Investigation of Vesicular Stomatitis Virus (VSV) as an immunotherapy in melanoma

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Contents

1. Abstract

Cutaneous melanoma is a form of skin cancer that arises from pigment producing cells called melanocytes. While melanoma accounts for a minority of skin cancer cases, they make up the majority of skin-cancer related deaths. Treatments for unresectable or metastatic melanoma were limited before the advances provided by immune checkpoint inhibitor (ICI) therapy, which is now standard of care for late-stage metastatic melanoma patients. Although ICI treatment has tremendously improved outcome for some patients, approximately half do not respond or develop resistance. A proposed mechanism of ICI resistance are mutations in the IFNγ-JAK-STAT pathway which governs immune and antiviral responses. Oncolytic viruses (OVs) are a promising immunotherapy agent for combination therapy with ICI due to their potential induction of host anti-tumor immune activity. T-VEC, a herpes simplex virus-1 (HSV-1) based OV, has received Food and Drug Administration (FDA) approval for treatment of melanoma, but the latest unpublished clinical trial combining T-VEC and ICIs has shown disappointing results. Our lab has published a study showing that melanomas with IFNγ-JAK-STAT mutations have increased sensitivity to OVs, and different OVs have different oncolytic potential. We hypothesized vesicular stomatitis virus (VSV) may be a better oncolytic virus to use in combination with ICIs due to the different melanoma intrinsic signaling pathways that respond to HSV-1 compared to VSV infection. Indeed, we found that a VSV-based OV showed greater oncolytic efficiency than an HSV-based OV in our melanoma models. Additionally, VSV increases human leukocyte antigen 1 (HLA-1) expression in melanoma lines with or without IFNγ signaling defects. Mechanistically, we observed retinoic acid-inducible gene I (RIG-I) was not the only regulator of VSV-induced HLA-1 increase. Since expression of HLA, the antigen presenting protein, and tumor associated antigens (TAAs) are known to play a key role in

mediating ICI responses, our work supports the use of VSV to be used in combination immunotherapy strategies as a melanoma treatment.

2. Résumé

Le melanome cutané est une forme de cancer de la peau survenant chez les cellules productrices de pigment appelées mélanocytes. Malgré le fait qu'ils ne comptent pour qu'une minorité des cancers, ils causent la majorité des décès reliés aux cancers de la peau. Le traitement pour le mélanome non résécable ou métastatique était limité avant les progrès effectués chez les thérapies d'inhibition de point de contrôle immunitaire (ICI), la norme de soin actuel. Bien que les ICIs aient nettement amélioré la survie de certains patients, une moitié n'y répond pas ou y développe une résistance. Un mécanisme de résistance connu sont les mutations dans la voie d'IFN-JAK-STAT qui gouverne les réponses immunitaires et antivirales. Les virus oncolytiques (OVs) sont une forme d'immunothérapie prometteuses en multithérapie avec les ICI grâce à leur potentiel d'induire une réponse antitumorale. T-VEC, un OV à base d'herpès simplex (HSV-1), a reçu l'approbation par la FDA pour le traitement du mélanome, mais le plus récent essai clinique inédit combinant T-VEC et les ICIs a montré des résultats décevants. Notre équipe a publié une étude montrant que les mutations dans la voie d'IFNγ-JAK-STAT causant la résistance aux ICIs rendent les cancers sensibles aux OVs, et différents OV ont différents potentiels oncolytiques. Nous émettons l'hypothèse que le virus de la stomatite vésiculeuse (VSV) serait un meilleur OV à utiliser en multithérapie avec les ICIs dû aux différentes voies de signalisation intrinsèques au mélanome répondant à une infection par HSV-1 comparées à VSV. En effet, nous avons trouvé qu'un virus à base de VSV démontre une plus grande efficacité oncolytique qu'un virus à base de HSV dans nos modèles de mélanome. De plus, VSV augmente l'expression de human leukocyte antigen 1 (HLA-1) chez les mélanomes ayant ou n'ayant pas de défauts reliés à IFNγ. Mécaniquement, nous avons vu que retinoic acid-inducible gene I (RIG-I) n'est pas le seul régulateur de l'augmentation de HLA-1 par VSV. Puisque l'expression de HLA-1, qui présente

les antigènes, et les antigènes associés aux tumeurs (TAAs) jouent un rôle clé dans la réponse médiée par les ICIs, notre étude supporte l'utilisation de VSV dans les stratégies d'immunothérapie combinées pour le traitement du mélanome.

3. Acknowledgements

First and foremost, I would like to thank my supervisor, Dr. Ian Watson, for his support throughout my graduate studies. His passion and dedication to the project, his enthusiasm, his encouragements, and his advice, but also his compassion as fellow human were what helped me push through even at times when things were going awry. I would also like to thank our lab manager, Mozhdeh Ahanfeshar-Adams, for her training, support and listening both in and outside of the lab. Last but not least, I would also like to thank the team members of the Watson lab, Rached Alkallas, Kayla Heney, Diana Berry, Marine Lingrand, Tiffany Lin, Aristophane Chay, Claris Gu, Rong Ma, Dr. Mathieu Lajoie and Lee Ann Chau Ramsay as well as past members, Shivshankari Rajkumar, Trieu Tan Nguyen, Dr. Alexandra Lissouba for their support and insights. My time at the lab would not have been the same without their cheerful and contagious energy. Graduate studies can get stressful and arduous, but their presence and companionship made it easier to go through. I would like to thank my Research Advisory Committee members, Dr. Tommy Alain, Dr. Selena Sagan, and Dr. Jose Teodoro for their advice, expertise, and questions that helped consolidate the project.

4. Preface and Contributions of Authors

Oncolytic viruses (OVs) are a type of immunotherapy that preferentially infects and lyses (oncolysis) cancer cells that have often disabled anti-viral signaling during the tumorigenesis process. This study aimed to provide insight into the potential of a Vesicular Stomatitis Virus (VSV)-based OVs as a potential melanoma therapy. Through *in vitro* immunoblot, real-time quantitative polymerase chain reaction (RT-qPCR), viability and fluorescence-activated cell sorting (FACS) assays, we provided mechanistic insight providing a rationale for utilizing VSV to improve patient responses to standard of care ICI therapy. All research conducted should be considered original content. I declare the invaluable contributions of Trieu Tan Nguyen in the making of the Colo857 JAK2 overexpression cell line as well as the YUMMER1.7 Jak2 KO cell lines. I declare my contributions for the rest of the experiments, including, but not limited to, the immunoblot, RT-qPCR, viability and FACS assays.

