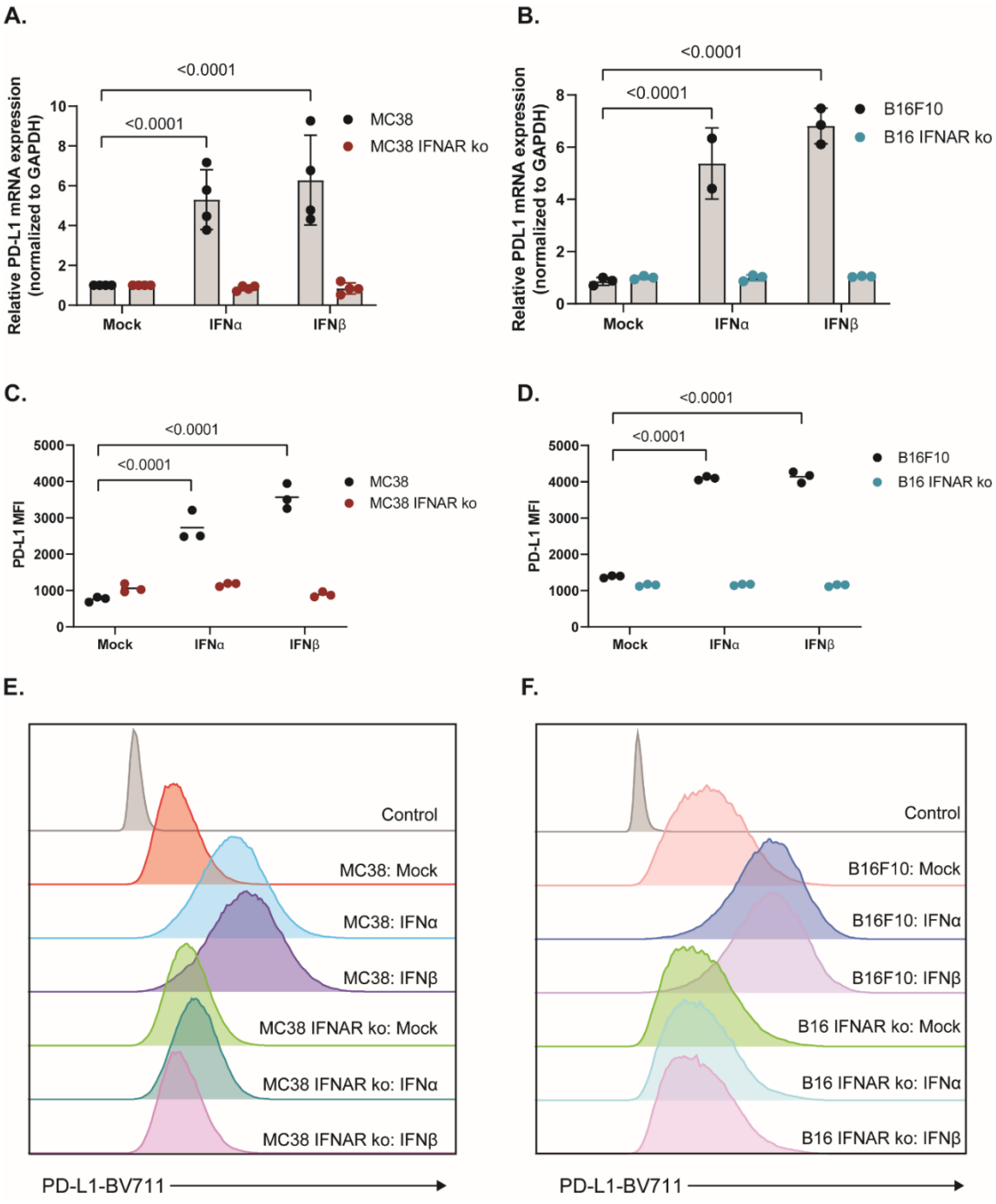


Figure 4.2. Type I IFN induces PD-L1 upregulation in murine cancer cell lines. MC38/MC38 IFNAR knockout cells and B16F10/B16F10 IFNAR knockout cells were treated with 100 U/mL of IFNα or IFNβ. **(A-B)** RNA was harvested and used to assess mRNA expression of PD-L1 via RT-PCR. **(C-D)** Cells were stained with anti-PD-L1-BV711 antibody and MFI was measured via flow cytometry. **(E-F)** Representative histograms of PD-L1-BV711 fluorescence intensity.

4.2.2 Virus-induced PD-L1 cell surface expression is dependent on IFN-I signaling

While type I IFNs induce PD-L1 expression through IFNAR, several reports have also demonstrated that PD-L1 can be induced via IFN-independent inflammatory pathways that generally converge on IRF1, a transcription factor involved in PD-L1 regulation [24–27]. We thus used IFNAR knockout cells to determine the dependence of VSV-induced PD-L1 upregulation on IFN-I signaling. While both MC38 and B16F10 IFNAR knockout cells showed an upregulation of PD-L1 when infected with VSV, the expression of PD-L1 mRNA in IFNAR knockout cells was significantly lower (Figure 4.3A and 4.3B). Despite a small increase in PD-L1 expression at the mRNA level, flow cytometry analysis of B16F10 cells failed to detect an increase in PD-L1 surface expression in VSV-infected IFNAR ko cells, while parental B16F10 cells demonstrated a significant increase in PD-L1 surface expression when infected with VSV (Figure 4.3C and 4.3D). GFP expression analysis confirmed similar initiation of infection in parental and knock-out cells (Figure S4.4A – S4.4C). Although data were consistent at the protein level in B16F10 cells, monitoring surface expression of PD-L1 on MC38 cells was difficult due to the cytotoxic nature of VSV infection (Figure S4.4D). As a result, we used Polyinosinic:polycytidylic acid

(Poly I:C) as a replacement of viral infection to initiate antiviral signaling while maintaining cell viability. Indeed, poly I:C transfection also induced an upregulation of PD-L1 at both the mRNA and protein levels on MC38 and B16F10 cells. While there was small increase in PD-L1 mRNA expression in IFNAR knockout cells stimulated with poly I:C, there was no significant change in surface expression of PD-L1 (Figure 4.3E – 4.3J). Altogether these data strongly suggest that the virus-induced expression of PD-L1 is largely dependent on IFN-I signaling.

