Overcoming Immune checkpoint inhibitors resistance using oncolytic viruses in melanoma

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March 2020

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Masters

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1. Abstract

Immune checkpoint inhibitors (ICIs) have produced significant clinical responses in metastatic melanoma patients, quickly becoming the standard-of-care treatment for this disease. Nevertheless, despite these unprecedented results, a large fraction of melanoma patients still do not benefit from this therapy. Recent whole exome sequencing studies of ICI non-responders have identified mutations in interferon (IFN)γ signaling pathway genes as possible mechanisms of resistance. In support, a number of functional CRISPR screens have identified key regulators of the IFN signaling pathway as ICI resistance genes. IFNs signal through the IFN receptors-JAK-STAT axis to upregulate interferon-stimulated genes (ISGs) that have anti-proliferative, adaptive immune, as well as antimicrobial and antiviral resistance properties. We hypothesize that ICI-resistant mutations in the IFNs signaling pathways would lead to a reduced ability for melanomas to mount an antiviral response, which could result in increased vulnerability to oncolytic virus (OV) therapy. OVs are natural or engineered viruses that preferentially target cancer cells. Currently, Talimogene laherparepvec (T-Vec), a modified OV strain of the herpes simplex virus1 (HSV1), is approved for the treatment of melanoma patients. In this thesis, we show, through patient-derived models, genetic manipulation, and pharmacological inhibition, that loss of IFN response sensitizes melanomas against different types of OVs. This study provides strong support for the use of OVs as a salvage therapy for ICI-resistant melanomas as well as precision medicine strategy for cancers with genetic defects in the IFN pathway. Finally, our study supports the combinatorial use JAK inhibitors with OVs as a therapeutic strategy for treatment-naïve melanomas.

2. Résumé

Les inhibiteurs immunitaires des points de contrôle (ICI) ont produit des réponses cliniques significatives chez les patients atteints de mélanôme métastatique, devenant rapidement le traitement de référence pour cette maladie. Néanmoins, malgré ces résultats sans précédent, une grande partie des patients atteints de mélanôme ne bénéficient toujours pas de cette thérapie. Des études récentes de séquençage de l'exome entier de non-répondeurs aux ICIs ont identifié des mutations dans les gènes de la voie de signalisation de l'interféron (IFN) γ comme mécanisme possible de résistance. Ainsi, un certain nombre de cribles CRISPR fonctionnels ont identifié des régulateurs clés de la voie de signalisation IFN en tant que gènes de résistance ICI. Les IFN transmettent des signaux via l'axe des récepteurs IFN-JAK-STAT pour réguler positivement les gènes stimulés par l'interféron (ISG) qui ont des propriétés immunitaires adaptatives anti-prolifératives, ainsi que des propriétés de résistance antimicrobienne et antivirale. En plus des ICIs, d'autres immunothérapies, y compris les virus oncolytiques (OV), se sont révélées très prometteuses pour le traitement du mélanôme métastatique. Les OV sont des virus naturels ou modifiés qui ciblent préférentiellement les cellules cancéreuses. Nous émettons l'hypothèse que les mutations résistantes aux ICIs dans les voies IFN conduiraient à une capacité réduite pour les mélanômes à monter une réponse antivirale, ce qui pourrait entraîner une vulnérabilité accrue à la thérapie OV. Déjà, Talimogene laherparepvec (T-Vec), une souche OV modifiée du virus de l'herpès simplex-1 (HSV1), est approuvée pour le traitement des patients atteints de mélanôme et nos recherches effectuées ici pourront être transmises en clinique. Dans cette thèse, nous démontrons, en utilisant des modèles dérivés des patients, ainsi que la manipulation génétique et l'inhibition pharmacologique, que la perte de réponse IFN sensibilise les mélanômes contre différents types de virus oncolytiques. Cette étude permettrait de développer une thérapie combinée entre les ICI et les virus oncolytiques pour lutter contre les problèmes de résistance.

3. Acknowledgments

I would like to firstly thank my supervisor, Dr Ian Watson, for his all support throughout my graduate studies. His expertise on the subject, his insightful questions, his passion for the project, his advice and encouragement when times are tough are what helped me keep going and power through. I would also like to thank my lab manager Mozhdeh Ahanfeshar-Adams, for her supervision and training. I would like to thank all my fellow lab members, Shivshankari Rajkumar, Marine Lingrand, Jaclyn Retallick, Emily Wu, Diana Berry, Kayla Heney, Rached Alkallas, Colton Strong, Farzaneh Jalili, Dr Mathieu Lajoie, LeeAnn Chau Ramsay, Dr Alexandra Lissouba, and past members for making my time in the lab a fulfilling and memorable experience. Graduate study is a difficult journey, and I am glad that they have always been right there with me. I would also like to thank my Research Advisory Committee members, Dr Sidong Huang, Dr Selena Sagan, and Dr Jose Teodoro for their feedbacks and advices on the project.