5. Abbreviations

APAF-1 – Apoptotic peptidase activating factor 1

APC – antigen presenting cell

B2M – beta-2-microglobulin

BTLA – B- and T-lymphocyte attenuator

C – cytosine

CAF – cancer-associated fibroblast

CCL4 – chemokine (C-C motif) ligand 4

CD – cluster of differentiation

CD4 – Cluster of differentiation 4

CD8 – Cluster of differentiation 8

CEACAM1 – Carcinoembryonic antigen-related cell adhesion molecule 1

CRISPR – clustered regularly interspaced short palindromic repeats

CTLA-4 – Cytotoxic T-lymphocyte associated protein 4

CXCL12 – CXC motif chemokine ligand 12

CXCL9 – CXC motif chemokine ligand 9

CXCR4 – CXC motif chemokine receptor 4

DAMP – damage-associated molecular pattern

DC – dendritic cell

DNA – Deoxyribonucleic acid

DR – Durable response

EGFR – epithelial growth factor receptor

eIF2-a – eukaryotic translation initiation factor 2a

ER – endoplasmic reticulum

ERK – extracellular signal-related kinase

FACS – fluorescence activated cell sorting

FDA – Food and Drug Administration

G – guanine

GDP – guanosine diphosphate

GTP – guanosine triphosphate

GTPase – guanosine triphosphatase

GEF – guanine nucleotide exchange factor

GM-CSF – Granulocyte-macrophage colony-stimulating factor

GSEA – gene set enrichment analysis

GTP – guanosine triphosphate

HLA – human leukocyte antigen

HSV-1 – Herpes simplex Virus 1

ICI – Immune Checkpoint Inhibitor

ICP – Immune checkpoint protein

ICP0 – infected cell protein 0

ICP34.5 – infected cell protein 34.5

ICP47 – infected cell protein 47

IDO – indoleamine 2,3-dioxygenase

IFN – Interferon

IFNGR2 – interferon gamma receptor

IL-10 – interleukin 10

IL-2 – interleukin2

IL-35 – interleukin 35

irAEs – immune related adverse effects

IRF1 – interferon regulatory factor 1

ISG – interferon stimulated gene

JAK – Janus Kinase

Kbps – kilo base pairs

KD – knockdown

KLRG1 – killer cell lectin-like receptor subfamily G member 1

KO – knock out

LAG-3 – lymphocyte-activation gene 3

LGP2 – laboratory of genetics and physiology 2, DHX58

LoF – loss of function

MAPK – mitogen-activated protein kinase

MAVS – mitochondrial antiviral-signaling protein

MCL-1 – induced myeloid leukemia cell differentiation protein

MDA5 – melanoma differentiation-associated protein 5

MDSC – myeloid derived suppressor cell

MEK – mitogen-activation protein kinase kinase

MHC – major histocompatibility complex

MMR – DNA mismatch repair

MOI – multiplicity of infection

mRNA – messenger ribonucleic acid

MSI – microsatellite instability

NF1 – Neurofibromin 1

NIS – sodium/iodide symporter

NK – natural killer

OR – overall response

ORF – open reading frame

OS – overall survival

OV – Oncolytic virus

PAMP – pathogen associated molecular pattern

PD-1 – Programmed cell death protein 1

PD-L1 – programmed death ligand 1

PFS – progression free survival

PI3K – phosphoinositide 3-kinase

PI3Ky – phosphoinositide 3-kinase y

PKR – protein kinase RNA-activated

POMC – Proopiomelanocortin

PTEN – phosphatase and tensin homolog

Q – glutamine

RIG-I – retinoic acid-inducible gene 1

RNA – ribonucleic acid

RT-qPCR – reverse transcriptase quantitative polymerase chain reaction

SHP-2 – src homology-2 domain containing protein tyrosine phosphatase-2

siRNA – small interfering ribonucleic acid

STAT – signal transducer and activator of transcription

T – thymine

TAA – tumor associated antigen

TAM – tumor associated macrophage

TAP – transporter associated with antigen processing

TAPBP – TAP binding protein

$TCID_{50}$ – median tissue culture infectious dose

TCR – T-cell receptor

TGF-b – transforming growth factor beta

TIGIT – T-cell immunoglobulin and ITIM domain

TIL – tumor infiltrating lymphocyte

TIM3 - T-cell immunoglobulin mucin-3

TMB – tumor mutational burden

TME – tumor microenvironment

TNF-a – tumor necrosis factor alpha

T-VEC – Talimogene Laherparepvec

UV – Ultraviolet

VEGF – vascular endothelial growth factor

VSV – Vesicular stomatitis virus

WES – whole exome sequence

WT – Wild type

YUMMER1.7 – Yale University Mouse Melanoma Exposed to Radiation

6. Comprehensive literature review

6.1 Melanoma

Melanoma is a cancer arising from melanocytes principally found at the basal layer of the epidermis. Melanocytes are also found at other anatomical sites such as hair follicles, the uvea, the mucosal epithelia, and meninges[1]. Cutaneous melanocytes are responsible for the production of melanin, which is the pigment responsible for the coloration of our skin[2]. While cutaneous melanoma accounts for a minority of skin cancer cases (5%), they make up the majority of skin cancer-related deaths[3]. Furthermore, the incidence of melanoma is on the rise. In 2020, there was an estimate of 1.5 million cases of skin cancers, and of those, melanoma accounted for approximately a fifth of them (325 000)[4]. While the pathogenesis of melanoma has not been linked to a single cause, multiple risk factors that have been identified. A major risk factor for development of melanoma is exposure to ultraviolet (UV) radiation namely from sunlight and tanning beds[5]. Although ultraviolet A (UVA) and ultraviolet B (UVB) are known to contribute to melanogenesis and progression in many ways, UVB is known to cause direct deoxyribonucleic acid (DNA) damage leading to somatic mutations through the generation of dipyrimidine photoproducts[6]. Indeed, cutaneous melanoma are known to harbor an increased base mutation load compared to almost all other solid tumors, which can be attributed to the high load of cytidine to thymine (C > T) transitions, a characteristic of UV-induced mutation[7]. For nearly two decades, it has been known that activating mutations in *BRAF* and *RAS* are found in nearly 50% and 20% of melanomas, respectively[8]. Both genes encode for key regulators of the mitogen-activated protein kinase (MAPK) RAS-RAF-mitogen-activated protein kinase kinase (MEK)-extracellular signal-regulated kinase (ERK) signaling cascade. Interestingly, hotspot mutations

that affect p.V600 found in *BRAF*, which accounts for ~90% of *BRAF* mutations, and Q61, G12 and G13 found in *NRAS*, are not characteristic UV-induced mutations[9]. Despite this, there is a durable epidemiological relationship between *BRAF* mutations and sun exposure, which is not completely understood. Melanomas arising on intermittent sun-exposed skin are more likely to contain *BRAF* mutations compared to those arising from glabrous skin and mucosal melanomas[10].

6.2 Melanoma Therapies

When diagnosed early before metastasizing to regional lymph nodes or distant parts of the body, melanomas is highly curable with surgical resection[11]. Historically, metastatic melanoma has been one of the most treatment-resistant cancers. For example, dacarbazine, a chemotherapeutic drug, was the standard of care for advanced or metastatic melanoma despite only having a 27% 1-year survival[12]. Currently, the standard of care includes targeted therapies and immunotherapy[13]. Targeted therapies such as BRAF and MEK inhibitors are used to treat BRAF p.V600 mutant melanoma patients by targeting the MAPK pathway, which regulates many cellular processes such as proliferation and differentiation. Under normal signaling conditions, cell surface receptor tyrosine kinases, once bound to their ligand, dimerize resulting in autophosphorylation, recruiting guanine exchange factors (GEFs) to the membrane. GEFs activates small guanosine triphosphatases (GTPases) by converting inactive RAS-guanine diphosphate (GDP) to the active RAS-guanine triphosphate (GTP) form[14]. Active RAS promotes hetero- and homo-dimerization of RAF kinases, BRAF and CRAF, activating them and initiating a cascade of phosphorylation events culminating in transcription of genes regulating cell proliferation and survival[14]. Mutations in *BRAF* lead to constitutive activation of MAPK signaling resulting in uncontrolled proliferation. Early clinical trials demonstrated BRAF

monotherapy improves overall survival to 6 to 7 months for patients with unresectable metastatic melanoma[15,16]. Currently, combination BRAF and MEK inhibitors are used as the standard-of-care targeted therapy regimen for *BRAF* p.V600 mutant melanoma patients to produce more durable survival benefits to patients compared to monotherapy, although resistance remains a challenge[17]. Conversely, immunotherapy produce more long-term durable benefits for melanoma patients[18].

6.3 High dose cytokines

The ability of the immune system to combat one's own cancer has been known since as early as the 19th century[19]. One of the first Food and Drug Administration (FDA) approved immunotherapies to treat metastatic melanoma was high-dose interferon (IFN)-a-2b, approved in 1996, which is a cytokine capable of exerting anti-tumor properties such as anti-proliferation, anti-angiogenesis, an increase in Natural Killer (NK) cell activity and an increase in tumor antigen presentation through human leukocyte antigen 1 (HLA-1) and human leukocyte antigen 2 (HLA-2)[20]. High dose interleukin-2 (IL-2) was another regimen approved by the FDA in 1996 for the treatment of advanced melanoma which was capable of increasing NK and T-cell proliferation[21]. Although these regimens could produce long-term survival benefits for a subset of patients, the low response rates, and the adverse immune related effects of these treatments limited its utility.

6.4 Immune Checkpoint Inhibitors (ICIs)

A crucial part of the antitumor effect exerted by the host's immune system relies on the proliferation and activation of NK and effector T-cells[22]. For a highly immunogenic cancer such as melanoma, tumor proliferation would presumably require the tumor microenvironment to

suppress such processes. This is thought to be achieved by cancer cells expressing immune regulatory proteins, such as programmed death-ligand 1 (PD-L1), which are normally expressed by regulatory T-cells to downregulate effector T-cell activity[23]. To counter this aberrant inactivation of the immune system, monoclonal antibodies targeting these proteins were developed, alleviating tumor mediated inhibition. The use of these antibodies to treat metastatic melanoma in clinical settings showed great promise. In 2010, results from a phase 3 clinical trial in patients with metastatic melanoma showed ipilimumab alone, an antibody against cytotoxic T-lymphocyte associated protein 4 (CTLA-4), improved overall survival (OS) by over 3 months compared to a melanoma-specific antigen vaccine which led to its FDA-approval for treatment of metastatic melanoma in 2011[24]. Shortly after, in 2015, results from a phase 3 clinical trial in patients with metastatic melanoma, when treated with pembrolizumab, an antibody against programmed death 1 (PD-1), compared to ipilimumab, showed an improved 6-month progression free survival (PFS) (46.4-47.3% vs 26.5%, respectively), estimated 1-year OS (68.4-74.1% vs 58.2%, respectively, and overall response (OR) (32.9-33.7% vs 11.9%, respectively)[25]. In the same year, results from a phase 3 clinical trial involving metastatic melanoma patients showed patients treated with nivolumab had a 1-year OS of 72.9%, another PD-1 antibody, compared to 42.1% when treated with dacarbazine, the only chemotherapy agent approved by the FDA for metastatic melanoma[26]. In 2018, James Allison and Tasuku Honjo would both receive the 2018 Nobel Prize in Physiology or Medecine for their discovery of CTLA-4 and PD-1, pioneering the research on immune checkpoint inhibitor therapies. With their immense success, these immune checkpoint inhibitors (ICIs) have since been approved for the treatment of a number of other liquid and solid tumors[27]. ICIs are now considered standard of care for the treatment of metastatic melanoma.

6.5 Anti-CTLA-4 monoclonal antibody

The discovery of CTLA-4 was made in the early 1980s, but it wasn't until 2011 that ipilimumab, a monoclonal antibody against CTLA-4, was approved by the FDA for the treatment of metastatic melanoma[27]. This checkpoint protein competes with cluster of differentiation 28 (CD28), both of which are found on the surface of CD4+ and CD8+ T-cells, to bind with B7-1 and B7-2, found on the surface of antigen-presenting cells (APCs)[28]. CTLA-4 and CD28 binding have antagonistic effects where CD28 binding mediates a co-stimulatory effect while CTLA-4 mediates a co-inhibitory effect[29]. Although the mechanism is now being revisited and contested, the current understanding of how CTLA-4 blockade exerts an antitumor effect lies in the early activation stages of T-cells[30]. Under normal circumstances, CTLA-4 binds to B7 on APCs in the tumor-draining lymph node with a higher avidity than CD28, leading to a dominant negative regulation resulting in decreased T-cell proliferation[31]. Preclinical and clinical data suggests that CTLA-4 blockade results in the enhanced activation of both CD4+ and CD8+ T-cells through an increased production of IL-2[30,32].

6.6 Anti PD-1/PD-L1 monoclonal antibody

PD-1 is another immune checkpoint protein that has been widely studied. PD-1 is predominantly expressed on the cell surface of activated T-cells, NK cells, B lymphocytes, macrophages and dendritic cells (DCs), acting as an inhibitor of both the innate and adaptive immune responses[33]. Binding of PD-1 to its ligands, PD-L1 and PD-L2, results in the recruitment of src-homology 2 domain-containing phosphatase 2 (SHP-2) which downstream effects translates to a decrease of tumor infiltrating lymphocytes (TIL) antitumor activity through increased T-cell apoptosis, decreased IL-2, IFNγ and tumor necrosis factor alpha (TNF-α)

production[34]. PD-L1 is normally found on regulatory T-cells, B-cells, macrophages and APCs, but it has been shown that it is commonly overexpressed in tumor cells allowing them to escape immune surveillance[35]. Whereas anti-CTLA-4 effects are mainly seen in the tumor-draining lymph node, the anti-PD-1/PD-L1 axis controls immune response in the tumor microenvironment[33]. A number of antibodies have been approved by the FDA targeting PD-1, which include nivolumab, pembrolizumab and cemiplimab, and against PD-L1, such as atezolizumab, avelumab and duravulumab[34].