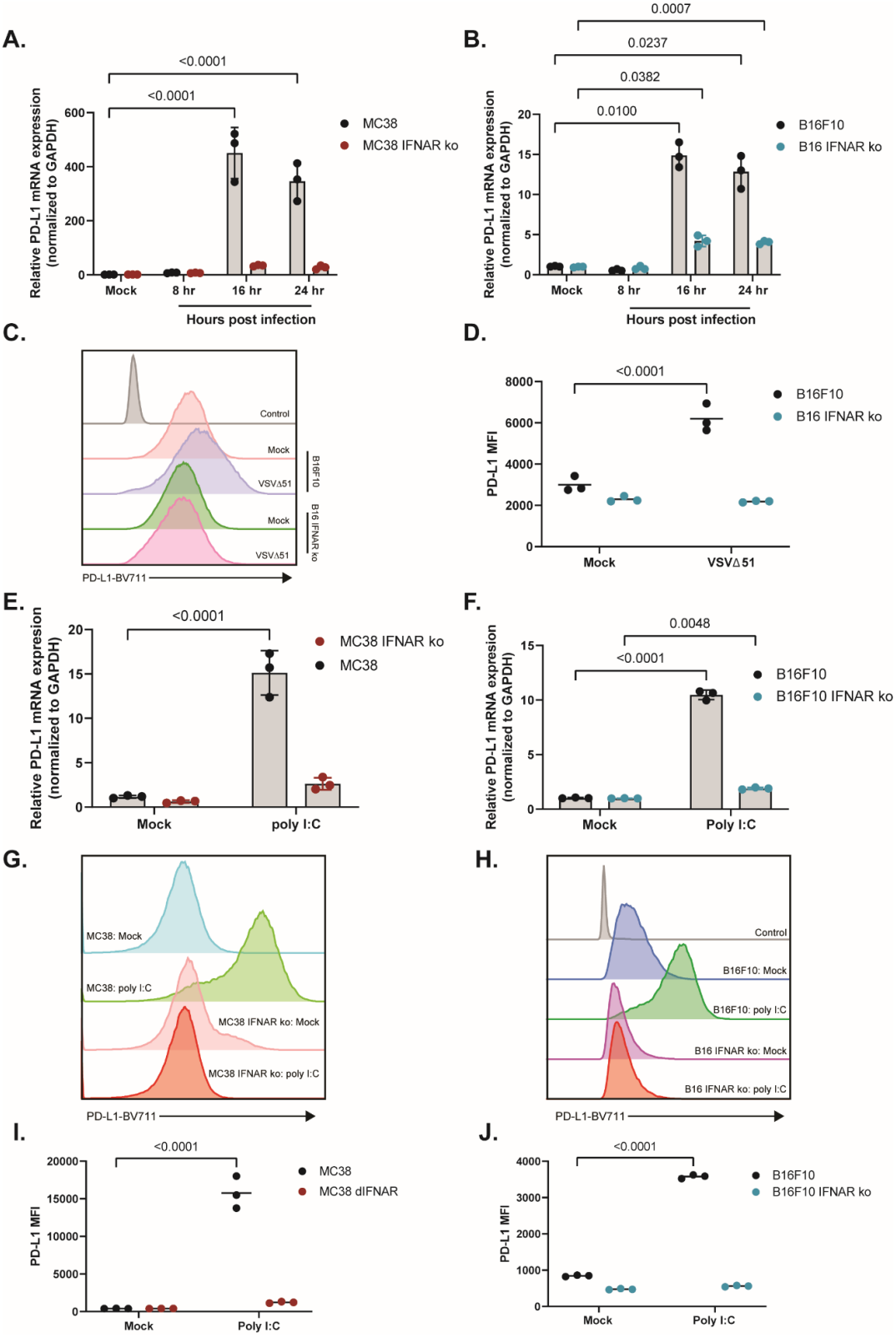

A.
<0.0001
<0.0001
Relative PD-L1 mRNA expression (normalized to GAPDH)
600
400
200
0
Mock
8 hr
16 hr
24 hr
Hours post infection
MC38
MC38 IFNAR ko
B.
0.0007
0.0237
0.0382
0.0100
Relative PD-L1 mRNA expression (normalized to GAPDH)
20
15
10
5
0
Mock
8 hr
16 hr
24 hr
Hours post infection
B16F10
B16 IFNAR ko
C.
Control
Mock
VSVΔ51
Mock
VSVΔ51
B16F10
B16 IFNAR ko
PD-L1-BV711
D.
<0.0001
PD-L1 MFI
8000
6000
4000
2000
0
Mock
VSVΔ51
B16F10
B16 IFNAR ko
E.
<0.0001
Relative PD-L1 mRNA expression (normalized to GAPDH)
20
15
10
5
0
Mock
poly I:C
MC38 IFNAR ko
MC38
F.
0.0048
<0.0001
Relative PD-L1 mRNA expression (normalized to GAPDH)
15
10
5
0
Mock
Poly I:C
B16F10
B16F10 IFNAR ko
G.
MC38: Mock
MC38: poly I:C
MC38 IFNAR ko: Mock
MC38 IFNAR ko: poly I:C
PD-L1-BV711
H.
Control
B16F10: Mock
B16F10: poly I:C
B16 IFNAR ko: Mock
B16 IFNAR ko: poly I:C
PD-L1-BV711
I.
<0.0001
PD-L1 MFI
20000
15000
10000
5000
0
Mock
Poly I:C
MC38
MC38 dIFNAR
J.
<0.0001
PD-L1 MFI
4000
3000
2000
1000
0
Mock
Poly I:C
B16F10
B16F10 IFNAR ko

Figure 4.3. Virus-induced PD-L1 expression is dependent on type I IFN signaling. Wild type and knock out MC38 and B16F10 cells were infected with VSVΔ51-GFP (VSV) at an MOI of 1. **(A-B)** RNA was harvested and used to assess mRNA expression of PD-L1 via RT-PCR. **(C)** Representative histogram and **(D)** MFIs of PD-L1 surface expression on B16F10 and B16 IFNAR ko were measured via flow cytometry. **(E)** MC38/MC38 IFNAR knockout cells and **(F)** B16F10/B16F10 IFNAR knockout cells were transfected with 1µg of poly I:C**.** RNA was harvested and used to assess mRNA expression of PD-L1 via RT-PCR. **(G-H)** Representative histograms and **(I-J)** MFIs of PD-L1 surface expression were measured via flow cytometry**.**

4.2.3 Oncolytic VSV-induced PD-L1 upregulation in murine tumors is partially dependent on type I IFN signaling

While our data strongly suggest that virus-induced PD-L1 expression is dependent on IFN-I signaling *in vitro*, the TME *in vivo* contains a plethora of other factors that may regulate the expression of PD-L1. This includes the presence of other cytokines and chemokines that can regulate PD-L1 expression [25,26]. To this end, we assessed the effect of VSV on PD-L1 expression *in vivo*, along with characterizing the role of IFN-I signaling. MC38 or B16F10 tumors were implanted subcutaneously and intradermally, respectively, into C57Bl/6 mice. One-week later tumors were treated with anti-IFNAR or isotype control antibody followed by intravenous administration of VSV. Tumors were harvested and PD-L1 surface expression was assess via immunohistochemistry 24 hours after treatment. In both MC38 and B16F10 tumors, treatment with VSV induced much higher PD-L1 expression relative to untreated mice. Pre-treatment with anti-IFNAR antibody decreased, but did

not eliminate, virus-induced PD-L1 expression. Next, we validated these data by comparing virus-induced PD-L1 expression in MC38 IFNAR knockout tumors. MC38 and MC38 IFNAR knockout cells were implanted subcutaneously, and mice were treated with VSV intravenously one week later. Consistent with anti-IFNAR antibody treatment, the loss of IFNAR in MC38 tumors reduced but did not eliminate PD-L1 expression following VSV treatment (Figure 4.4B). These data suggest that early virus-induced expression of PD-L1 in the TME is partially dependent on IFN-I signaling.

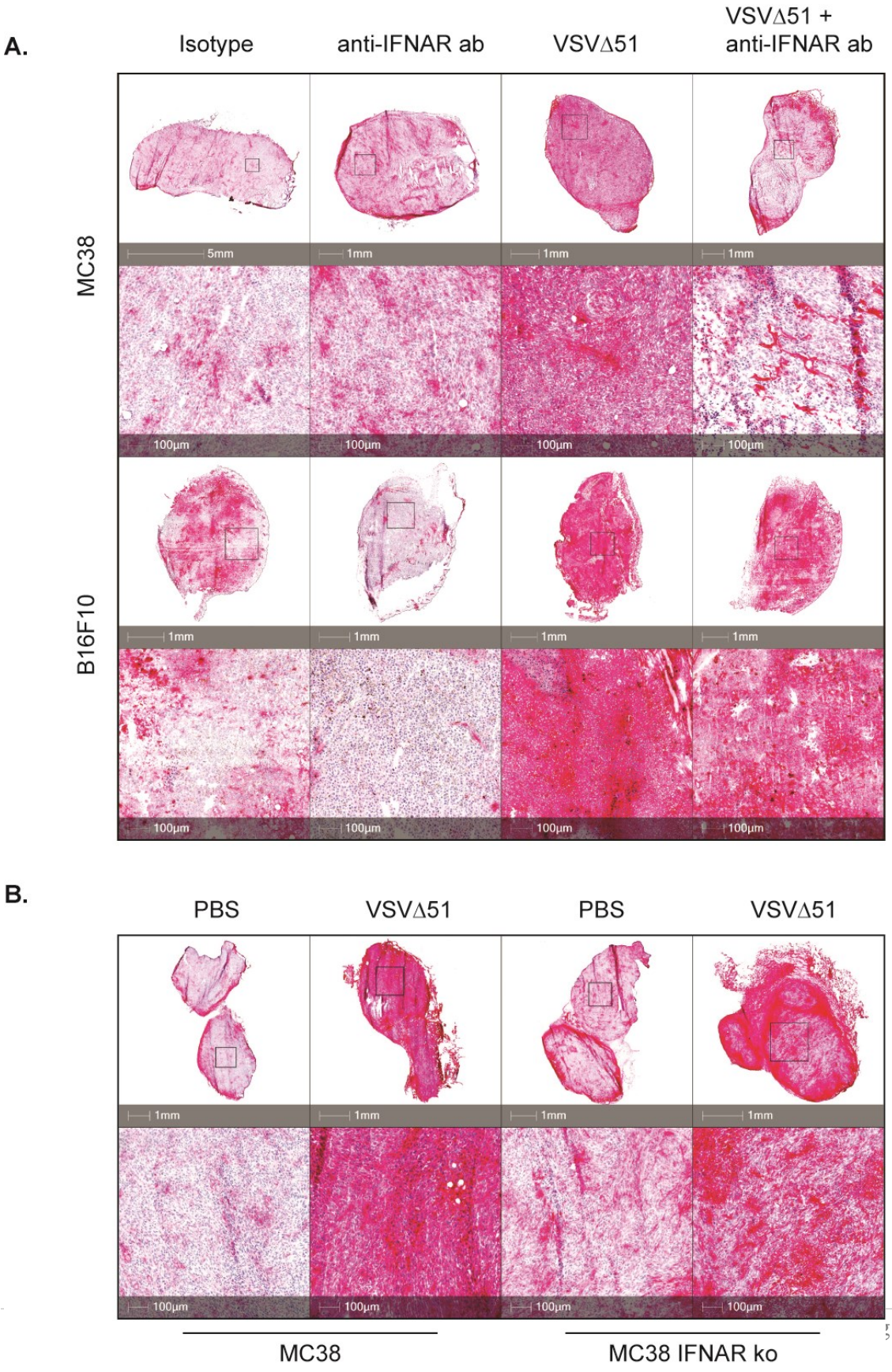

A.
Isotype
anti-IFNAR ab
VSVΔ51
VSVΔ51 +
anti-IFNAR ab
MC38
B16F10
5mm
1mm
100μm
B.
PBS
VSVΔ51
PBS
VSVΔ51
MC38
MC38 IFNAR ko

Figure 4.4. IFNAR blockade reduces virus-induced PD-L1 expression in the tumor. **(A)** MC38 and B16F10 cells were implanted into C57Bl/6 mice subcutaneously and intradermally, respectively. Mice were treated with 1mg anti-IFNAR antibody by i.p. followed by i.v. injection of 2 x 10^8 pfu of VSVΔ51 (VSV) 2 hours later. **(B)** MC38 and MC38 IFNAR knockout cells were implanted into C57Bl/6 mice subcutaneously. Mice were treated with VSV by i.v. injection. 24 hours after VSV administration, tumors were harvested and stained for PD-L1 expression by immunohistochemistry.