4. Preface and Contributions of Authors

This project aims to examine the resistance mechanisms to immune checkpoint inhibitors (ICIs) and exploit one such mechanism, defects in the IFNs response pathway, by employing the use of oncolytic viruses. Through the use of several *in vitro* models, we hope to set the stage for further clinical combination therapies that will benefit melanoma patients. All research conducted for this thesis should be considered original content. I declare the contribution of Génome Québec Innovation Centre for all Sanger Sequencing. I declare my contributions to the rest of the experiments, including but not limited to immunoblots, virus propagation, and cytotoxicity assays.

5. Abbreviations

APC – Antigen presenting cell

bp – base pair

B2M – beta-2-microglobulin

CCL4 – C-C motif Ligand 4

CD – Cluster of Differentiation

CNS – Central nervous sytem

CTLA4 – Cytotoxic T-lymphocyte-associated protein 4

CRISPRs – Clustered Regularly Interspaced Short Palindromic Repeats

DC – Dendritic cell

DMSO – Dimethyl Sulfoxide

eIF – eukaryotic Initiation Factor

ED_{50} – 50% Effective Dose

FDA – Food and Drug Association

GADD 34 - Growth arrest and DNA damage-inducible protein 34

GDP – Guanosine diphosphate

GEM – genetically engineered mouse

GFP – Green Fluorescence Protein

GM-CSF – Granulocyte-macrophage Colony-stimulating Factor

GTP – Guanosine triphosphate

ICD – Immunogenic Cell Death

ICP – Infected Cell Protein

IL – Interleukin

HIV – Human Immunodeficiency Virus

HSPG – Heparan Sulfate Proteoglycan

HSV1 – Herpes Simplex Virus 1

HVEM – Herpevirus Entry Mediator

JAK – Janus Kinase

ICAM-1 – Intercellular Adhesion Molecule 1

ICIs – Immune Checkpoint Inhibitors

IFN – Interferon

IFNAR – Interferon Alpha Receptor

IFNGR – Interferon Gamma Receptor

IRF – Interferon Regulatory Factor

ISGF – Interferon-stimulated Gene Factor

IU – international unit

LAG-3 – Lymphocytes Activation Gene 3

LDL – low-density lipoprotein

LoF – Loss of Function

MAPK – Mitogen Activated Protein Kinase

MDSC – Myeloid-derived suppressor cell

MEK – Mitogen-activated protein kinase kinase

MHC – Major Histocompatibility Complex

MOI – Multiplicity of Infection

NK – Natural Killer

NOD-scid mice – Nonobese Diabetic – severe combined immunodeficiency mice

Nup98 – Nucleoporin 98

OV – Oncolytic virus

PAK – p21-activated Kinase

PD-1 – Programmed Cell Death - 1

PD-L1 – Programmed Death Ligand 1

PDX – Patient-derived xenograft

PFU – Plaque-forming unit

PI-3K – Phosphoinositide-3 kinase

PKR – Protein Kinase R

PTEN – Phosphate and Tensin Homolog

PTPN2 – Protein Tyrosine Phosphatase Non-receptor Type 2

Rae-1 – Retinoic Acid Early inducible 1

Rb – Retinoblastoma protein

Rux - Ruxolitinib

SHP-2 – Src Homology Domain 2

STAT – Signal Transducer and Activator of Transcription

TAA – Tumor-associated antigen

TAP – Transported associated with Antigen Processing

TCR – T-cell receptor

TCF/LEF – T-cell Factor/Lymphoid Enhancer-binding Factor

TIM-3 – T-cell immunoglobulin and Mucin domain 3

TME – Tumor microenvironment

Treg – Regulatory T cell

Teff – Effector T cell

T-vec – Talimogene Laherparepvec

VISTA – V-domain Immunoglobulin-containing Suppressor of T-cell Activation

VEGF – Vascular Endothelial Growth Factor

VSV – Vesicular Stomatitis Virus

UVR – Ultraviolet radiation

6. Comprehensive literature review

6.1. Melanoma

Melanoma is a type of cancer that arises from the melanin-producing melanocytes residing in the basal layer of the epidermis. Melanoma is the deadliest form of skin cancer, accounting for approximately 70% of skin cancer-related deaths in Canada[1]. Exposure to ultraviolet radiation (UVR) remains a major risk factor for melanoma. Cells exposed to UVR are susceptible to cytosine to thymine transition mutations at dipyrimidines[2]. Thus, melanoma exhibits a unique UV-induced mutation signature, characterized by the highest mutation rate in solid tumors and predominantly YC>YT mutations[3]. With advance sequencing, researchers have