6.7 Clinical ICI efficacy in melanoma

While ICIs have been shown to be one of the best therapeutic strategies to treat melanomas, over 50% of patients do not benefit from long-term survival benefits, either due to innate or acquired resistance[36]. In a recent follow-up of the Checkmate-067 clinical trial comparing ipilimumab and nivolumab monotherapy and combination therapy, the 6.5-year (OS) rate for ipilimumab monotherapy and nivolumab monotherapy were 26% and 42%, respectively, while combination therapy increased OS to 49%[37]. As for pembrolizumab, from the Keynote-006 phase 3 trial comparing pembrolizumab and ipilimumab monotherapy, a 5-year OS rate of 38.7% was achieved following pembrolizumab monotherapy[25]. It should be noted that immune-related adverse events (irAEs) occur in up to 90% and 70% of the patients treated with anti-CTLA-4 and anti-PD-1/PD-L1, respectively[24,38,39]. In light of the significant portion of unresponsive patients and commonly occurring irAEs, a clear need has emerged to identify resistance mechanisms to better personalize prescription of ICI therapy to optimize response and limit side effects.

6.8 Immune checkpoint inhibitor (ICI) resistance

A number of studies have examined and found overlapping mechanisms of resistance to first-line ICI treatment (primary resistance) or in responders who relapse after a period of objective response (acquired resistance)[40]. Resistance may arise from tumor-intrinsic factors that include absence of antigen presentation or genetic alterations within tumor cells that help diminish the immune response. Resistance can also arise from tumor-extrinsic factors, which include an immune-suppressive tumor microenvironment.

6.9 Tumor-extrinsic Resistance factors

ICIs utilize the ability of the immune system to combat one's cancer by inhibiting the immunosuppressive signaling pathways affecting immune effector cells, effectively lifting the "brakes" of the immune response[41]. Therefore, lack of immune effectors (i.e., the target of ICIs), or self-suppression of the immune response has been linked to poor ICI responses. For example, lack of PD-L1 expression, lack of TIL recruitment, secretion of immunosuppressive cytokines and chemokines and the presence of immunosuppressive immune derived cell types are all tumor-extrinsic resistance mechanisms that have been reported in the literature[42–45].

6.10 Lack of TIL recruitment through non-genetic basis

Cancer development and progression is known to be heavily influenced by the surrounding cells and tissues, such as the immune cells, blood vessels, the extracellular matrix, fibroblasts, etc., which is termed as the tumor microenvironment (TME)[46]. One way the TME evolves to evade immune surveillance is by preventing T-cell infiltration. An increase in activated, cancer-specific T-cells following ICI treatment cannot target tumors if they cannot get in physical contact with the tumor cells[42]. For example, cancer associated fibroblasts (CAFs)

promote immune exclusion through two methods. CAF-mediated stiffening of the stromal matrix by dense collagen matrix formation has been shown to physically hinder T-cell motility, preventing their localization to the tumor region[47]. The chemokine C-X-C motif chemokine 12 (CXCL12), secreted by CAFs, was also found to localize to cancer cells, excluding T-cells from the same regions, a phenotype which can be reversed following C-X-C motif chemokine receptor 4 (CXCR4) inhibition, the receptor for CXCL12[48]. The importance of T-cell inclusion limiting ICI response was shown in a melanoma clinical trial employing single agent anti-PD-1, pembrolizumab, where patients who benefitted from treatment showed higher CD8+ T-cell density in the tumor margin and inside the tumor compared to those who progressed[49].

6.11 Lack or overexpression of immune checkpoint proteins (ICPs) ligand in TME

Anti-PD blockade mainly functions through the disruption of the anti-PD-1/PD-L1 immune inhibitory axis. Therefore, lack of expression of either protein in the TME has been linked to anti-PD-1 resistance due to the absence of the therapeutic target[44]. This has been termed "target-missing" resistance, where either PD-L1, TILs or both are missing from the TME[50]. Conversely, overexpression of alternative immune checkpoint proteins (ICPs) promote resistance through their direct immunosuppressive effects. Studies have shown that lymphocyte-activation gene-3 (LAG-3), an immune checkpoint ligand, is increased following anti-PD-1 treatment as compensation mechanism to promote resistance through immune evasion[51]. Conversely, co-inhibition resulted in better clinical outcome in mice with a complete regression within 50 days in 8 out of 10 mice compared to 4 out of 10 mice with anti-PD-1 treatment alone[52]. Increased expression of numerous other alternative checkpoint protein, such as T-cell immunoglobulin mucin-3 (TIM-3), B- and T-lymphocyte attenuator (BTLA), T-cell immunoglobulin and ITIM domains (TIGIT) and carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1),

has been correlated with disease progression and poor T-cell function restoration which can we reversed with co-inhibition of the respective upregulated checkpoint protein[53–56].

6.12 Immunosuppressive Cells

A subset of immune cell types possess functions to restrict the immune response, normally playing a crucial role in self-tolerance and restricting autoimmune responses, but have been found to contribute to immune escape[43]. FoxP3 positive regulatory T-cells (T reg) suppress effector T-cell activity by secreting inhibitory cytokines, such as TGF-β, IL-10 and IL-35, resulting in decreased proliferation and activation of T-cells[57,58]. T reg cells also directly kill T-cells and APCs through granzymes and perforin and competitively interact with major histocompatibility complex (MHC) II through LAG-3 suppressing DC maturation[57,58]. Murine studies show that T reg depletion restores/enhances anti-tumor immunity and that response to anti-CTLA-4 treatment was associated with a high ratio of effector to regulatory T-cells[59,60]. Myeloid derived suppressor cells (MDSCs) have also been described as major regulators of the immune response. It has been reported that patients exhibiting low frequencies of MDSCs are more likely to respond to anti-CTLA-4 treatment[61]. Independent reports also showed that inhibition of phosphoinositide 3-kinase y (PI3Ky), a key player in MDSCs recruitment, combined with anti-CTLA-4, improve anti-tumor responses in mice bearing tumors compared to anti-CTLA-4 treatment alone[62]. Similar results were shown for PI3Ky inhibition where combination treatment with anti-CTLA-4 and anti-PD-1 resulted in increased tumor regression and long-term survival compared to combination treatment alone in mice[63]. Tumor associated macrophages (TAMs), both M1 and M2, are another subset of immune regulatory cells thought to influence ICI response. In addition to secreting cytokines and chemokines as previously

mentioned, M2 macrophages can directly suppress T-cell function through the PD-L1 and B7-H4 signaling pathways in hepatocellular carcinoma and ovarian cancer, respectively[64,65].

6.13 Resistance caused by tumor-intrinsic factors

In addition to the immune suppressive tumor microenvironment, adaptations within the tumors affect immune therapy responses[43]. Despite ICIs targeting the immunological "brakes", if the immune system cannot recognize self from non-self, such therapies will be limited. A number of studies have investigated how tumor cells evade immune surveillance through multiple cell intrinsic mechanisms that alter immune recognition.

6.14 Alterations in signaling pathways

Dysregulation of a multitude of pathways have been implicated as mechanisms of ICI resistance. For example, MAPK mutations are found in approximately 85% of melanomas[40]. Activation of MAPK signaling leads to production of vascular endothelial growth factor (VEGF),interleukin 6 (IL-6) and interleukin 10 (IL-10), which are known to inhibit T-cell recruitment and function[66]. Similarly, loss of phosphatase and tensin homolog (PTEN) signaling, also commonly found in melanoma, leads to increased phosphoinositide 3-kinase PI3K/AKT signaling[67]. Loss of PTEN correlates with inferior outcomes to anti-PD-1 therapy through decreased IFNγ and granzyme B expression, T-cell mediated autophagy, and thus, tumor cell clearance and decreased T-cell trafficking to the tumors[68]. One study also showed an increase of PD-L1 following PTEN loss, contributing to the inactivation of effector T-cells[69] Independent of AKT, oncogenic activation of PI3K signaling pathway was shown to be able to suppress HLA expression and antigen presentation[70].

The B-catenin/Wnt signaling cascade, a tumor cell intrinsic pathway, has been identified as a potential resistance mechanism to ICI treatment. Notably, the stabilization of B-catenin

leads to the constitutive activation of the Wnt signaling pathway resulting in decreased expression of chemokine C-C motif ligand 4 (CCL4), inhibiting CD103+ DCs, and therefore, DC mediated T-cell recruitment[71]. Conversely, tumors lacking B-catenin expression have improved ICI responses[71]. Interestingly, non-canonical Wnt signaling pathway activation through Wnt5a in melanoma has been shown to increase indoleamine 2,3-dioxygenase (IDO) activity and decrease IL-6 and IL-12 expression, increasing regulatory T-cell development in the tumor microenvironment[72]. Consequently, in mouse melanoma models, IDO knockout mice treated with anti-CTLA-4, as well as with anti-PD-1, showed delay in melanoma growth and overall survival compared to wild-type mice[72,73]. Pharmacological inhibition of IDO in combination with anti-CTLA-4 treatment was also able to synergistically hinder melanoma progression in mice[74].

Finally, a large body of evidence has revealed the importance of the interferon γ (IFNγ)-Janus Kinase (JAK)- signal transducer and activator of transcription (STAT) pathway in mediating ICI response[40]. Canonical IFNγ signaling starts with the binding of cytokine IFNγ to the extracellular domain of its receptor composed of subunits interferon gamma receptor 1 (IFNGR1) and IFNGR2[75]. JAK1 and JAK2 kinases are associated to the intracellular domains of IFNGR1 and IFNGR2, respectively[75]. This causes the dimerization and autophosphorylation of both JAK subunits, activating them and allowing them to subsequently phosphorylate downstream STAT1. When phosphorylated, STAT1 homodimerizes and translocates to the nucleus to act as a transcription factor (TF) of wide array of genes involved in antiviral, antiproliferative and immunomodulatory responses, including increased MHC expression and immune recruitment[75]. Tumor-intrinsic loss of IFNγ-JAK-STAT signaling has been linked to decreased T cell killing and ICI antitumor effects. For example, in a small cohort of 16

melanoma tumors from patients treated with anti-CTLA-4, 9 out of the 12 non-responders showed defects in the IFNγ signaling pathway while none of the responders showed any similar defects[76]. Furthermore, two studies implicated *JAK1* and *JAK2* mutations in primary and acquired resistance. In 2016, whole exome sequencing (WES) analysis of match pre- and post-anti-PD-1 treatment biopsies from a small cohort found acquired *JAK1* and *JAK2* mutations in treatment resistant patients[77]. Furthermore, homozygous loss of function mutation in *JAK1* were found in published case reports in patient that had primary resistance [78]. We should note that larger meta-analyses have suggested mutations in the IFN signaling pathway are found in patients that respond to anti-PD-1[79], and therefore more work is needed to understand the role of the tumor intrinsic IFN signaling in mediating anti-PD1 response. However, conversely, gene set enrichment analysis (GSEA) has consistently revealed IFNγ gene signatures are predictive of ICI responsiveness in melanoma patients[80].