4.2.4 Oncolytic VSV induces PD-L1 upregulation in circulating leukocytes

Expression of PD-L1 on T cells, B cells, macrophages and other leukocytes can enhance immunosuppression and promote tumor tolerance [28–30]. In this regard, we investigated whether PD-L1 expression on circulating leukocytes is upregulated following OV treatment, and if early expression of PD-L1 is dependent on IFN-I signaling. We used tumors and virus expressing gp33, an immunodominant lymphocytic choriomeningitis virus antigen, as a surrogate antigen. Mice harboring B16F10-gp33 tumors were treated with anti-IFNAR antibody followed by intravenous delivery of VSVΔ51-gp33 (VSV-gp33). Peripheral blood mononuclear cells (PBMCs) were isolated and PD-L1 surface expression was assessed via flow cytometry 24 hours later. We found that there was a substantial increase in the percent of PD-L1 positive T cells and B cells after treatment with VSV-gp33. Moreover, pre-treatment with IFNAR blockade partially abrogated virus-mediated upregulation of PD-L1 on these cells (Figure 4.5A and 4.5B). Interestingly, this trend was not entirely consistent among different cell types. While monocytes and macrophages also demonstrated VSV-mediated PD-L1 expression, IFNAR blockade only mildly prevented

virus-mediated expression (Figure 4.5C and 4.5D). The percent of PD-L1 positive neutrophils was substantially increased following VSV-gp33 treatment, however unlike the other immune cell types, neutrophils demonstrated a further increase in %PD-L1 positive cells after IFNAR blockade (Figure 4.5E). Finally, circulating natural killer (NK) cells demonstrated a mild reduction in %PD-L1 positive cells following treatment with VSV-gp33 (Figure 4.5F). Collectively, these data suggest that the level of dependence of VSV-induced PD-L1 expression on IFN-I signaling varies between cell types. Virus-mediated upregulation of PD-L1 on T and B lymphocytes is moderately dependent on IFN-I signaling, while similar PD-L1 upregulation on monocytes and macrophages seems to be largely independent of IFN-I signaling, or even dampened by IFN-I signaling.

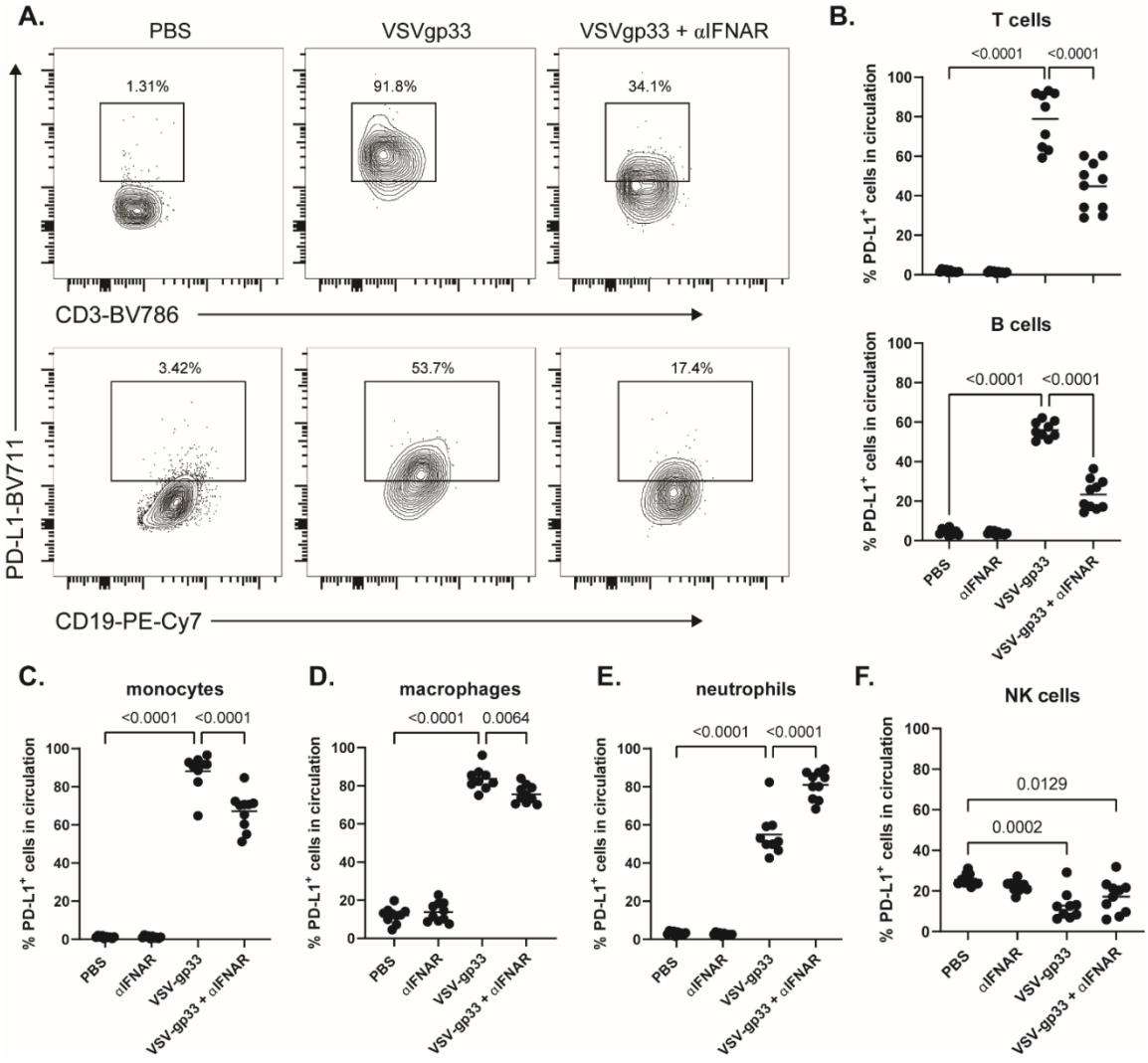


Figure 4.5. Blocking IFNAR signaling alters virus-mediated PD-L1 expression on circulating leukocytes. 10^5 B16F10-gp33 cells were implanted intradermally in C57Bl/6 mice. Mice were treated with 1mg anti-IFNAR by i.p. followed by i.v. injection of 2 x 10^8 pfu of VSVΔ51-gp33 2 hours later. Expression of PD-L1 on circulating mono-nuclear cells was assessed 24 hours after VSV treatment via flow cytometry. **(A)** Representative flow plots of PD-L1 expression on circulating T cells (top) and B cells (bottom). **(B)** Precent of

circulating T cells (top) and B cells (bottom) expressing PD-L1. **(C-F)** Percent of circulating monocytes, macrophages, neutrophils, and NK cells expressing PD-L1.

4.2.5 IFNAR blockade potentiates activation of antitumor $CD8^+$ T cells and prevents up-regulation of exhaustion markers

IFN-I signaling is required for priming antitumor $CD8^+$ T cells and can promote effector functions in $CD8^+$ T cells [31,32]. Therefore, we decided to investigate the effect of IFNAR blockade on the activity of antitumor $CD8^+$ T cells and determine if IFNAR blockade dampens the generation of effector $CD8^+$ T cells. We used tumors and virus expressing gp33 as a surrogate antigen to assess the priming and magnitude of antitumor T cell responses. C57BL/6 mice harboring B16-gp33 tumors were treated with anti-IFNAR or anti-PD-L1 antibodies followed by vaccination with VSV-gp33. PBMCs were isolated 7 days later and stimulated with gp33 peptide. The magnitude of the gp33-specific T cell response was measured by assessing IFN-γ production by intracellular cytokine staining. As expected, vaccination with VSV-gp33 increased the capacity of gp33-specific $CD8^+$ T cells to produce IFN-γ and was improved further by the addition of anti-PD-L1 therapy. Interestingly, IFNAR blockade was substantially more effective than PD-L1 blockade at improving IFN-γ production when combined with VSV-gp33 (Figure 4.6A and 4.6B). Next, we decided to further characterize the T cells by assessing the expression of CD44 and CD62L, which are surface markers used to differentiate between naïve ($CD44^-$, $CD62L^+$), central memory (CM; $CD44^+$, $CD62L^+$), effector memory (EM; $CD44^+$, $CD62L^-$) and double negative (DN; $CD44^-$, $CD62L^-$) cells (Figure 4.6C). We found that vaccination with VSV-gp33 resulted in a higher fraction of EM $CD8^+$ T cells (T_{EM} cells) in circulation.

Addition of either PD-L1 or IFNAR blockade further increased the percentage of T_{EM} cells (Figure 4.6C and 4.6D). This trend was consistent when assessing the absolute number of T_{EM} cells in circulation, with PD-L1 and IFNAR blockade substantially increasing the number of T_{EM} cells when combined with VSV-gp33 (Figure 4.6E). Finally, we assessed the level of T cell exhaustion characterized by the overexpression of Programmed Death-1 (PD-1) and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3). These markers can be upregulated following T cell effector functions and chronic antigen stimulation, leading to exhaustion and dysfunctional T cell activity [33]. While vaccination with VSV-gp33 marginally increased the absolute number of PD-1^+ and TIM-3^+ $CD8^+$ T cells in circulation, the addition of PD-L1 blockade significantly increased the number of $CD8^+$ T cells expressing exhaustion markers (Figure 4.6F – 4.6H). Impressively, the combination of IFNAR blockade with VSV-gp33 resulted in a negligible increase in PD-1^+ and TIM-3^+ T cells compared to VSV-gp33 monotherapy. Taken together, these data demonstrate that IFNAR blockade can promote tumor-specific $CD8^+$ T cell activation and T_{EM} cell proliferation in a manner similar to that of PD-L1 blockade, while maintaining lower expression of exhaustion markers such as PD-1 and TIM-3.