been able to elucidate the compendium of driver mutations responsible for melanomagenesis. Specifically, activating mutations in *BRAF* and *NRAS* are found in 50% and 20% of melanoma patients respectively[4]. BRAF and NRAS are components of the mitogen activated protein kinase (MAPK) signaling pathway regulating cellular proliferation and motility. Interestingly, the common *BRAF* p.V600 and *NRAS* p.G12, G13, and Q61 hotspot mutations are not characteristic UV-induced signature mutations. More recent sequencing studies have identified UVR-induced mutations in other genes such as *TP53, RB, PTEN, RAC1* and non-coding mutations in *TERT* that cooperate with BRAF and NRAS to promote melanoma[3, 5-8]. The current standard of care therapies for treating metastatic melanoma include the use of BRAF (Vemurafenib, Dabrafenib) and MEK (Trametinib, Cobimetinib) inhibitors as well as immune checkpoint inhibitors (ICIs)[9].

6.2. Immune checkpoint inhibitors (ICIs):

The concept of immunotherapy that makes use of the body's own antitumor defense mechanism to fight cancer has been around since the late 19th century[10]. IFNα-2b was one of the first cytokines to be studied for cancer treatment for its role in activating various cells of the

immune systems including T cells, B cells, and dendritic cells (DCs). IFNα-2b was approved by the U.S Food and Drug Administration (FDA) in 1995 as adjuvant therapy for resected stage IIB/III melanoma[11]. Similarly, Interleukin (IL)-2, another cytokine capable of driving T cells' growth, was approved by the FDA in 1998 for the treatment of metastatic melanoma. Unfortunately, their clinical effectiveness was underwhelming, and many patients suffered from adverse immunologic adverse events[9]. Through basic science research discoveries, we have witnessed rapid advances in the development of immunotherapy, culminating into the discovery of immune checkpoints.

To evade the host's immune response, cancers take advantage of co-inhibitory immune checkpoint pathways that normally function to regulate the immune response within a normal physiological range[12]. Thus, antibodies were developed to target these immune checkpoints, thereby re-invigorating the host's immune response against tumors. Currently, ICIs are the most commonly prescribed immunotherapy, leading the way in the battle against cancer[13].

6.2.1. Anti CTLA-4 monoclonal antibody inhibitors

The first ICI to be developed and approved by the FDA for the treatment of melanoma was ipilimumab, an anti-Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) inhibitor antibody[14]. CTLA-4 is a surface receptor present on T cells, that supresses T cells' function through its competition with a co-stimulatory receptor, cluster of differentiation 28 (CD28)[15]. While both CTLA-4 and CD28 recognize their ligands B7 protein 1 and 2 (B7-1 and B7-2) present on antigen presenting cells (APCs), CTLA-4 has a much higher affinity, thus limiting T cell activation by CD28. CTLA-4 acts during the early T cells priming phase in lymph nodes, as its ligands are mostly expressed on APCs[16].

6.2.2. Anti PD-1 / anti PD-L1 monoclonal antibody inhibitors

Programmed cell death protein 1 (PD-1) is another immune checkpoint that negatively regulates the immune response[17]. PD-1 binds to programmed death-ligand 1 (PD-L1) and PD-L2 present on the surface of APCs and tumor cells, and the interactions lead to recruitment of Src homology domain 2 phosphatase (SHP-2) that inactivates CD28 signaling[18]. While essentially leading to the same effect of inhibiting an immune response, CTLA-4 and PD-1 have notable temporal and spatial differences. While CTLA-4 is active during the early priming phase in lymph nodes, PD-1 acts mainly during the effector phase within peripheral tissues[19]. There are several inhibitors targeting the PD-1/PD-L1 axis currently approved for treatment of melanoma, which include Pembrolizumab, Nivolumab, and Cemiplimab (anti-PD-1), as well as Atezolizumab, Avelumab, and Durvalumab (anti PD-L1)[20].