6.15 Lack of tumor associated antigen

Immune-mediated clearance of cancer cells relies on the distinction and recognition by T-cells of self from non-self peptides[81]. In the context of cancer, aberrant oncogenic protein expression or activation and tumor suppressor inhibition arising from somatic mutations are often the first steps in tumorigenesis[82]. However, these now mutated proteins are distinct from their wild-type counterparts, giving rise to tumor-associated antigens (TAAs) that can be potentially recognized by the immune system to specifically target tumor cells. One of the first biomarkers of Similar findings were seen for anti-CTLA-4 treatment where whole exome sequencing (WES) analysis of 110 patients with metastatic melanoma treated with ipilimumab showed that nonsynonymous tumor mutation burden (TMB) and neoantigen load was significantly associated with clinical response[83]. Furthermore, patients displaying mis-match

repair (MMR) deficiency, resulting in increased TMB and thus higher likelihood of TAA presence, showed greater immune-related OR as well as immune-related PFS (40% and 78%, respectively) following anti-PD-1 treatment compared to those who displayed MMR proficiency (0% and 11%, respectively)[84]. In support, a meta-analysis of WES of more than 1000 ICI treated patients spanning 7 different cancer types showed that the strongest predictor to ICI response was clonal TMB, which are nonsynonymous mutations found in the whole population of tumor cells, closely followed by total TMB, which are nonsynonymous mutations found in the bulk tumor[85]. However, it should be noted that there are exceptions to this predictor. In a study looking at microsatellite instability (MSI), among MSI-high patients, all of which had high TMB, less than half responded to anti-PD-1/PD-L1 treatment[86]. Conversely, patients with Hodgkin's lymphoma, which carries little to no TMB, are very sensitive to anti-PD-1 treatment. An objective response was reported in 87% of treated patients, 17% of which reported a complete response[87].

6.16 Lack of antigen presentation

In order for an antigen to be recognized by the immune system, it must first be presented at the cell surface through a MHC molecule, or HLA in the case of humans[88]. The HLA system is mainly composed of HLA class I and class II complexes. Class I HLA (HLA-1) presents endogenous antigens and class II (HLA-2) presents exogenous antigens[88]. HLA-2 is mostly found on APCs while HLA-1 is found on most cells including tumor cells, in this case potentially presenting TAAs[88]. Before TAAs are presented to the cell surface, oncogenic or mutated proteins must first be digested by the proteosome and then transported to the endoplasmic reticulum (ER) through the transporter associated with antigen processing (TAP), which is embedded in the ER. In the ER, TAP forms the loading complex along with "empty"

HLA-1 and other chaperones such as TAP binding protein (TAPBP). TAPBP is a dedicated chaperone which loads peptides onto the HLA-1 molecule, stabilizing the peptide-loading complex[89]. Once stably loaded, the HLA-1 complex moves through the ER, into the Golgi apparatus and finally to the cell surface[88]. T-cells recognize these cell surface HLA-1 molecules through TCR binding, leading to their activation[90]. As the main recognition method by CD8+ T-cells, loss of antigen presentation, through any of the steps covered above, has been linked to immune escape and resistance to ICIs[91]. Studies have widely reported that inactivating and truncating mutations in the *B2M* gene encoding a subunit of the HLA-1 complex are associated with low levels of HLA-1 and lead to lack of recognition by CD8+ T-cell[77,92,93]. In a CRISPR screen, loss of IRF2, and IFN regulatory factor, was associated with lack of HLA-1 presentation on tumor cells, and subsequently, decreased T-cell killing[94]. Specifically in ICI treatment resistance, *MEX3B*, a gene involved in downregulation of HLA-1, was found to be expressed at higher levels in non-responders to anti-PD-1 compared to responders[95]. Similarly, transcriptional downregulation of HLA-1 was associated with primary resistance to anti-CTLA-4 treatment[96]. Together, work to date has identified a number of tumor-intrinsic and -extrinsic mechanisms of immune escape with implications for immunotherapy strategies to overcome treatment resistance.

6.17 Oncolytic Viruses

Oncolytic viruses (OVs) are an important form of immunotherapy. Reports dating back as early as the 19th century observed viral infection associated with concomitant spontaneous tumor regression, mainly in leukemia and lymphoma[97–99]. More recently, reports have described regression of leukemia, Hodgkin's lymphoma disease and Burkitt's lymphoma linked to measles infection supporting the notion that viral infections under specific have anti-tumor effects[100–102].

As a result, significant resources have been invested in OV research. Notably, toxicity and limited anti-tumor properties have limited the use of OVs[103]; however, advances in virus engineering, are addressing many of these challenges.

Treating malignant gliomas in mice using Herpes simplex virus (HSV) lacking its thymidine kinase gene, a neurovirulence gene, was one of the first successful applications of OV[104]. The deletion of the thymidine kinase rendered HSV unable to replicate in nondividing cells and in the human nervous system while retaining replication in dividing cells[104]. This provided newfound appreciation for the oncolytic potential of engineered viruses. In recent years, efforts have been made to attenuate the virulence of OV virulence in healthy cells and increasing their oncolytic potential and their cancer specificity.

OVs exhibit a dual mechanism of action towards tumor cell clearance. First, OVs selectively infect and lyse cancerous cells while limiting harm to healthy cells[105]. Different viruses will inherently have different tropism for distinct cell types. For example, rabies virus will selectively infect neurons, while hepatitis B virus will selectively infect hepatocytes. However, almost all viruses will exhibit tropism for tumor cells due to their inherent resistance for apoptosis and host cell translation suppression[105]. They are also able to infect tumor cells through specific viral entry receptors which are expressed on tumor cell surface[106]. In parallel, cancer cells often harbor driver mutations, such as constitutively active RAS signaling or defective antiviral IFN type 1 signaling, which supports selective replication of viral particles[107,108]. While the cells are infected, they also limit the tumor cells' ability to proliferate as it hijacks the host cellular translational machinery for viral replication[109]. Second, selective virus-mediated rupture of tumor cells promotes immunogenic cell death releasing cytokines, TAAs, damage-associated molecular patterns (DAMPs) and pathogen-associated molecular

patterns (PAMPs) resulting in a robust anti-tumor immune response[110]. TAAs are phagocytosed by APCs and the released cytokines, such as IFNs, TNF-a and ILs, stimulate their maturation and guide tumor recognition through antigen presentation to prime CD4+ and CD8+ T-cells against tumor cells[111–113]. Interestingly, this priming of CD4+ and CD8+ T-cells against TAAs promotes both local and systemic immune response that can overcome resistance to ICI treatment[114]. In addition to T-cell activation, type 1 IFNs and DAMPs are also able to prime NK cells, further boosting the anti-tumor immune response[115].

6.18 Herpes Simplex Virus 1 (HSV-1)

A widely studied OV and extremely widespread virus is HSV-1. It is a prototypical lipid bilayer enveloped, linear double stranded DNA virus in the Herpesviridae family. Its viral genome is approximately 152 kbps in length, encoding for approximately 80 open reading frames (ORFs)[116]. As an OV, HSV-1 is an attractive vector due to its natural lytic capabilities, a large viral genome that includes approximately 30 kb of nonessential genes, allowing for ease of genetic manipulation, its inability to cause insertional mutagenesis to host cells and the existence of specific antiviral drugs against it[117]. To date, a number of HSV-1 OV variants with differential genetic profiles have been studied such as NV1020, G207 and HSV-1716, but the most extensively studied strain is talimogene laherparepvec, or T-VEC[117–120].

6.19 Talimogene Laherparepvec (T-VEC, Imlygic, OncoVex)

T-VEC is a modified HSV-based OV and it is first and only FDA-approved OV for the treatment of melanoma[121]. It has been modified to be less virulent in healthy cells, through the deletion of herpes ICP34.5, and to be more immunogenic through the deletion of ICP47 and the addition of human GM-CSF[121]. Upon HSV-1 infection, activation of interferon-induced PKR

allows PKR to phosphorylate host eIF2-a, resulting in its inactivation and consequently host protein translation arrest. To bypass this arrest, HSV-1 expresses ICP34.5 protein which inhibits PKR, resulting in eIF2-a reactivation[122]. As PKR signaling is usually lost in tumor cells, double deletion of ICP34.5 allows healthy cells to shut off protein translation upon infection, whereas tumor cells, having hindered PKR signaling, would not shut off protein translation, therefore increasing tropism to tumor cells[123]. To increase immunogenicity, the gene encoding for ICP47 has also been deleted. ICP47 mediates immune evasion by binding to TAP, preventing viral peptides translocation to the ER, thereby preventing their loading onto HLA-1 molecules and, ultimately, their presentation on the cell surface[124]. Additionally, the gene encoding for human GM-CSF has been added to its viral genome. GM-CSF is an immune stimulatory cytokine which has been shown to have many proinflammatory effects in a granulocytes and lymphocytes including pro-developmental and -maturation effects in DCs as well as proliferative and activating effects in T-cells, increasing both DC- and T-cell-mediated anti-tumor response[125]. Clinically, the first successful trial compared intralesional T-VEC and subcutaneous GM-CSF in patients with unresected stage IIIB to IV melanoma, where durable response (DR) and OR in T-VEC (16.3% and 26.4%, respectively) was significantly higher than GM-CSF alone (2.1% and 5.7%, respectively)[126]. These results led to a phase III clinical trial studying T-VEC in combination with pembrolizumab (MASTERKEY-265). However, the MASTERKEY-265 study reported that although there were some improvements, T-VEC did not significantly improve PFS, or OS compared to placebo combination therapy[127]. However, T-VEC has shown to play an important role in treating in-transit metastases where patients present with multiple locoregional metastatic lesions which are difficult to treat. In a 2018 single institution study by Perez et al. of 27 patients with unresectable stage IIIB-IV melanoma, a CR of 44% and a ORR 57% over

median follow-up of 8.6 months was achieved[128]. On a larger scale, in a 2019 multi-institutional evaluation by Louis et al. of 80 patients with advanced melanoma, a 39% CR and 57% ORR was determined over a median follow-up of 9 months[129].