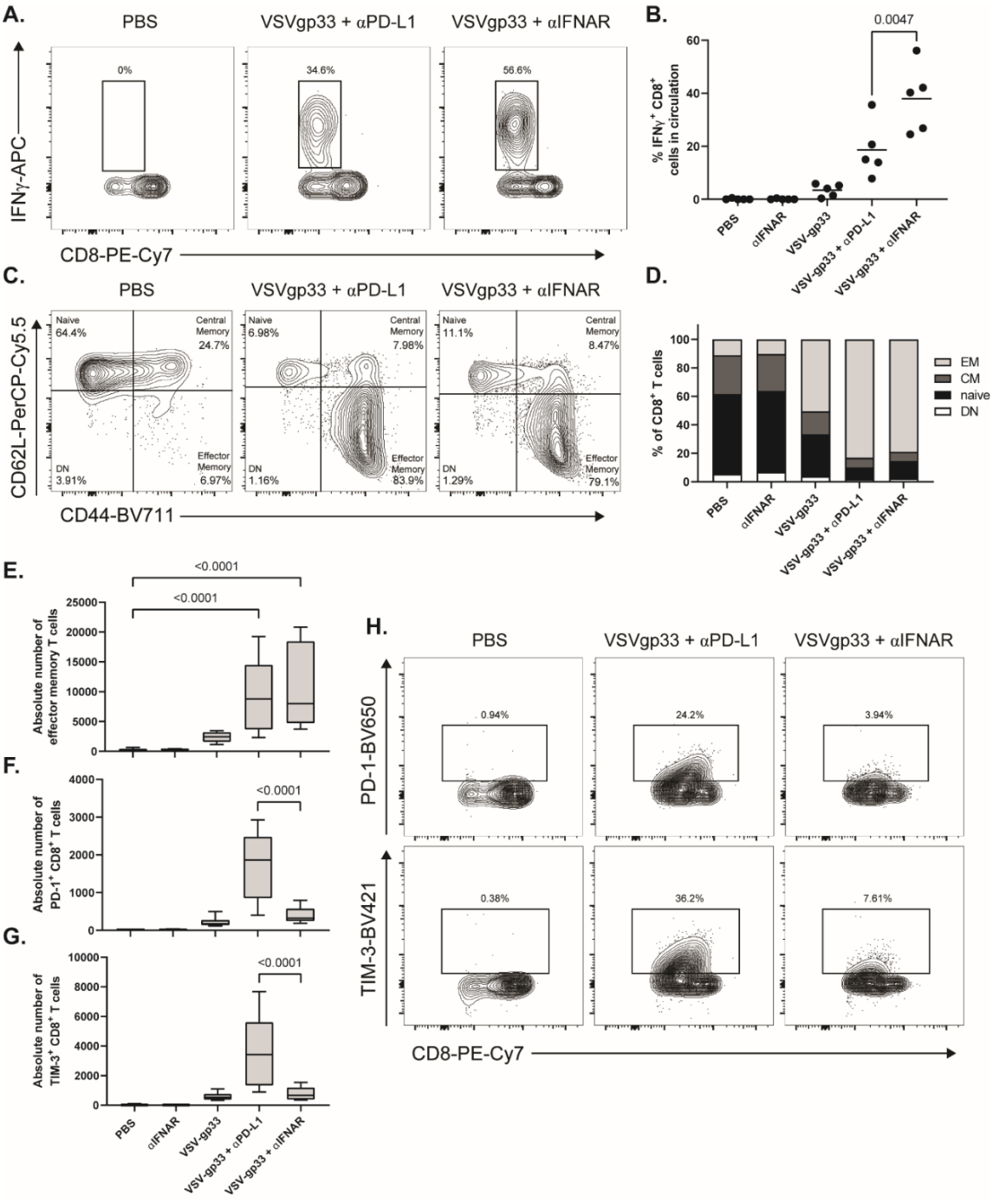


Figure 4.6. IFNAR blockade promotes tumor-specific CD8 T cell activation and the generation of T_{EM} cells while maintaining lower expression of exhaustion markers. 10^5 B16F10-gp33 cells were implanted intradermally in C57Bl/6 mice. Mice were treated with

1mg anti-IFNAR or 250µg anti-PD-L1 antibodies by i.p. followed by i.v. injection of 2 x 10^8 pfu of VSVΔ51-gp33 2 hours later. PBMCs were isolated 7 days post treatment and re-stimulated with gp33 peptide and IFNγ production was assessed by ICS. **(A)** Representative contour plots showing the percent of CD8 T cells that are IFNγ$^+$. **(B)** Percent of IFNγ$^+$ CD8 T cells were graphed, lines represent the means. PBMCs were isolated 7 days post treatment and expression of T cell markers was assessed by flow cytometry. **(C)** Representative contour plots showing the percentage of Naïve, central memory (CM), effector memory (EM), and double negative (DN) CD8 T cells. **(D)** Quantification of the T cell populations for each group. **(E)** Box plot showing the absolute number of EM CD8 T cells. Box plots showing absolute numbers of **(F)** PD-1$^+$ and **(G)** TIM-3$^+$ CD8 T cells. **(H)** Representative contour plots showing percentage of PD-1$^+$ and TIM-3$^+$ CD8 T cells.

4.2.6 IFNAR blockade synergizes with oncolytic VSV to improve therapeutic outcomes in murine melanoma model

Our data show that IFNAR blockade can limit early virus-induced expression of PD-L1 in the tumor and promote tumor-specific T cell activity. To further establish the relevance of these observations in the context of therapeutic efficacy, we assessed the therapeutic potential of IFNAR blockade when combined with VSV-gp33 in tumor-bearing mice. To this end, we used B16F10 tumors due to their documented resistance to ICI therapy [34]. C57BL/6 mice were implanted intradermally with B16F10-gp33 cells and treated with anti-IFNAR and/or anti-PD-L1 antibodies 10 days later. After 2 hours, the mice were then treated with VSV-gp33. Tumor volumes were monitored and anti-PD-L1 treatments were administered every 3 days according to a previously established therapeutic regimen

(Figure 4.7A). VSV-gp33 monotherapy significantly delayed tumor progression relative to the control, however the combination of VSV-gp33 and anti-PD-L1 resulted in a significant delay in tumor progression and prolonged survival (Figure 4.7 B-D). Of interest, the combination of VSV-gp33 and a single dose of anti-IFNAR antibody was comparable to VSV-gp33 + continual anti-PD-L1 administration (Figure 4.7 B-D). Finally, the addition of anti-PD-L1 to VSV-gp33 + anti-IFNAR treatment did not improve therapeutic outcomes further (Figure 4.7 B–D). These data suggest that IFNAR blockade has comparable therapeutic efficacy to PD-L1 blockade in combination with VSV-gp33.

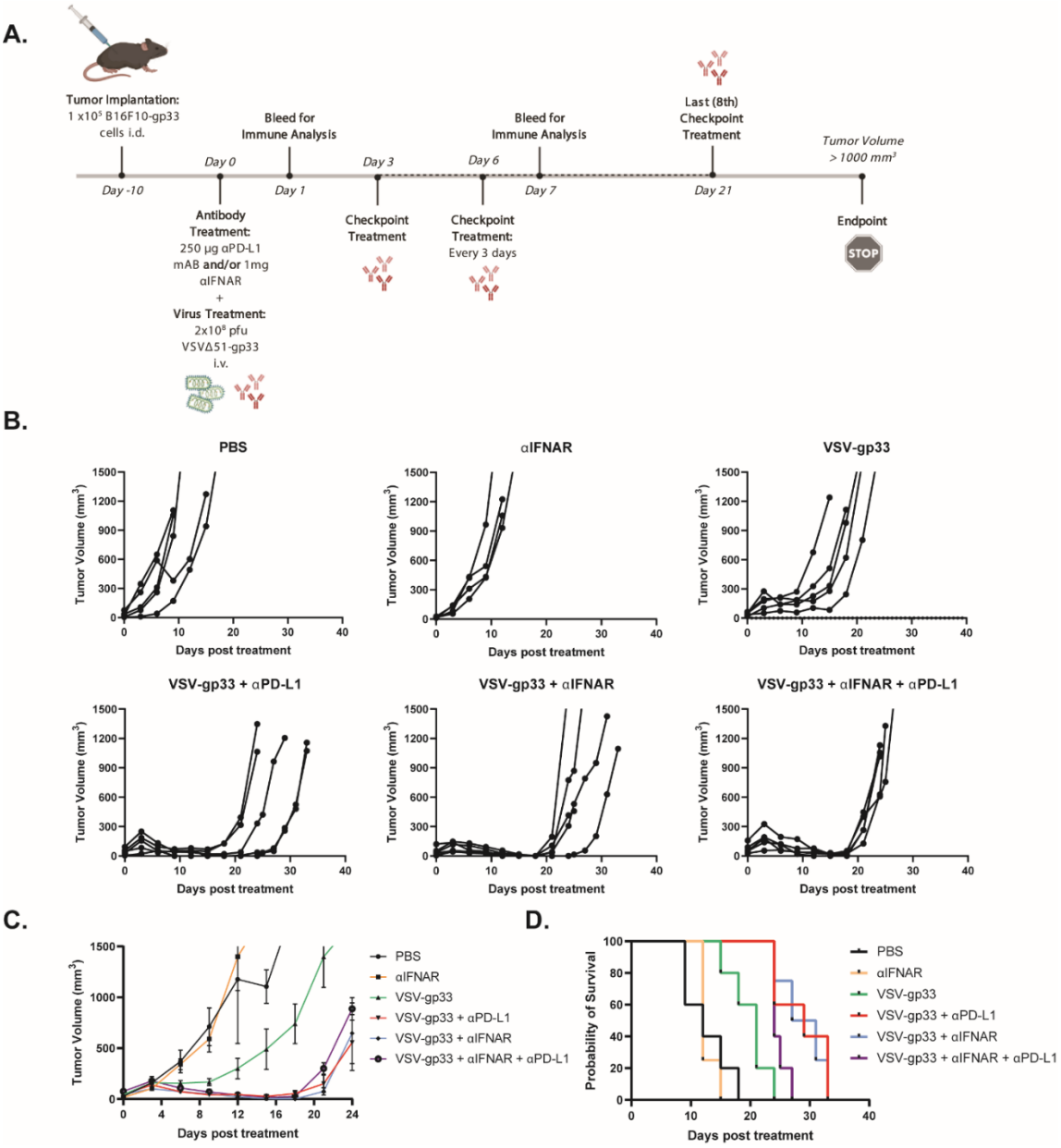


Figure 4.7. IFNAR blockade synergizes with oncolytic VSV to improve therapeutic outcomes in tumor-bearing mice. **(A)** Schematic representation of the treatment regimen used. 10^5 B16F10-gp33 cells were implanted intradermally in C57Bl/6 mice. Mice were treated with 1mg anti-IFNAR and/or 250µg of anti-PD-L1 (checkpoint) antibody by i.p. followed by i.v. injection of 2 x 10^8 pfu of VSVΔ51-gp33 2 hours later. Tumor volumes were