6.2.3. Efficacy of ICIs

ICIs and BRAF inhibitors are used as first-line treatment options for metastatic melanoma patients and are also being used in the neoadjuvant and adjuvant setting [21, 22]. However, over 50% of patients still do not derive long-term survival benefits from ICIs, owing to innate or acquired resistance to treatment[23]. 5-year results from the recent phase 3 Checkmate 067 trial showed overall survival rate of 44% for nivolumab group and 26% in the ipilimumab group. While combination of nivolumab and ipilimumab increases survival rate (52%), more than half the patients (59%) suffered treatment-related adverse events[24]. In light of these results, a major ongoing effort is to identify resistances mechanisms of ICIs.

6.3. Reported mechanisms of resistance to Immune Checkpoint Inhibitors (ICIs):

A number of reported tumor-intrinsic and -extrinsic resistance mechanisms have been reported from analysis of patients who do not respond to initial therapy (primary resistance) or in

patients who develop resistance after a period of response (acquired resistance)[25]. Tumor-intrinsic factors include genetic alterations within the tumors that allow for immune invasion by limiting mechanisms that attract and activate antitumor T-cell functions. Tumor-extrinsic factors include factors not limited to tumors, such as upregulation of alternative checkpoints which can exert inhibitory effects to the antitumor immune response.

6.3.1. Tumor extrinsic factors:

6.3.1.1. Suppressive tumor microenvironment

Tumors do not grow in isolation. Rather, they interact with the surrounding environment consisting of immune and stromal cells, vasculature structures, cytokine expression, presence of extracellular matrix, and a number of other factors[26]. A number of studies have clearly demonstrated the important role of the tumor microenvironment (TME) affecting all aspects of tumor biology[27-29]. Due to the success of ICIs in the treatment of metastatic melanoma, a significant global research effort is underway to identify factors that mediate an immunotherapy response [30]. A number of correlates have been linked to improved ICI response, which include tumor mutation burden, lymphocytic infiltration and specific tumor intrinsic pathways that mediate immune cell exclusion [31-33]. Furthermore, a major ongoing challenge is how to collectively consider all factors associated with an ICI response into a predictive algorithm[34]. For example, various factors can affect response of ICIs despite high CD8+ T cells infiltrations.

Regulatory T cells (Tregs) are a subtype of CD4+ T cells that negatively modulate activity of effector CD8+ T cells (Teffs) via direct contact or by secretion of cytokines such as IL-35, IL-10, and granzyme B[35-37]. In some cases, Tregs are able to infiltrate TME and create an immunosuppressive environment for infiltrating CD8+ T cells. Thus, the ratio of Teff:Tregs usually indicates poor prognosis in cancers[38]. A recent study has demonstrated that depleting Tregs

cells using anti-CD25 potentiates anti-PD-1 blockade in murine models[39]. In another study, response to anti-CTLA4 blockade was found to correlate with ratio of Teff:Tregs[40].

Myeloid suppressor derived cells (MDSCs) is another regulator of the antitumor immune response. MDSCs are a heterogenous population of immune cells from the myeloid lineage that expand rapidly during chronic inflammation and cancer[41]. MDSCs act to suppress not just T cells, but also DCs and natural killer cells. MDSCs within TME negatively regulate T cells through various mechanisms, including L-arginine depletion, production of reactive oxygen species, and nitrosylation of important amino acids[42]. Therefore, high level of MDSCs was shown to correlate with poorer outcome in anti CTLA-4 treated patients[43].

6.3.1.2. Alternative immune checkpoints

In addition to CTLA-4 and PD-1, other coinhibitory immune checkpoints are upregulated in response to increased immune activation[44]. Overexpression of T cell immunoglobulin, mucin domain-3 protein (TIM-3), lymphocyte activation gene 3 (LAG-3), V-domain immunoglobulin-containing suppressor of T-cell activation (VISTA), and others have functions in induced T cell exhaustion. In the case of ICIs, blocking one immune checkpoint, such as anti-PD-1, results in upregulation of others checkpoints, leading to acquired resistance[45, 46]. As a result, significant investment has been made to study and develop antibodies targeting these additional immune checkpoints, and clinical trials are underway to test how best to combine these new inhibitors with the established ICIs[47-49].