6.20 Vesicular Stomatitis Virus (VSV)

VSV is a prototypical single-stranded, negative sense RNA virus from the Rhabdoviridae family[130]. Its entire genome is 11 kbps long, containing 5 genes encoding for their nucleocapsid protein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G) and large polymerase protein (L)[131]. As an OV, VSV is attractive due to its high titer and potent lytic activity that allows this virus to spread at a faster rate, infecting more cells before immune recognition and clearance by the host immune system[132]. VSV induces apoptosis through multiple mechanisms, proving its potent lytic activity. VSV M protein promotes apoptosis through apoptotic peptidase activating factor 1 (APAF-1)-mediated activation of caspase-9[133]. Additionally, VSV decreases levels of induced myeloid leukemia cell differentiation protein (MCL-1), a known anti-apoptotic factor, through proteasomal degradation[134]. Transient ER stress and infection of tumor vasculature was also seen following VSV infection, resulting in increased apoptosis[135,136]. Two variants of VSV, VSV-IFNβ and VSV-IFNβ-NIS, have been used in phase I clinical trials[132]. Interestingly, in a trial studying VSV-IFNβ-NIS treatment in canine cancers, all canines showed measurable tumor regression while not shedding any viral particles[137]. To date few studies have investigating the role of VSV in melanoma.

7. Hypothesis and summary of aims

As the latest phase 3 clinical trial (MASTERKEY-265) failed to meet its primary endpoint of improved PFS for combination pembrolizumab treatment with an HSV-based OV, T-VEC, compared to placebo, we hypothesized that VSV would be a better OV to use in combination with ICIs due to the different melanoma intrinsic signaling pathways that respond to HSV-1 compared to VSV infection. To address our hypothesis, we first assessed the oncolytic capabilities of both OVs in melanomas *in vitro* with or without intact IFN signaling. We also examined intrinsic signaling differences between VSV- and HSV-based OV on IFN signaling and key downstream biomarkers of ICI response, including HLA-I. Next, since the only FDA-approved OV for melanoma is T-VEC, and HSV-based OV that expresses exogeneous GM-CSF, we examined the effects of GM-CSF on HLA-I expression in mice. Finally, in preparation of future combination anti-PD-1 studies in mice, we examined intracellular effects of VSV and HSV in mouse melanoma cell lines.

8. Results

8.1 VSV-Δ51 is more efficient at oncolysis and increases IFN signaling compared to HSV-1-dICP0 in melanoma.

Inactivating mutations in the IFNγ-JAK-STAT pathway have been linked to modulating ICI responses in patients[77,78]. Our lab previously showed that JAK1 and JAK2 loss renders melanomas sensitive to OVs[138]. One observation made in this study was that VSV was more cytotoxic compared to HSV in melanomas lines with and without a functional IFNγ-JAK-STAT pathway. To confirm these results, we performed dose-response cytotoxicity assays with VSV and HSV-1-based OVs in the JAK2-null melanoma line, Colo857 and following restoration of stable overexpression of JAK2 following lentiviral expression. Cells were pre-treated with 200 IU/mL IFNγ for 24 hours prior to infection by either VSV-Δ51 or HSV-1-dICP0 at increasing concentrations for 3 hours. After 72 hours, cell viability was measured using Cell Titer Glo. As expected, melanomas with or without JAK2 defects showed increased sensitivity to VSV-Δ51 compared to HSV-1-dICP0 as measured by Tissue Culture Infectious Dose ($TCID_{50}$), the concentration of the number of infectious viral particles per cells in a well at which 50% of the cells show cytopathic effects (CPE). The $TCID_{50}$ of VSV-Δ51 in the JAK2-null Colo857 was 22 times lower than the $TCID_{50}$ of HSV-1-dICP0 (Fig. 1A). When we did the same viability assay with Colo857 overexpressing JAK2, again, we observed a 19-fold difference in $TCID_{50}$ when treating with VSV-Δ51 compared to HSV-1-dICP0 (Fig. 1B). These results demonstrated the increased VSV-Δ51 cytotoxicity requiring less viral particle to induce cytopathic effects in melanomas compared to HSV-1-dICP0 and being the most cytotoxic to JAK2-null melanomas.

In addition to direct oncolysis, a second anti-tumoral mechanism of OVs, and of particular importance when considering OV utility in combination with ICIs, is the ability to induce a potent immune response by releasing DAMPs, PAMPs and cytokines upon cell lysis, increasing recruitment of immune cells. As such, we sought to investigate the ability of either OVs in activating the IFNγ-JAK-STAT pathway, one that governs immune responses. Colo857 and Colo857 JAK2 overexpression cells were treated with IFNγ as control, or infected with VSV-Δ51 or HSV-1-dICP0 and gene expression changes of interferon stimulated genes (ISGs), such as STAT1, IRF1 and CXCL9, and mRNA expression fold-changes were assessed using RT-qPCR. As expected, IFNγ had no effect on ISG expression in the JAK2-null Colo857 line, whereas IFNγ treatment in the Colo857 JAK2 overexpression line increased ISG expression (Fig. 1C, 1D, 1E). Next, we compared ISG expression following low and high dose infection with VSV-Δ51 and HSV-1-dICP0. In either cell lines, when infecting with low concentration of VSV-Δ51, IRF1 expression was slightly increased, and this increase was comparable to both low and high concentrations of HSV-1-dICP0 (Fig. 1D). Similarly, low concentration VSV-Δ51 slightly increased expression of STAT1 and CXCL9 which was comparable to both concentrations of HSV-1-dICP0 (Fig. 1C, 1E). A one-way ANOVA was performed to compare the effects of the two OVs on mRNA-fold change. Changes in IRF1 (p = .22) and STAT1 (p = .16) were not statistically significant but show a consistent trend. Changes in CXCL 9 were statistically significant (p = 0.038). Interestingly, in both cell lines, high concentration of VSV-Δ51 increased expression of the ISGs, STAT1 and IRF1, to comparable levels to IFNγ treatment in Colo857 JAK2 (Fig. 1C, 1D). Furthermore, high concentration of VSV-Δ51 increased expression of the ISG, CXCL9, to higher levels than observed with 200 IU/ml of IFNγ (Fig. 1E).

Taken together, these results demonstrate VSV-Δ51 can induce ISG expression to greater levels than HSV-1-dICP0 even in melanomas that lack a functioning IFNγ-JAK-STAT pathway.

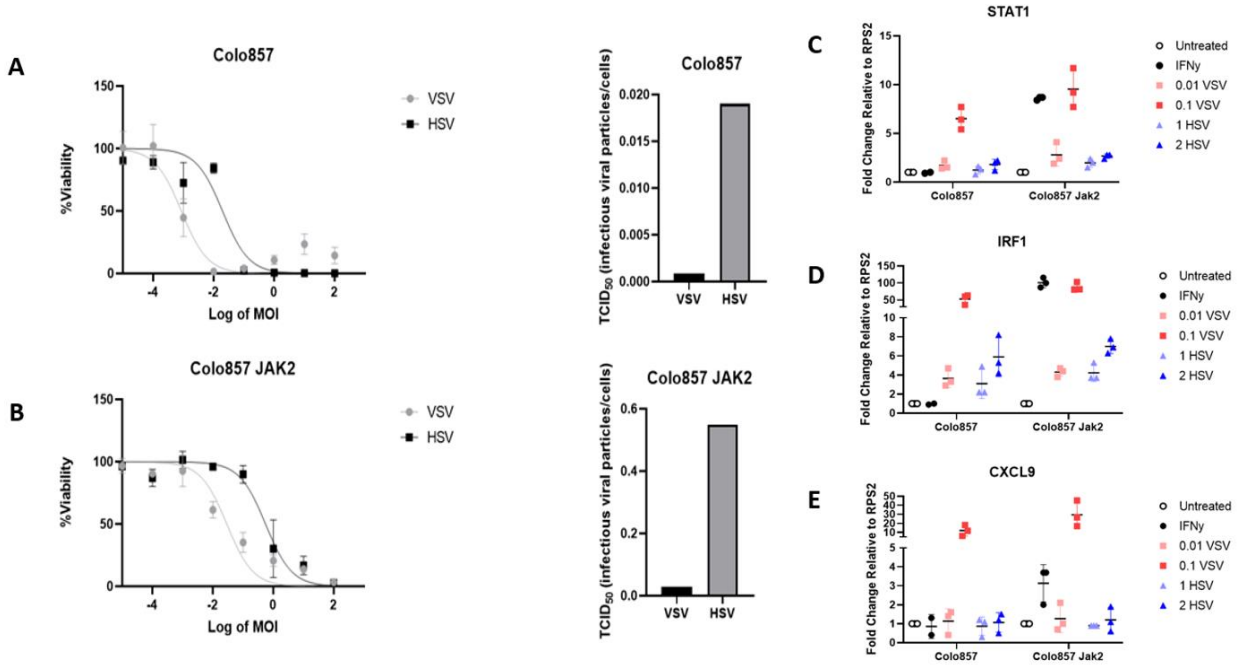


Figure 1. VSV-Δ51 treatment is more cytotoxic and increases ISGs in melanomas compared to HSV-1-dICP0. Log-transformed dose-response curve in A) Colo857, JAK2-null melanoma, and B) Colo857 overexpressing JAK2 following VSV-Δ51 treatment and HSV-1-dICP0 and their respective $TCID_{50}$ values. Cells pre-treated with IFNγ (200 IU/mL) were infected with OVs for 3h and incubated for 72h. Cell viability was measured following incubation. Relative mRNA fold-change, normalized against RPS2, of C) STAT1, D) IRF1 and E) CXCL9 were measured using RT-qPCR following VSV-Δ51 (0.1 or 0.01 MOI), HSV-1-dICP0 (1 or 2 MOI) or IFNγ (200 IU/mL) treatment. RT-qPCR experiments were done in triplicates.

8.2 VSV-Δ51 treatment increases antigen presentation protein HLA-1

High TMB and expression of HLA has been linked to favorable ICI responses[81,83]. Interestingly, it was recently shown that IFN-resistant melanomas are still able to upregulate HLA-1, an IFN regulated gene, through RIG-I following dsRNA challenge[139]. Since VSV-Δ51 is an RNA virus and HSV-1-dICP0 is a DNA virus, we sought investigate their ability to induce HLA-1 expression. To that end, we assessed IFN signaling and protein levels of HLA-1 through immunoblot analysis of Colo857 and Colo857 JAK2 overexpression cells following OV treatment. As expected, independent of JAK2 status, VSV-Δ51 treatment increased levels of phosphorylated STAT1 and total STAT1 to similar levels to IFNγ treatment in the JAK2

competent melanoma line (Fig. 2A). This was not seen following HSV-dICP0 treatment (Fig. 2A). VSV-Δ51 infection also increased HLA-1 protein levels to a higher extent than IFNγ treatment at 200 IU/ml (Fig. 2A). This was not seen following HSV-dICP0 treatment (Fig. 2A). As T-VEC is a modified strain of HSV that expresses GM-CSF, we next sought to assess the capability of GM-CSF to increase HLA-1. We treated Colo857 and Colo857 JAK2 cells with an increasing amount of GM-CSF and performed immunoblot assays. GM-CSF treatment, at physiologically relevant concentrations, did not increase HLA-1 protein expression (Fig. 2B). Together, our results demonstrate that VSV-Δ51 increases HLA-I and antigen presentation, but HSV-1-dICP0 and GM-CSF has no effect or even decreases HLA-I expression.