monitored every 2-3 days until endpoint (1000 mm^3). **(B)** tumor volumes graphed for each mouse over time. **(C)** Average tumor volumes over time. **(D)** Kaplan-Meier survival curves.

4.3 Discussion

Oncolytic viruses (OVs) have gained traction as a potent cancer immunotherapy in the past decade. The presence of an actively replicating virus in the TME can cause substantial upregulation of IFN-I production; however, the role of IFN-I signaling in immunotherapy has become a topic of controversy. In this study, we show that IFNAR blockade is an effective therapeutic strategy when combined with oncolytic VSV. Efficacy of this combination in tumor-bearing mice is comparable to that of PD-L1 blockade and oncolytic VSV. Furthermore, addition of PD-L1 blockade does not improve therapeutic efficacy any further. This observation suggests that the efficacy of IFNAR blockade could be dependent on inhibition of PD-L1 upregulation. Furthermore, we use IFNAR knockout cells *in vitro* to show that OV-mediated upregulation of PD-L1 is highly dependent on IFN-I signaling. Interestingly, this observation is consistent *in vivo*, where a variety of other factors can be involved in the expression of PD-L1. Namely, IFNγ is a key player for cancer immunotherapy, and is known as a potent inducer of PD-L1 [21,22]. In the context of OV therapy, however, IFNα/β is induced as early as 5 hours after OV treatment [15], while IFNγ is detected in circulation a few days post-infection. Our data suggest that early induction of PD-L1 expression in the TME following OV therapy is at least partially dependent on IFN-I signaling. Our results match similar findings which show that sustained IFN-I and IFN-II confer resistance to immunotherapy and induce expression of inhibitory ligands [35].

However, they demonstrate that IFN-mediated resistance is only partially dependent on PD-L1 upregulation, and that upregulation of other inhibitory ligands including galectin 9, HVEM and MHCII contribute to IFN-mediated resistance [35]. This observation means that IFNAR blockade could potentially function as a multimodal inhibitor of several immune checkpoints.

Expression of PD-L1 in the TME is not restricted to cancer cells. Indeed, studies have demonstrated that PD-L1 knockout tumor models can still benefit from anti-PD-L1 therapy [36,37]. Furthermore, expression of PD-L1 on immune cells can also suppress anti-tumor immune responses. Indeed, one study by Diskin and colleagues demonstrated that PD-L1 expression on T cells can promote tumor tolerance and suppression of effector T cells [30]. Expression of PD-L1 on antigen-presenting cells can also attenuate tumor antigen presentation and priming CD8 T cells [37–39]. In this study we show that OV-mediated PD-L1 expression on T cells, B cells and monocytes is partially dependent on IFN-I signaling. We also demonstrated that IFNAR blockade can promote the generation of T_{EM} cells and enhance IFNγ production from tumor-specific T cells in manner similar to PD-L1 blockade. This finding suggests that blocking IFN-I signaling promotes priming of tumor-specific T cells, however the relevance of inhibiting PD-L1 expression on other immune cells has yet to be determined. Interestingly, the dependance of PD-L1 expression on IFN-I signaling varies by cell type. While OV-mediated PD-L1 expression on T cells, B cells and monocytes is partially dependent on IFN-I signaling, PD-L1 upregulation on macrophages is IFN-I-independent. Furthermore, IFNAR blockade caused an upregulation of PD-L1 on circulating neutrophils, suggesting that IFN-I signaling negatively regulates PD-L1

expression on neutrophils. While we demonstrate that IFNAR blockade can enhance anti-tumor T cell activity, it has been well established that IFN-I signaling is important for promoting effector functions in CD8 T cells [31,32]. Interestingly, existing reports have demonstrated that specifically prolonged IFN-I signaling is detrimental to antitumor immunity [35]. Another study shows that IFN-I can still confer therapeutic benefit when combined with PD-L1 blockade to counteract the IFN-mediated upregulation of PD-L1 [40]. Future studies should continue to focus on the kinetics of IFN-I signaling in the TME to establish the effects of acute vs sustained signaling on resistance to immunotherapy.

Finally, IFNAR blockade as a therapeutic strategy has unique implications for OV therapy. Several OVs induce expression of PD-L1 in the tumor, and so OVs are often combined with anti-PD-1/PD-L1 therapy to improve therapeutic outcomes [41–44]. Our study demonstrates that IFNAR blockade can prevent the onset of IFN-mediated PD-L1 expression and synergize with oncolytic VSV in a similar manner to PD-L1 blockade. Unlike anti-PD-L1 therapy, however, IFNAR blockade has the added benefit of potentially enhancing OV replication in the TME. Indeed, several groups have shown that inhibition of IFN-I signaling can enhance OV replication and promote therapeutic efficacy in several OV platforms [17,18,45]. Furthermore, OVs can function as effective vectors for transgenes, including pro-inflammatory chemokines and tumor antigens that can further boost the generation of a robust antitumor response. Promoting OV replication by inhibiting IFN-I signaling will, in turn, increase the production of encoded transgenes. Therefore, our development of an IFNAR blockade strategy has broad clinical implications for the future of OV therapy.

4.4 Materials and Methods

Cell Lines

MC38 cells (ATCC) were maintained in Dulbecco's modified Eagle's media (DMEM) supplemented with 5% fetal bovine serum (FBS, ATCC 30-2020) 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco). B16F10 (ATCC) and B16-gp33 cells (B16F10 cells stably transfected with a minigene corresponding to the gp33 peptide) were maintained in minimum essential medium with Earle's salts (MEM-Earle's) supplemented with 1x MEM vitamin solution (ThermoFisher Scientific, 11120052), 10% FBS, 2 mmol/l L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin. MC38 and B16F10 IFNAR knockouts were obtained from the lab of Dr. John Bell (OHRI, Ottawa, ON) and cultured under the same conditions as their respective wild-type cell lines.

Virus propagation

VSVΔ51 is an oncolytic attenuated variant of the VSV Indiana strain. VSV was propagated, purified and quantified on Vero cells as described previously [48]. Briefly, virus stocks were purified from cell culture supernatants by filtration through a 0.22 µm Steritop filter (Millipore) and centrifugation at 30,000 × g before resuspension in PBS. The VacV used in this study is the wild-type Copenhagen strain and was produced in HeLa cells and quantified in U2OS cells. For VacV purification, virus was collected by repeated freeze-thaw cycles. Further purification was done by centrifugation at 20,700 × g through a 36% sucrose cushion before resuspension in 1 mM Tris, pH 9.

In vivo experiments

Mice were maintained at the McMaster University Central Animal Facility and all the procedures were performed in full compliance with the Canadian Council on Animal Care and approved by the Animal Research Ethics Board of McMaster University. MC38 tumors: 2 x 10^5 cells were implanted subcutaneously into the left flank of 6–8-week-old female C57/Bl6 mice (Charles River Laboratories, Wilmington, MA). B16F10 tumors: 1 x 10^5 cells were implanted intradermally into C57Bl6 mice. Mice were treated when 7 days post B16F10 tumor challenge or 10 das post MC38 tumor challenge. 1mg of αIFNAR (InVivoMab, clone MAR1-5A3) and/or 250µg of αPD-L1 (InVivoMab, clone 10F.9G2) antibodies were administered by i.p. injection. Experimental groups receiving αPD-L1 followed a dosing schedule of 250µg treatments every 3 days for a total of 8 doses. 2 hours after αPD-L1 and/or αIFNAR treatment, 2 x 10^8 pfu of VSVΔ51 was administered via tail vein injection. ~150 µl of blood was collected via retro-orbital bleed 1- and 7-days following treatment for immune analysis (described in another section). Tumor volumes were monitored and measured every 2-3 days until they reached their endpoint volume (1000 mm^3).