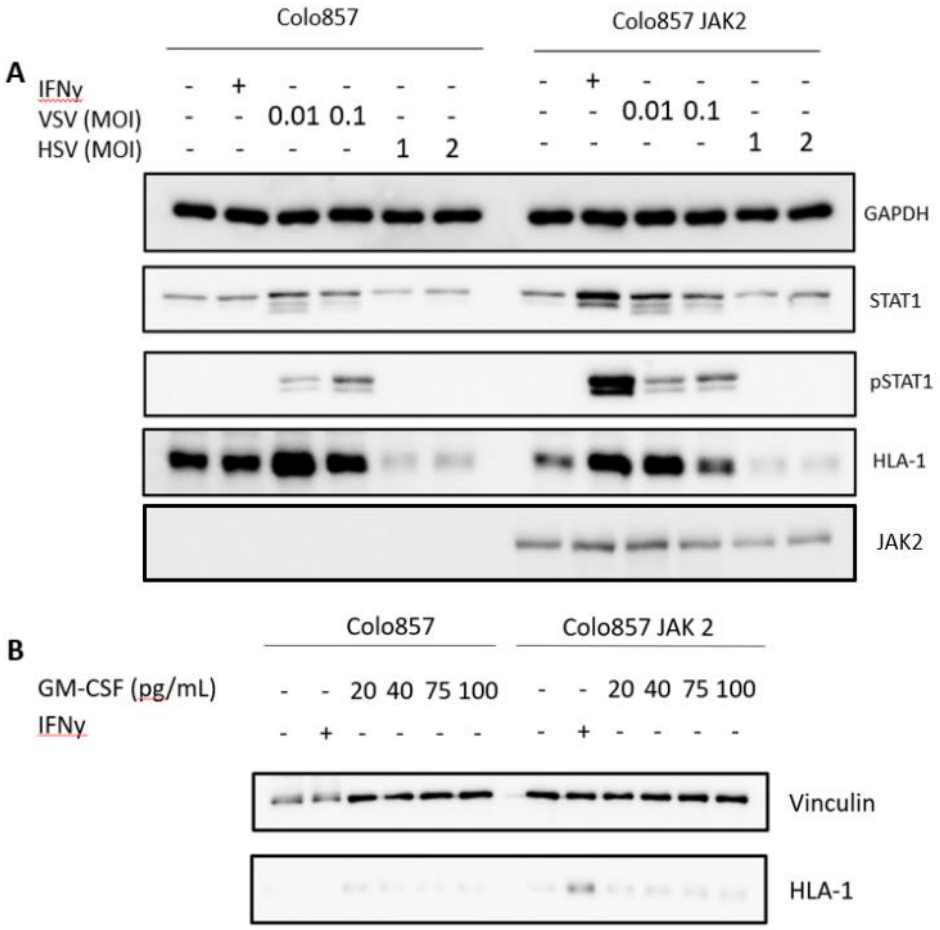


Figure 2. VSV-Δ51 treatment increases HLA-1 protein expression. A) Immunoblot analysis of Colo857 and Colo857 JAK2 following IFNγ or OV treatment. Cells were treated with VSV-Δ51 or HSV-1-dICP0 for 1h and incubated for 24h before protein extraction. B) Immunoblot analysis following GM-CSF treatment. Cells were treated with media supplemented with increasing amounts of GM-CSF for 24h before protein extraction and immunoblot assay.

8.3 VSV-Δ51 mediated HLA-1 increase does not depend on RIG-I

RIG-I plays an important role regulating HLA-I in response to dsRNA and viral infections. To determine if the increased expression of HLA-I upon infection of VSV-Δ51 was dependent on RIG-I, we performed siRNA knockdown experiments. Colo857 and Colo857 JAK2 overexpressing melanoma cell lines treated with siRNA against RIG-I were subsequently infected with VSV-Δ51 at 0.1 MOI for immunoblot analysis. As expected, IFNγ treatment increased HLA-1 protein levels in the JAK2 competent melanoma line, but not in JAK2 null line (Fig. 3A). VSV-Δ51 treatment increased HLA-1 to similar degree as IFNγ treatment. However,

transient knockdown of RIG-I had no effect on the VSV-Δ51-mediated increase in HLA-1, demonstrating this effect is not dependent on RIG-I.

8.4 Similar phenotype consistent in mouse melanoma models

We next examined the effects of VSV-Δ51 and HSV-1-dICP0 on H2-Kb, the mouse counterpart to HLA-1. These *in vitro* studies were performed to determine the feasibility of performing future combination OV and ICI studies. Therefore, we performed OV experiments on the YUMMER (Yale University Mouse Melanoma Exposed to Radiation) 1.7 cell line, which is a mouse line derived from a C57Bl/6J *BrafV600E CA/+; Pten lox/flox; Tyr-CRE-ERT2*

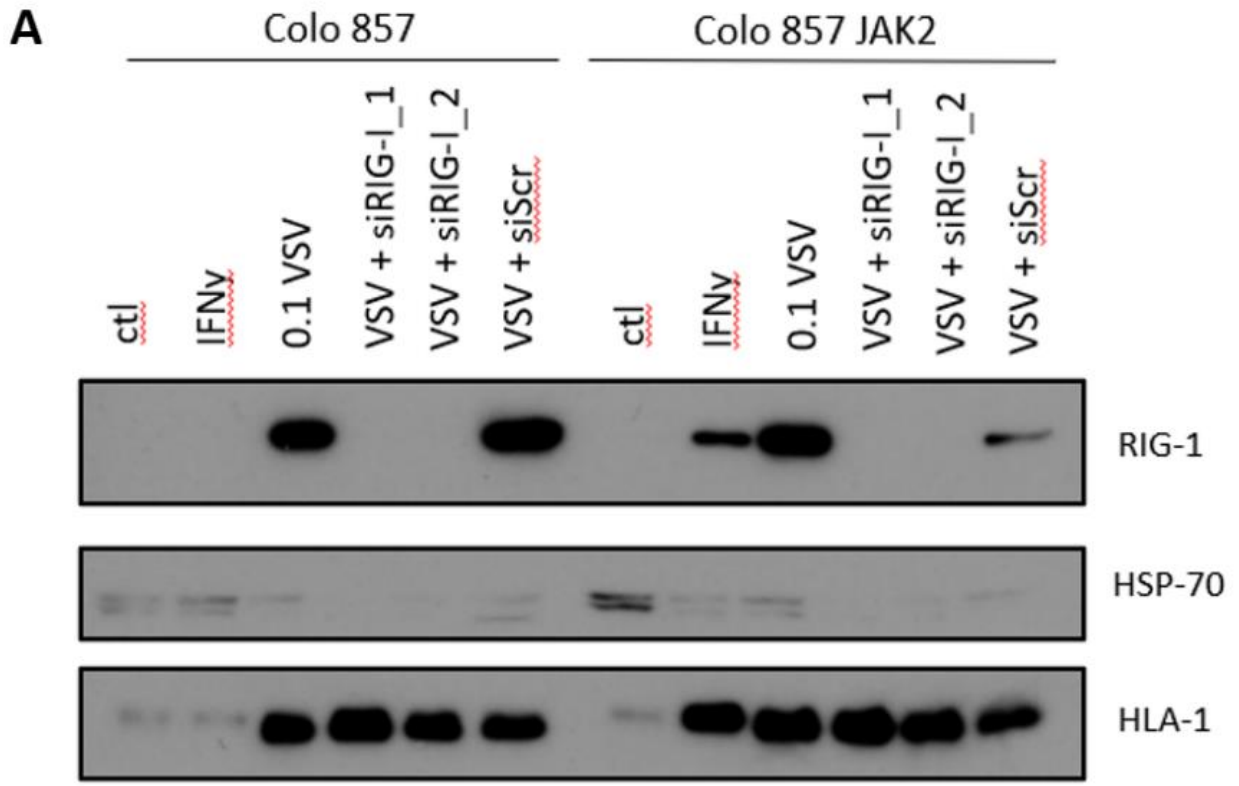


Figure 3. VSV-Δ51 treatment following siRNA knockdown of RIG-I. A) Immunoblot analysis of Colo857 and Colo857 JAK2 following transient knockdown of RIG-I and VSV-Δ51 treatment. Cells were treated with siRNA against RIG-I for 24h followed by VSV-Δ51 treatment for 1h. IFNγ supplemented media (200 IU/mL) was applied before the incubation in IFNγ condition. Cells were incubated for 24h following viral treatment.

melanoma[140,141]. This line has also been UV-irradiated to model human sun-exposed melanomas. We treated YUMMER 1.7 and YUMMER 1.7 *Jak2* KO cells with VSV-Δ51, HSV-1-dICP0 or IFNγ and performed immunoblot assays to assess IFN signaling and FACS assays to assess cell surface expression of H2-Kb. As expected, following IFNγ treatment increased Stat1, pStat1 and Pd-l1 protein levels (Fig. 4A). Consistent with our findings in human melanoma lines, these proteins were increased following VSV-Δ51 treatment, but not HSV-1-dICP0 (Fig. 4A). Following IFNγ treatment, H2-Kb levels were increased as seen by the increase of APC fluorescence (Fig. 4B). Consistent with our data using human melanomas, after VSV-Δ51 infection, cell surface H2-Kb levels were increased to a similar degree as IFNγ treatment when compared to the untreated population, independent of Jak2 status (Fig. 4B, 4C). Cell surface H2-Kb levels between untreated and HSV-1-dICP0 treated cells were comparable. Interestingly, uninfected cells from the VSV- treated plate also had higher surface H2-Kb levels,

demonstrating a stronger systemic pro-immune response following VSV-Δ51 compared to HSV-

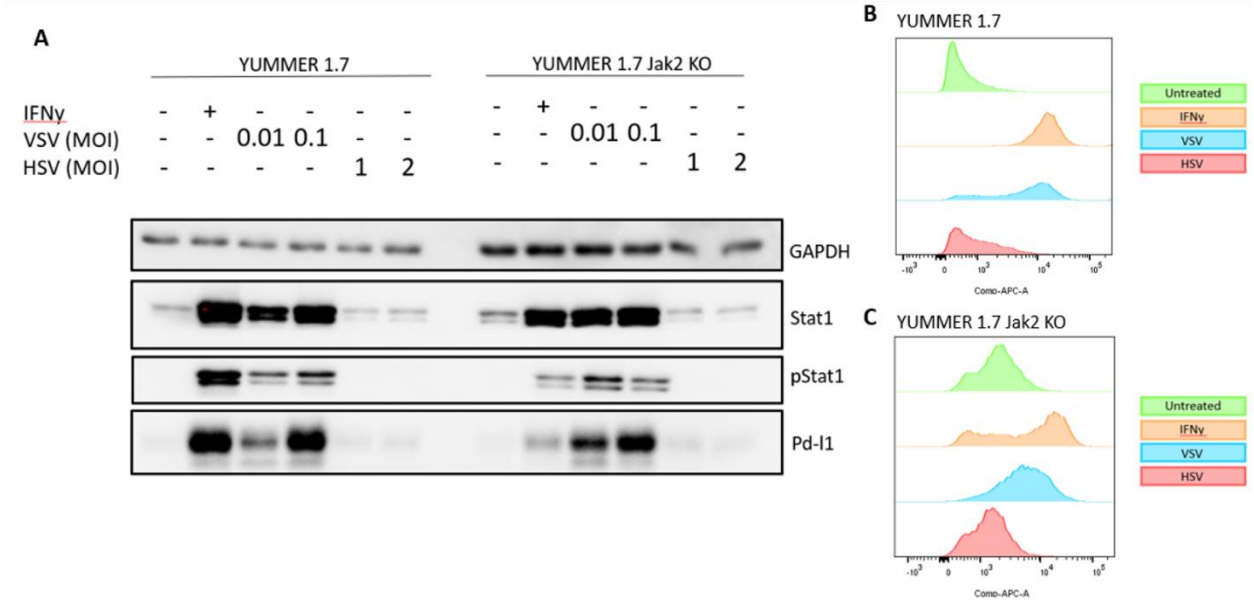


Figure 4. VSV-Δ51 mediated phenotype is consistent in mouse melanoma models. A) Immunoblot assays of YUMMER1.7 and YUMMER1.7 Jak2 KO following IFNγ (200 IU/mL), VSV-Δ51 (0.01 or 0.1 MOI) or HSV-1-dICP0 (1 or 2 MOI). Fluorescence activated cell sorting assays of B) YUMMER1.7 and C) YUMMER1.7 Jak2 KO following IFNγ (200 IU/mL), VSV-Δ51 (0.01 MOI) or HSV-1-dICP0 (1 MOI). H2-Kb cell surface levels were assessed via APC-linked H2-Kb Ab. Dead cells were excluded. Uninfected cells were excluded from virus treated populations. FACS assays were done in triplicates (n=3).

1-dICP0 (Supp. Fig. 1).

9. Discussion

9.1 Conclusions and Future directions

In this thesis, we provide evidence to support VSV as a potential melanoma therapy. Furthermore, compared to HSV-1, VSV induces different melanoma intrinsic signaling compared to HSV, that warrants further investigation of VSV in combination ICI studies. OVs work through a dual mechanism to carry out its anti-tumor effects. They selectively infect and lyse tumor cells and, upon lysis, release factors such as TAAs, cytokines, DAMPs and PAMPs which attract immune cells to induce a potent immune antitumoral response[115]. Using viability assays, we demonstrated that VSV-Δ51 has a higher melanoma oncolysis effect compared to HSV-1-dICP0. Additionally, through RT-qPCR assays, we demonstrated that VSV-Δ51 induces mRNA levels of STAT1, IRF1 and CXCL9 to similar levesl as exogenous IFNγ treatment, whereas HSV-1-dICP0 does not. Using immunoblot assays we saw that only VSV-Δ51 treatment increased protein levels of STAT1 and its phosphorylation, regardless of JAK2 status. The same was seen in our mouse melanoma lines. Importantly, treatment with VSV-Δ51 induced protein levels of HLA-1 in human melanomas and cell surface levels of H2-Kb, the mouse MHC protein, in mouse melanomas. When treating with GM-CSF, an immune maturation factor expressed by T-VEC, at physiologically relevant concentrations, did not affect HLA expression. Together, our data suggests that VSV may be a better OV to use in combination with ICIs compared to HSV-based OVs such T-VEC as it shows more efficient oncolysis and a better potential at inducing systemic immune antitumoral response through increase of ISGs and HLA-1, the two arms of action of OVs.

Future studies based on this work will need to confirm our observations in additional human and mouse models. We believe our *in vitro* data on the YUMMER1.7 mouse melanoma

line serves as a foundation to test both VSV-Δ51 and HSV-1-dICP0 with *in vivo* experiments. The YUMMER1.7 are derived from C57Bl/6J mice to enable *in vivo* xenograft studies. Future studies from our lab will test OV treatment with and without combination ICIs treatment to determine potential applicability of such regimens in the clinic. Additionally, the use of immunodeficient mice, such as $Rag1^{-/-}$ C57Bl/6J mice, would help differentiate between oncolysis-mediated tumor regression and immune-related tumor regression. Humanized patient-derived xenograft model would also provide a clinically relevant platform, especially with patients who do not respond to or developed resistance to first-line ICI treatment. Identifying changes in the TME would also be an important next step in understanding of OV mechanisms to better devise treatment options for patients. We plan on characterizing the immune changes mediated by OV treatment using multiplexed imaging strategies to identify immune-related biomarkers of response[142–147].

We believe the observation that VSV-induced HLA-1 protein expression that is not seen with HSV-1 is clinically relevant. Sequencing studies will be useful to determine the type of antigens presented on the resulting HLA-1 proteins as it could be a double-edged sword. For example, increased tumor-associated antigen presentation would likely work synergistically with ICIs, whereas increased viral antigen presentation would result in rapid clearance of the OV, eliminating it from the TME and reducing the efficacy of OV.

Mechanistically, we observed siRNA-mediated knockdown of RIG-I did not affect VSV-Δ51-mediated increase of HLA-1, demonstrating that RIG-I is not the sole regulator of VSV-mediated HLA-1 increase. As RIG-I is part of a larger family of RNA sensors, future studies should investigate other RIG-I like receptors (RLRs) such as MDA5 and LGP2 or downstream effectors like MAVS which interacts with both RIG-I and MDA5[148].

Clinically, our research is particularly relevant as oncolytic viruses are a promsing avenue of immunotherapy with advances in genetic engineering. OVs are being tested in combination with ICIs in a number of clinical trials accoss multiple cancer types. However, the only currently FDA approved OV for melanoma treatment is T-VEC, which has recently produced disappointing results in combination immunotherapy clinical trials. The most recent phase III clinical trial testing T-VEC in combination with pembrolizumab (MASTERKEY-265) saw no improvement of progression free survival (PFS) or overall survival (OS) between T-VEC and the placebo control. Of note, there is an abundance of clinical trials studying HSV-1, which includes T-VEC and other variants across multiple histological cancer types, whereas VSV clinical trials remain limited with only two ongoing melanoma trials (NCT04291105, NCT03865212). However, the thesis presented here demonstrates VSV oncolysis and ability to induce HLA regarding of IFNg/JAK/STAT status that supports its further investigation as OV to be used in combination ICI studies. ICIs are the standard of care treatment for late-stage metastatic melanomas; unfortunately, approximately half of patients either do not respond or develop resistance[149] . Thus, there is a clear need to develop additional treatment strategies to improve ICI responses. We believe our study here provides support to examine VSV-based OVs in melanoma that has a number of advantages compared to T-VEC.

Although HSV-based OVs are generally extremely safe, known limitations include the risk of reversion, meaning the re-acquisition of virulent genes, and their known latency[117]. HSV is infects human hosts, which is commonly seen around the world. Acquisition of neurovirulence at any point would allow HSV to infect peripheral neurons and result in the loss of tumor specificity, decreasing the oncolytic potential of the virus. Additionally, latency is a known feature of herpesviruses, potentially allowing oncolytic HSV to enter latency during or after

treatment, and reappearing at later times, causing yet unknown adverse effects in previously treated patients[150]. Conversely, while VSV is a well-studied mammal virus, VSV infections in humans are known to be generally asymptomatic or lead to mild illness[151]. In addition, studies have shown that VSV replication is extremely sensitive to IFNs, owing to their interaction with PKR and eIF-2a, which provides an accessible method of containing VSV replication in the case of uncontrolled replication[152]. Thus, VSV may be an overall safer alternative to HSV as an OV template.

9.2 Limitations

One limitation of our study lies in the fact that T-VEC contains a deletion in the ICP47 gene, which normally mediates immune evasion by binding to TAP, the antigen transporter, preventing loading of antigens onto the HLA-1 complex, resulting in its localization in the ER membrane[124]. Our model of HSV-1 still expresses the ICP47 protein, suggesting that HLA-1 presentation might be impaired. However, we saw a decrease of HLA-1 protein using a cell lysis method that solubilizes cytoplasmic, membrane and nuclear proteins, allowing us to look at whole protein expression of a cell[153]. HLA-1 protein levels seen in our immunoblot assays would therefore not be affected by their localization, suggesting that HSV-1-dICP0 does not increase HLA-1 protein levels in the same manner as VSV-Δ51 treatment, resulting in decreased antigen presentation.

In summary, our current study supports VSV as a superior OV compared to HSV for combination studies with ICIs due to its improved oncolysis and its potential induce an immune response. Although more research is needed, our study supports further inverstigation of VSV as a treatment for melanoma patients. Prioritizing VSV-based OVs may yield meaningful clinical

impacts, such as better treatment options, which may affect an increasing number of melanoma patients.

10. Materials and Methods

10.1 Cell Culture

Colo857 cells were maintained in RPMI 1640 medium (Wisent Bioproducts) supplemented with 5% heat-inactivated fetal bovine serum (Gibco, ThermoFisher Scientific) and 1% penicillin/streptomycin (ThermoFisher Scientific). Murine YUMMER1.7 and Vero cells were maintained in DMEM medium (Wisent Bioproducts) supplemented with 5% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin. All cell lines were kept at 37°C in a humidified 5% CO_2 incubator. Vero line was a gift from Dr Martin Richer.