Immune analysis and flow cytometry

Following blood collection, red blood cells were lysed with ACK buffer and peripheral blood mononuclear cells (PBMCs) were transferred to a round-bottom 96-well plate. For *in vitro* cell lines, confluent cells were treated with TrypLE Express Enzyme (ThermoFischer Scientific), resuspended in PBS then transferred to a round-bottom 96-well plate. The cell suspensions were stained with fixable viability stain 510 (BD Biosciences) for 30 min at room temperature then treated with anti-CD16/CD32 (Fc block; BD

Biosciences) for 15 min at 4 degrees. Cell surface staining was done for 30 min at 4 degrees. Intracellular staining was done using cytofix/cytoperm Fixation/Permeabilization kit (BD Biosciences). For analysis of gp33-specific T cells, PBMCs were treated with 1µg/ml of gp33 peptide and incubated for 1 hour at 37 degrees, 5% CO_2 followed by treatment with a protein transfer inhibitor (GolgiPlug, BD Biosciences) and incubated for another 3.5 hours. The cells were then stained as described above. Data acquisition was done on the LSRFortessa (BD) and data were analyzed using FlowJo.

RT-PCR

RNA extraction was performed using RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's protocol. 500 ng of purified RNA was reverse transcribed using iScript gDNA Clear cDNA Synthesis Kit (Bio-Rad). Real-time PCR reactions were performed with Ssofast EvaGreen kit (Bio-Rad) and data were acquired on a 7500 Fast Real-Time PCR system (Applied Biosystems). Relative mRNA expression was normalized to GAPDH and fold induction was calculated relative to the untreated/uninfected controls using the Pfaffl method [49]. Primer sequences are as follows:

PD-L1 F: CAGCAACTTCAGGGGGAGAG

PD-L1 R: TTTGCGGTATGGGGCATTGA

IFIT1 F: GCC TAT CGC CAA GAT TTA GAT GA

IFIT1 R: TTC TGG ATT TAA CCG GAC AGC

IFN-α F: CGGAATTCTCTCCTGCCTGAAGGAC

IFN-α R: AAGGGTACCACACAGTGATCCTGTGGAA

IFN-β F: AGC TCC AAG AAA GGA CGA ACA

IFN-β R: GCC CTG TAG GTG AGG TTG AT

GAPDH F: AATGGATTTGGACGCATTGGT

GAPDH R: TTTGCACTGGTACGTGTTGAT

Immunohistochemistry

Tumors were harvested and flash frozen in liquid nitrogen. Frozen tissue sections were cut at 5 µm onto coated slides. Sections were air dried overnight and then fixed in 10% NBF for 5 min before being treated with 1% H_2O_2 in dH_2O for 15min at room temperature. Slides were then wash in dH_2O to remove excess H_2O_2. Slides were rinsed in Bond Wash (Leica) and placed on the Leica Bond Automated stainer. The slides were stained with Rat primary PDL-1 (EBio 13-5982-52) 1:500 in Animal Free Diluent (Vector Labs SP-5035). The BOND Polymer Refine Red Detection kit (Leica) was used according to the manufacturer's protocol. The slides were then digitized according to a previously described protocol [50].

ELISA

Supernatants from cells were collected 8-, 16- and 24-hours post-infection and treated with a protease inhibitor cocktail (ThermoFisher Scientific) and centrifugated for 10 min at 4 degrees. Supernatants were transferred to a new tube and either frozen at -80 degrees or used immediately for ELISA. Verikine mouse IFN beta ELISA kit (PBL Assay Science) and Verikine mouse IFN alpha ELISA kit (PBL Assay Science) were used for data acquisition according to the manufacturer's protocol. Levels of secreted cytokines (pg/mL) were interpolated from experimental standard curves.

Statistical Analysis

Results are presented as means ± standard deviation. Log-rank (Mantel-Cox) tests were used to analyze the statistical significance between treatment groups for Kaplan–Meier survival graphs. Ordinary one-way ANOVA was used to determine the statistical significance between means of treated groups according to the normality of their distributions. In all cases the null hypothesis was rejected when p values < 0.05. All statistical analysis was performed using GraphPad Prism 9.

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Author contributions

Conceptualization, N.E., S.W., K.M.; Methodology, N.E., S.W., K.A., Y.W., K.M.; Investigation, N.E., S.W., A.V., A.R.; Writing, N.E.; Review/editing, N.E., S.W., Y.W., K.M.; Funding acquisition, K.M.; Resources, Y.W., K.M.; Supervision, K.M., S.W., Y.W.

4.5 Supplementary Figures

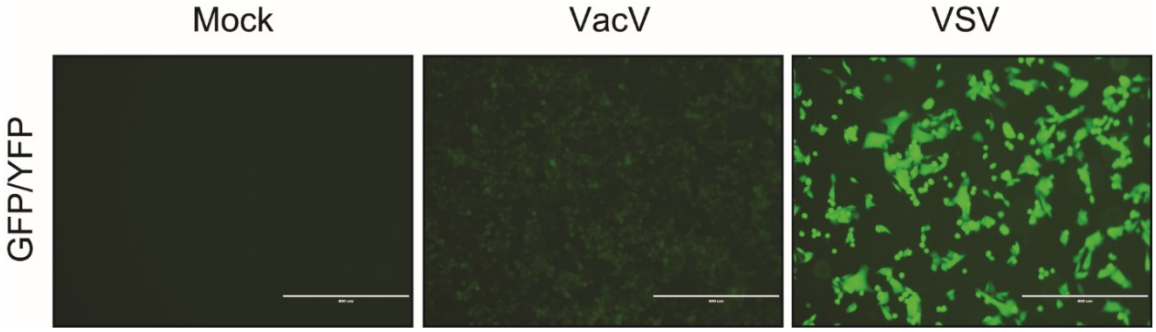

Figure S4.1. GFP and YFP expression following VSVΔ51 and VacV infection. B16F10 cells were infected with VSV-GFP (VSV) or VacV-YFP (VacV) at MOI 1 for 24 hours then imaged using a fluorescence microscope.

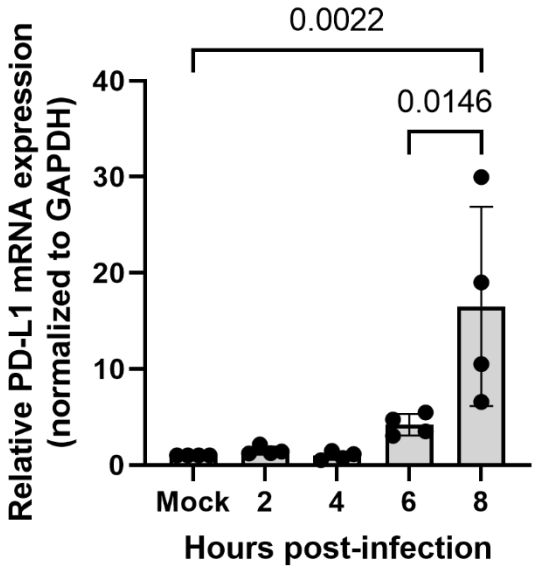

Figure S4.2. VSV-induced PD-L1 upregulation in MC38 cells. MC38 cells were infected with VSVΔ51 for 2, 4, 6 and 8 hours before RNA was harvested and used to assess PD-L1 mRNA expression via RT-PCR.

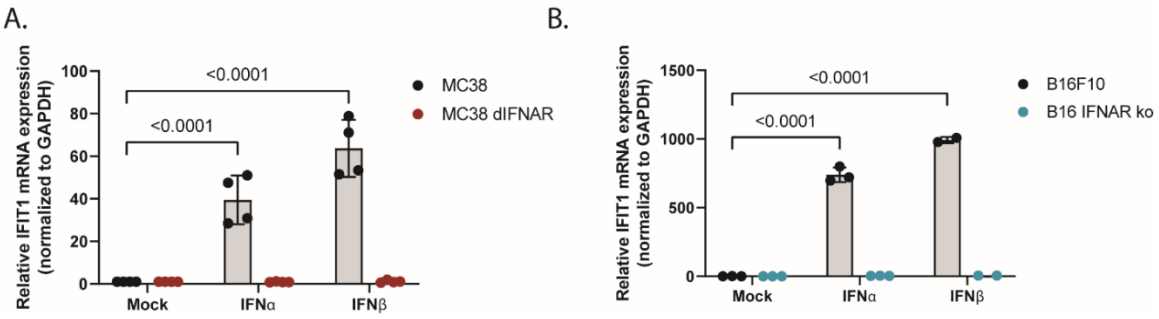

Figure S4.3. Type I IFN induces IFIT1 upregulation. **(A)** MC38 and **(B)** B16F10 parental and IFNAR knockout cells and were treated with 100 U/mL of IFNα or IFNβ. RNA was harvested and used to assess mRNA expression of IFIT1 via RT-PCR.