Colo857 JAK2 overexpression cell line, inducible Cas9 and sgJAK2 plasmids were generated by Tan Trieu Nguyen in accordance with the method from his original publication[138]. YUMMER1.7 Jak2 KO cell line was generated by Tan Trieu Nguyen. Inducible Cas9 plasmid was subcloned into YUMMER1.7 and infected cells were selected using gentamicin (ThermoFisher Scientifics). RFP-expressing plasmid containing sgJAK2 was subcloned into inducible Cas9 YUMMER1.7 cells and successfully infected cells were selected using fluorescence activated cell sorting. Lastly, Cas9 protein was induced by doxycycline to knock out Jak2.

10.2 Oncolytic virus propagation and purification

GFP-expressing VSV-Δ51 contains a deletion of the methionine 51 of the M protein. GFP-expressing HSV-1-dICP0 contains a deletion of the infected cell protein 0 gene. Once confluent, Vero cells were infected with VSV-Δ51 or HSV-1-dICP0 at 0.01 MOI for one hour. After 24h or 72hr for VSV-Δ51 or HSV-1-dICP0, respectively, cells and medium were collected. Cell lysis was induced through 3 cycles of freeze-thaw. Following lysis, the media was centrifuged at 4000 RPM for 20 minutes and the supernatant was filtered through a 0.45µM

filter. Viral particles were purified by ultracentrifugation at 20 000 RPM for 1 hour or 15 000 RPM for 90 minutes for VSV-Δ51 or HSV-1-dICP0. Pelleted virus was resuspended in 100 µL neat DMEM media and aliquoted. Stocks were maintained at -80°C. VSV-Δ51 and HSV-1-dICP0 were a gift from Dr. Nahum Sonenberg.

10.3 Plaque Assay

To measure viral titer, standard plaque assays were performed. Vero cells were seeded one day in advance and were treated with increasing MOI for 3 hours. After three hours, viral media was removed, and 0.5% agarose-DMEM solution was overlaid onto the wells. After 3 or 5 days for VSV-Δ51 or HSV-1-dICP0, respectively, or after small plaques could be seen, cells were fixed at room temperature with neutral buffered 10% formalin (Sigma-Aldrich) for one hour. Cells were then dyed with 0.1% crystal violet solution, and plaques were counted to determine viral titer according to the following formula: Plaque Forming Unit (PFU)/mL = (Number of plaques counted) / (Volume of viral media (mL) * dilution factor)

10.4 Virus Treatment

Cells were treated with VSV-Δ51 (0.1, 0.01 MOI) or HSV-1-dICP0 (1, 2 MOI) for one hour and incubated for 18-24h, when VSV-Δ51 and HSV-1-dICP0 plates showed similar levels of GFP expression under light microscope. Treatment was stopped and cells were used for further experiments.

10.5 siRNA Knockdown

siRNA against RIG-I and scramble negative control were purchased from Dharmacon. siRNA transfection was performed according to the manufacturer's protocol prior to virus infection and subsequent experiments.

Non-targeting siRNA: UAAGGCUAUGAAGAGAUAC

siRNA RIG-I #1: CAGAAGAUCUUGAGGAUAA

siRNA RIG-I #2: GCACAGAAGUGUAUAUUGG

10.6 Cytotoxicity Assay

Colo857 cells were seeded at a density of 5000 cells/well in a 96-well plate. After IFNγ pre-treatment (200 IU/mL) for 24h, cells were treated with an increasing MOI of VSV-Δ51 or HSV-1-dICP0 for 3h. Plates were incubated for 72h, after which the viability of the cells was measured using the Cell Titer Glo Assay. Luminescence was read using the instrument FLUOstar Omega Luminometer at 2000 gains. The log-response curves were plotted using GraphPad with reading of untreated cells set as 100% survival and reading cell-free well set as 0% survival.

10.7 Immunoblot

Cells were lysed with Pierce radioimmunoprecipitation assay (RIPA) buffer (ThermoFisher Scientific) supplemented with Halt protease inhibitor (ThermoFisher Scientific). Protein pellets were obtained by centrifugation at 15 000 RPM for 10 minutes and protein concentrations were calculated using colorimetric DC Protein Assay (Biorad). Proteins were denatured using 4X NuPAGE LDS Sample Buffer (Invitrogen) and heat at 95°C for 5 minutes. Samples were run on 10% Bis-Acrylamide SDS-PAGE gels (Biorad) at 180V for 2 hours, or until the dye front has run out. Proteins were transferred onto a nitrocellulose membrane (Biorad) using the Trans-blot Turbo Blot Transfer System (Biorad). Nitrocellulose membranes were blocked in 5% milk protein and incubated with primary antibody overnight. Antibodies against human HSP70 (1:2000; #4872), GAPDH (1:10000; #2294), JAK2 (1:1000; #3230), STAT1 (1:2000; #9172) and phosphoSTAT1(Y701) (1:2000; #7649) were purchased from Cell Signaling Technologies. Antibody against HLA-1 heavy chain (1:2000; MUB2037P) was

purchased from Nordic MUBio. Antibody against Vinculin (1:2000; P18206) was purchased from Abcam. Antibody for mouse PD-L1 (1:2000; 10F.9G2) was purchased from BioLegend. Following primary antibody incubation, membranes were incubated in secondary HRP-linked antibody for an hour before imaging using the ChemiDoc Imaging System (Biorad). HRP-linked Anti-rabbit IgG (1:2000; #7074), anti-rat IgG (1:2000; #7077) and anti-mouse IgG (1:2000; #7076) secondary antibody were purchased from Cell Signaling Technologies.

10.8 RT-qPCR

Following virus treatment, RNA was extracted using the commercial RNeasy mini kit, according to their protocol, from Qiagen Sciences. Extracted RNA was quantified using Nanodrop 2000 from ThermoFisher Scientific. cDNA was synthesized using iScript cDNA synthesis kit from Biorad. RT-qPCR primers were made using the public resource PrimerBank from Harvard University. Expression of IRF1, STAT1, CXCL9 were quantified by mRNA expression using RT-qPCR and normalized against RPS2 mRNA expression. Primer sequences were as follows: IRF1 Forward, 5'-CTGTGCGAGTGTACCGGATG-3'; IRF1 Reverse, 5'-ATCCCCACATGACTTCCTCTT-3'; STAT1 Forward, 5'-CGGCTGAATTTCGGCACCT-3'; STAT1 Reverse, 5'-CAGTAACGATGAGAGGACCCT-3'; CXCL9 Forward, 5'-CCAGTAGTGAGAAAGGGTCGC-3'; CXCL9 Reverse, 5'-AGGGCTTGGGGCAAATTGTT-3'; RPS2 Forward, 5'-CGTCGGTCTGGGTGTTAAGTG-3'; RPS2 Reverse, 5'-GGCTTGCCGATCTTGTTCC-3'. RT-qPCR was performed using iTaq Universal SYBR Green Supermix and cycles were performed using CFX Connect Optics Module from Biorad. The reactions were made as follows: 5 µL SYBR Green Universal Mix, 1µL primer mix containing both reverse and forward primers to make final primer concentration 400nM for each primer and 4µL cDNA template. RT-qPCR run was programmed as follows: 95°C for 3 minutes followed

by 39 cycles of 95°C for 10s, 60°C for 30s and 72°C for 30s, ending with an increase from 65°C to 95°C in increments of 0.5°C to generate a melt curve. Results were analyzed using the ΔΔCt method for relative mRNA expression against RPS2 reference gene.

10.9 hGM-CSF Treatment

Colo857 cells were treated with maintenance RPMI medium supplemented with an increasing amount of recombinant human GM-CSF (R&D systems) for 24h. Following 24h, cells were harvested, and protein was extracted to perform immunoblot.

10.10 IFNγ Treatment

Cells were treated with their respective maintenance medium supplemented with IFNγ (200 IU/mL), purchased from PBL Assay Science. for 18h or 24h before cells were harvested and protein or RNA was extracted to perform immunoblot or RT-qPCR.

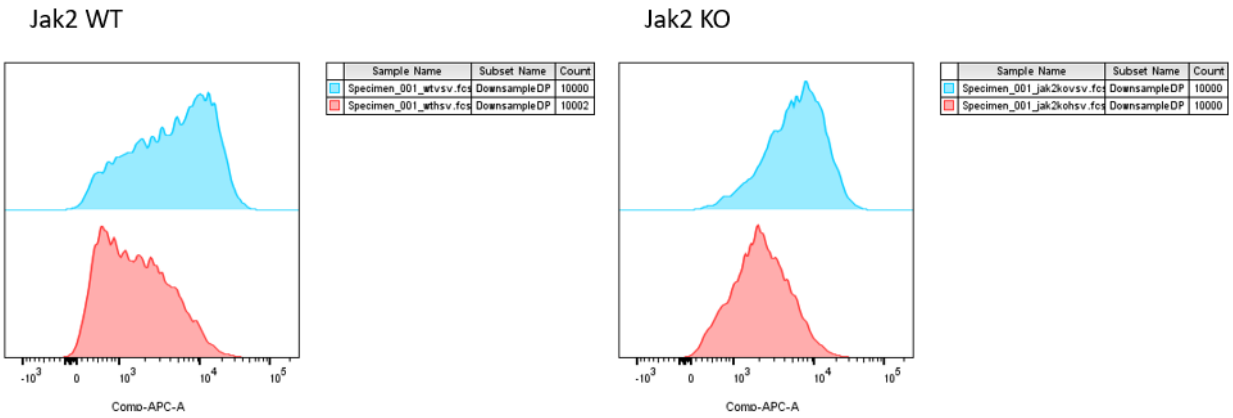
10.11 FACS

Following virus or IFNγ treatment, YUMMER1.7 cells were detached, filtered through a 0.45µM filter and washed with ice cold FACS buffer made from 1X D-PBS (Wisent) supplemented with 5% fetal bovine serum. Cells were then treated with either APC-linked mouse H2-Kb antibody (BioLegend; AF6-88.5) or APC-linked mouse IgG2a isotype (BioLegend; MOPC-173) as control. Propidium iodide (PI) (ThermoFisher Scientifics) stain was used as viability dye. Stained cells were sorted on a FACS Canto II instrument (Becton Dickinson Biosciences) and raw data was visualized using the FACSDiva software (BD Biosciences). Dead cells, PI positive cells, were excluded from analysis. Analysis was performed using FlowJo (FlowJo LLC) software.

10.12 Statistical Analysis

One-way ANOVA statistical analyses were performed using GraphPad Prism. *p*-values were considered significant at $p < 0.05$.

11. Supplemental Figures



Supplemental Figure 1. Cell surface H2-Kb of uninfected cells following viral treatment. FACS assay where dead cells and infected, GFP-positive, cells were excluded leaving live uninfected cells from the same dish plate.

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