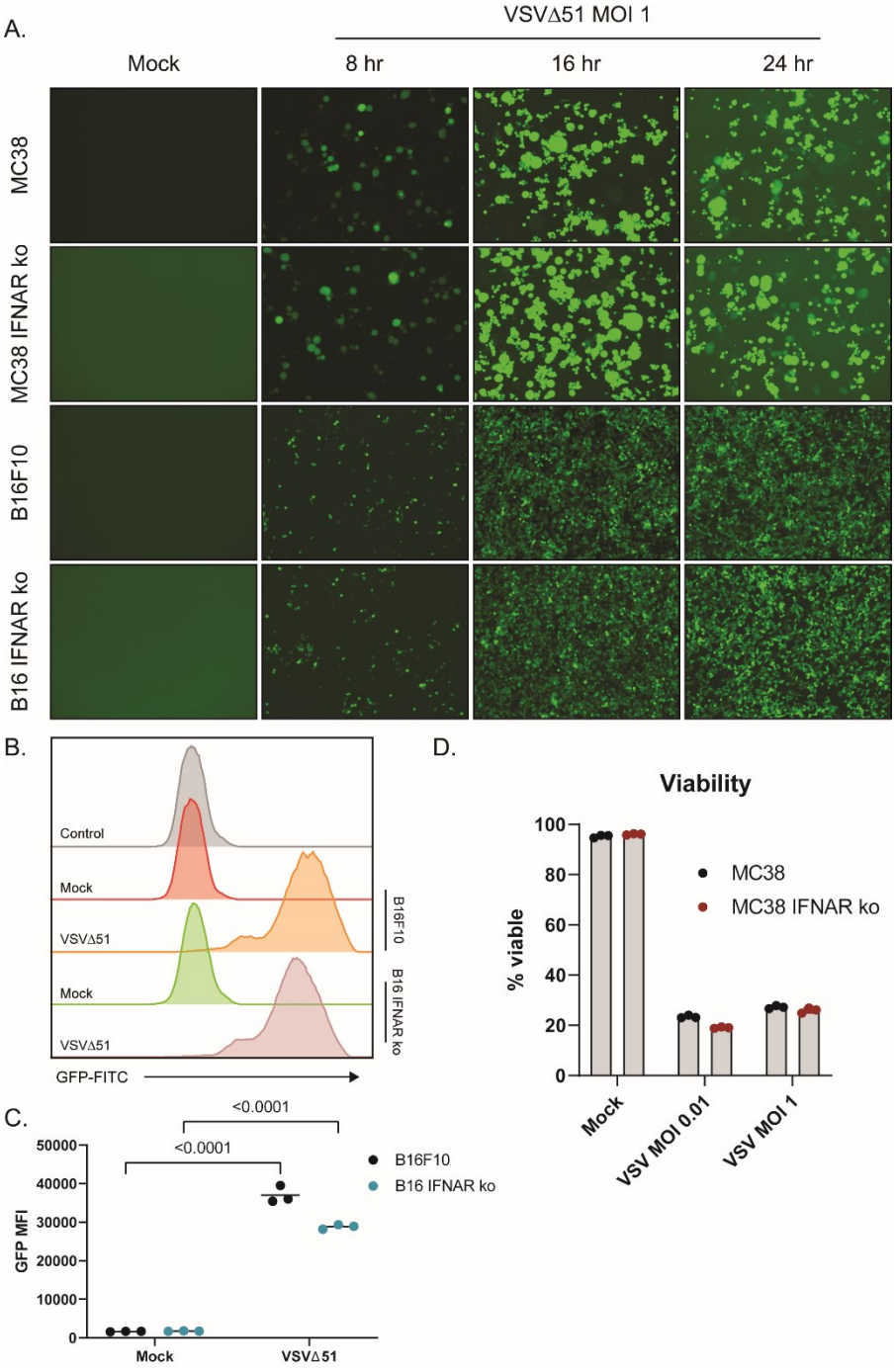

Figure S4.4. Validation of VSV initiation of replication. **(A)** MC38 and B16F10 parental and IFNAR knockout cells were infected with VSVΔ51-GFP at MOI 1 for 8, 16 and 24 hours then imaged using a fluorescence microscope. B16F10 parental and IFNAR

knockout cells were infected with VSVΔ51-GFP at MOI 1 for 24 hours then GFP fluorescence was measured by flow cytometry. **(B)** Representative histogram and **(C)** MFIs of GFP expression in B16F10 parental and IFNAR ko cells 24 hours post infection. **(D)** MC38 parental/IFNAR ko cells were infected with VSVΔ51-GFP at MOI 1 for 24 hours then viability was measured by flow cytometry.

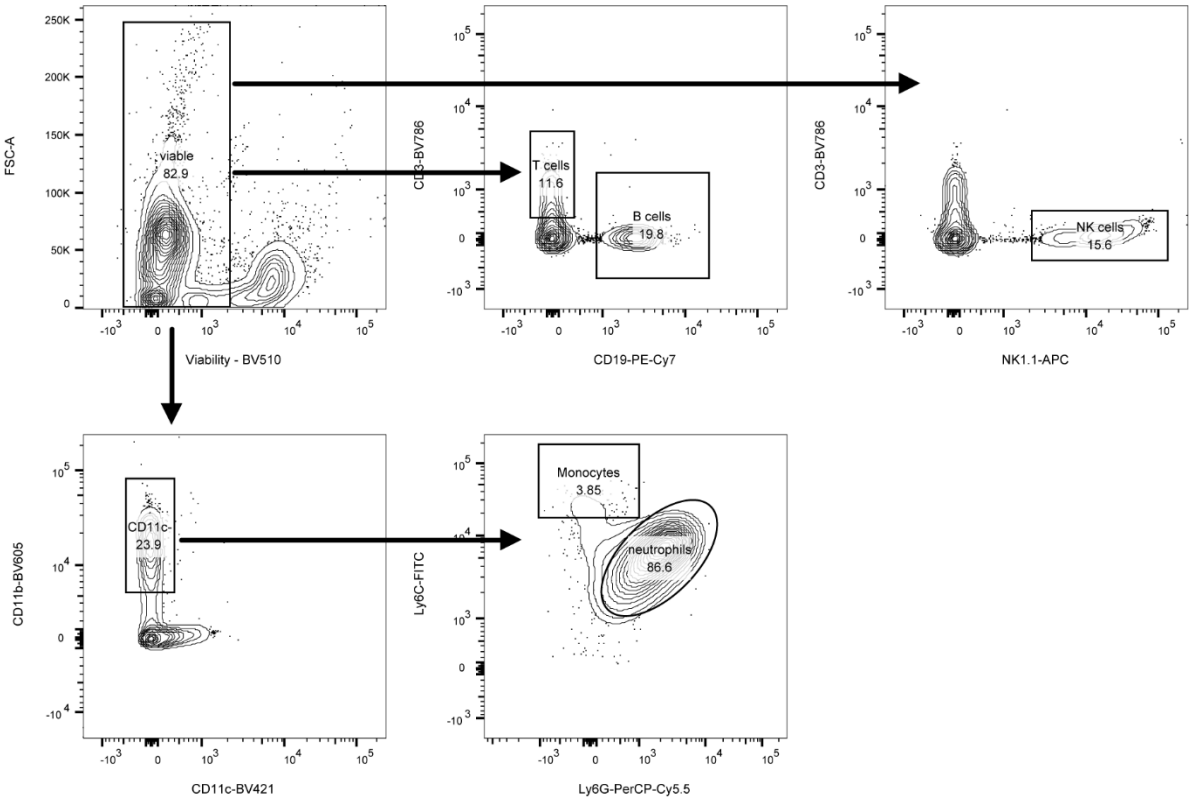

Figure S4.5. Gating strategy used to characterize different leukocytes from PBMCs to assess PD-L1 surface expression (corresponds to data from Figure 5).

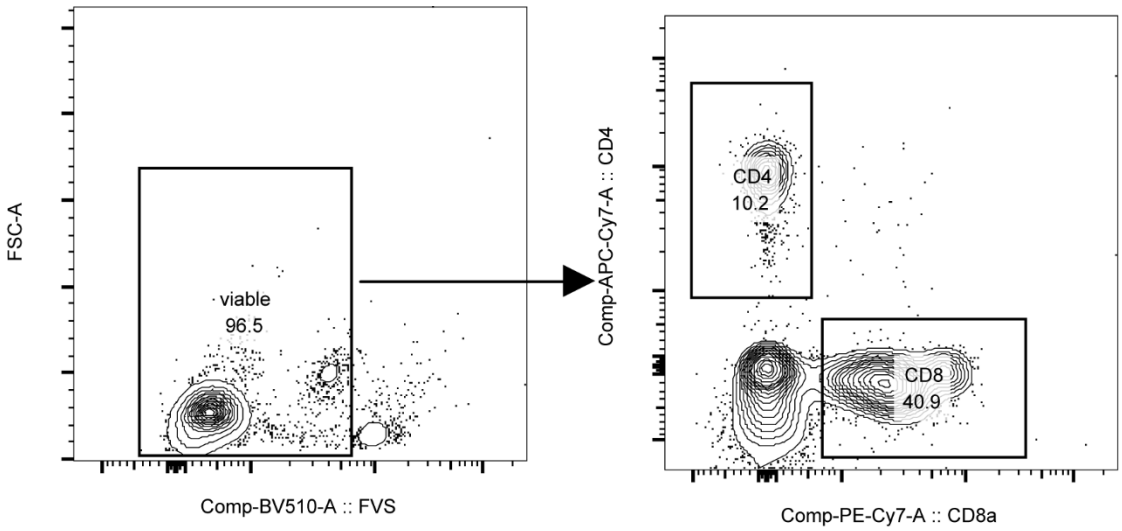

Figure S4.6. Gating strategy used to characterize T cells from PBMCs (corresponds to data from Figure 6).

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Chapter 5 : Conclusion and Future Directions

5.1 Combination of low-dose chemotherapy and oncolytic virotherapy sensitizes MC38 tumors to immune checkpoint inhibitor therapy in a cDC1-dependant manner.

Immune checkpoint inhibitors have demonstrated tremendous promise in the clinic. In particular, solid tumors with high mutational burdens demonstrate the greatest response to ICI therapy. High mutational burden contributes to the antigenicity of tumors, as frequent mutations increase the chances of acquiring neoantigens that can break immune tolerance. For this reason, mutational burden is used as a predictive biomarker for ICI therapy [1,2]. While some patients demonstrate unprecedented long-term remission, most patients fail to respond to ICI therapy [3]. Therefore, it is imperative that we develop a better understanding of factors in the TME that influence response to ICI therapy. While antigenicity is important, initiation of an immune response against pathogens or cancers also requires sufficient adjuvanticity, characterized by the release of DAMPs and inflammatory cytokines and the activation of myeloid cells [4]. Pre-clinical research suggests that enhancing adjuvanticity by inducing ICD can enable a therapeutic response to ICI therapy [5].

Several types of cancer therapy can induce ICD. Conventional chemotherapy has been shown to induce ICD and initiate an antitumor immune response in a variety of cancers [6]. For example, Hodge and colleagues demonstrated that docetaxel induced ICD and enhanced tumor killing by cytotoxic T cells [7]. Induction of ICD promotes the activation of myeloid cells that initiate inflammation through the secretion of cytokines and

chemokines [4]. Indeed, ICD can induce the maturation, activation, chemoattraction, and antigen presenting capacity of cDC1s [8].

Our lab has previously utilized combinations of chemotherapy and oncolytic HSV to induce ICD and sensitize breast adenocarcinoma to ICI therapy [9,10]. In **Chapter 3** we show that the combination of mito + oHSV can sensitize dMMR CRC to immune checkpoint therapy. We found that the combination enhances adjuvanticity in the tumor based on the increased tumor infiltration of myeloid subsets, including cDC1s. This highlights the role of adjuvanticity in enabling a response to ICI therapy and validates existing literature on the requirement of cDC1s for the initiation of antitumor immunity.

5.2 IFNAR blockade synergizes with oncolytic virotherapy to prevent virus-mediated PD-L1 expression and promote antitumor T cell activity.

The role of IFN-I signaling in cancer immunotherapy has been controversial in recent years. IFN-I is required for the activation of myeloid cells and an important component for antigen presentation by dendritic cells [11,12]. Furthermore, IFN-I signaling leads to the upregulation of MHC-I on tumor cells [13]. However, IFN-I signaling can also induce the upregulation of immunosuppressive ligands such as PD-L1 and galectin 9 on tumor cells and immune cells [14–17]. Liang and colleagues utilized this knowledge to develop a strategy in which they exploit the benefits of IFN-I signaling while simultaneously overcoming IFN-I-induced immunosuppression by arming anti-PD-L1 antibodies with IFNα [18]. However, other reports demonstrate that IFN-I-driven resistance can be independent of PD-L1 expression [19].

Even in the context of OVs, there has been debate on the benefits and drawbacks of IFN-I signaling. Perhaps counterintuitively, IFN-I can synergize with OVs to enhance antitumor immunity [20]. To this end, some OVs were designed to express IFNβ, which enhanced the efficacy and safety of the OV platform [21,22]. On the other hand, temporary inhibition of IFN-I signaling using small molecule inhibitors was also shown to enhance the antitumor activity of OVs [23,24].

In **Chapter 4** we demonstrate that OV-induced expression of IFN-I potentiates resistance to OV therapy. In particular, OVs induced the expression of PD-L1 on tumor cells and immune cells in an IFN-I-dependent manner. We demonstrate that IFNAR blockade with monoclonal antibodies synergizes with OV therapy to enhance tumor-specific T cell effector functions and improve on OV-mediated tumor control. IFNAR blockade is a strategy that is particularly effective in the context of OV therapy due to its potential to enhance transgene expression while simultaneously preventing IFN-I-induced expression of immunosuppressive ligands.

5.3 Study limitations

In **Chapter 3** we demonstrate that mito + oHSV + ICI induces the infiltration of several myeloid subsets including cDC1s. We also show that the presence of cDC1s is required for therapeutic efficacy. However, we have yet to identify the role of cDC1s in driving a response to ICI therapy in this model. Furthermore, we have yet to understand how mito + oHSV + ICI impacts the biological functions of cDC1s, including their antigen-presenting capacity.

Another limitation to this study is the limited number of CRC models used. The MC38 tumor model recapitulates the microsatellite instability of mismatch repair-deficient colon adenocarcinoma [25]. However, we have yet to determine if our findings are consistent among other dMMR CRC models, and if we would observe differences in CRC models with stable microsatellites and lower mutational burden. Furthermore, we use a transplantable tumor model which fails to recapitulate the immunoediting process involved in spontaneous tumor formation. To this end, other CRC murine models can be used to supplement this study. These models include carcinogen-induced tumors and conditional (in)activation of tumor suppressor genes and oncogenes [26]. Additionally, high mortality rates in CRC patients are directly associated with the occurrence of liver metastasis. Our model of ectopic tumor transplantation does not recapitulate the metastatic potential of CRC. To address this, we could make use of orthotopic models that result in rapid formation of metastasis in the liver, lymph node and spleens [26]. This can be achieved by subserosal injection of tumor cells, or by surgical orthotopic implantation of tumor fragments [26].

In **Chapter 4** we demonstrate that OVs can induce upregulation of PD-L1 in an IFN-I-dependent manner. We also demonstrate that blocking IFNAR signaling synergizes with OV therapy in a murine melanoma model. One limitation to the study is the lack of mechanistic insight into this synergy. While the data suggest that the synergy occurs as a result of inhibiting IFN-induced expression of PD-L1, we have yet to show this definitively. This can be achieved by testing the combination of IFNAR blockade and VSV-gp33 in PD-L1 knockout tumors and/or mice.

Unlike the study performed in chapter 3, this study is performed in an orthotopic model of murine melanoma. While B16 tumors can form lung metastasis [27], we have not utilized the metastatic potential of this model. Another limitation to this study is the over-expression of gp33 in the tumors. While gp33 acts as an effective surrogate antigen for assessing tumor-specific immunity, the antigen is highly immunogenic and renders tumors susceptible to treatment. Indeed, initial tumor regression was observed in all our treatment groups, which makes it difficult to assess the synergy of therapeutic combinations.

5.4 Future directions

In **Chapter 3** I provided insight into the requirement of cDC1s for enabling a response to ICI therapy in a model of murine CRC. However, we have yet to elucidate the effects of mito + oHSV on the maturation and activation of cDC1s. In previous models, we established that mito + oHSV sensitizes tumors to ICI therapy in a necroptosis-dependent manner [28], however we have yet to characterize the role of necroptosis in potentiating cDC1 infiltration and activation. Furthermore, mito + oHSV highly induced infiltration of neutrophiles into the tumor, however we haven't characterized their phenotype or their involvement in antitumor immunity. Future studies should focus on the following three points: 1) Characterizing the role of mito + oHSV in the maturation, activation, and antigen-presenting capacity of cDC1s; 2) Investigating the phenotype and role of tumor-infiltrating neutrophils; 3) Investigating the role of necroptosis in driving the infiltration and activation of cDC1s.

Maturation and activation of cDC1s can be characterized by assessing the expression of surface markers by multi-color flow cytometry. Assessment of antigen-presentation

can be done using MC38 cells expressing a surrogate antigen, such as gp33. After treatment with combinations of mito/oHSV and/or ICI, CFSE-stained p14 T cells can be adoptively transferred into the mice. Draining lymph nodes can then be processed to assess proliferation of p14 T cells as an indication of antigen presentation. Differentiation of neutrophil subsets can be done via transcriptomic analysis [29]. Necrostatin-1, an inhibitor of necroptosis, can be used to determine the requirement of necroptosis for enhancing the tumor infiltration of myeloid subsets including cDC1s. It can also be used to determine the involvement of necroptosis in promoting tumor antigen presentation in the MC38 tumor model.

Chapter 4 was focused on assessing the potential immunosuppressive properties of IFN-I in the context of OV therapy. Future studies should focus on 1) establishing the dependence of IFN-I immunosuppression on the expression of PD-L1; 2) comparing acute vs sustained IFN-I signaling in regard to resistance to immunotherapy and OV therapy. We can begin assessment of the first point by assessing the efficacy of IFNAR blockade and OV therapy in knockout tumor/mouse models. The second point can be investigated by comparing acute and sustained IFN-I signaling *in vitro* and *in vivo* to assess expression of immunosuppressive ligands and resistance to immunotherapy and OV therapy.

In the broader context of the field, the work presented in this dissertation alongside recent published findings emphasizes the role of adjuvanticity in enabling a response to immunotherapy. Future research should continue to unravel mechanisms that enable the initiation and maintenance of a robust antitumor immune response. As a result of tumor heterogeneity, the TME will vary between patients and even within an individual patient.

To this end, the development of accurate biomarkers based on knowledge of the TME could aid in choosing therapeutic combinations with the highest chance of success. While mutational burden is an effective biomarker of antigenicity, biomarkers of adjuvanticity should also be incorporated to predict favorable outcomes.

5.5. Concluding remarks

The Mossman lab studied oncolytic HSV as a cancer therapy for many years. Mechanistic studies comparing oncolytic HSVs led to the discovery that immunogenic oncolysis, not replicative potential, shapes the therapeutic response to OVs [30]. Using this knowledge, the Mossman lab has specialized in the development of combinations of chemotherapy and OVs to induce ICD and initiate antitumor immunity. To build upon the existing research in the Mossman lab, I aimed to characterize immune-tumor interactions that were modulated by oncolytic viruses. I hypothesized that OVs can enable response to ICI therapy by improving tumor immunogenicity though the induction of stimulatory cytokines and chemokines. Indeed, the combination of chemotherapy and oncolytic HSV induced the expression of chemokines associated with the recruitment and activation of myeloid subsets required of initiation of antitumor immunity. However, we also show that OV-induced expression of IFN-I can potentiate resistance to therapy.

Altogether this work demonstrates that OV platforms can further sensitize antigenic tumors to ICI therapy. Furthermore, it highlights a potential role of OVs in inducing favorable inflammation that results in the tumor infiltration of myeloid subsets required for initiating antitumor immunity. This work also shows that IFNAR blockade can synergize with OV therapy to enhance the effector functions of tumor-specific T cells and control tumor

progression. It also sheds light on the immunosuppressive functions of IFN-I upregulation following OV infection.

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