6.3.2. Tumor intrinsic factors:

6.3.2.1. Activation of oncogenic pathways

Dysregulation of many oncogenic signaling pathways that confer growth advantage also helps tumors evade immunosurveillance[50]. MAPK pathway is the most commonly mutated

pathway in melanoma[4]. Recognition and subsequent binding of the epidermal growth factor (EGFR) to its receptor trigger the exchange of RAS-bound GDP for GTP, thus activating RAS[51, 52]. Active RAS promotes dimerization of RAF, leading to subsequent phosphorylation of downstream MEK and ERK kinases, controlling cell proliferation and survival[53]. In the context of immunotherapy, MAPK pathway activation also leads to upregulation of soluble factors like vascular endothelial growth factor (VEGF) and IL-8 that function to suppress T cell proliferation and activation[54, 55]. Therefore, clinical trials have been created to evaluate the efficacy of combining MAPK pathway inhibitors with ICIs, but adverse side effects present a problem to this strategy[56, 57].

The phosphoinositide 3-kinase (PI3K) pathway is an important growth signal transduction pathway that is frequently dysregulated in cancers that may play a role in mediating immunotherapy response[58]. In preclinical mouse models, a loss of phosphatase and tensin homolog (PTEN) expression, a negative regulator of the PI3K pathway, promotes resistance to anti PD-1 therapy[59]. Furthermore, PI3K inhibitors combined with anti PD-1 improves survival rates in melanoma-bearing mice[60].

The Wnt-β-catenin signaling is another common oncogenic pathway in cancers[61]. In the canonical pathway, binding of Wnt leads to accumulation and translocation of β-catenin into the nucleus, where it acts as co-activator to members of the T-cell Factor/Lymphoid Enhancer-binding Factor (TCF/LEF) family of transcription factors to regulate genes involved in cell proliferation, migration, and differentiation[62]. In melanoma, hyperactivation of Wnt pathway results in a more invasive and metastatic phenotype[63]. In addition, Wnt signaling in tumors plays a role in T cell exclusion from the TME. By inhibiting transcription of CCL4 in CD103+ DCs and increasing Tregs survival via β-catenin, Wnt pathway activation leads to lower CD8+ T cells proliferation

and infiltration[64]. In clinical studies, a serine threonine kinase, PAK4, which enhances β-catenin stabilisation through phosphorylation, was found to be upregulated in non-responders to anti PD-1 compared to responders[12]. Importantly, PAK4 inhibition seemed to overcome resistance in mouse models[12].

6.3.2.2. Loss of antigen presentation

For T cells to exert their antitumor effects, they must first recognize tumors as foreign[65]. T-cells are activated when the T cell receptor (TCR) binds their cognate antigens on the surface of cancer cells presented by the major histocompatibility complex (MHC). Thus, mutations that disrupt this antigen presentation pathway, whether through the proteasome complex that process antigens, transporter associated with antigen processing (TAP), or the MHC itself, renders tumors resistant against ICIs [66]. A recent study discovered mutations in a component of the MHC class I, beta-2 microglobulin (B2M), in 29.4% of melanoma patients with primary or acquired resistance to ICIs in the analysis of a 17 patient cohort[67].

6.3.2.3. Loss of interferon response,

IFNs, including type I IFNs (α, β, ω) and type II IFNs (γ), function to modulate the immune system[68]. Type I and II IFNs signal through Janus kinase (JAK) and signal transducer and activator of transcription (STAT) families[69]. Binding of type I IFNs to IFN-α/β receptor (IFNAR) triggers auto-phophosphorylation of TYK2 and JAK1[70]. This signal is amplified by subsequent TYK2/JAK1-mediated phosphorylation and heterodimerization of STAT1 and STAT2. Heterodimerized STAT1/2 together with IFN regulatory factor 9 (IRF9), form the IFN-stimulated gene factor 3 (ISGF3) acting as transcription factor[71]. Conversely, IFNγ binds to interferon gamma receptor (IFNGR) and phosphorylates JAK1 and JAK2. Phosphorylated STAT1s homodimerize and act as transcription factor[72]. While having distinct biochemical properties and structures, type

I and type II IFNs do activate overlapping signal transduction pathways and regulate a common set of genes[73] **(Fig. 1)**. In the context of cancer, IFNs function to increase tumor antigen presentation, release of cytokines that recruit immune cells, and have antiproliferative effects on cancer cells[74]. These events lead to an effective antitumor response. Conversely, IFNs are also involved in cancer's adaptive immune resistance mechanism by inducing PD-L1 expression on the surface of tumor cells[75]. This is thought to render PD-1/PD-L1 therapy all the more effective in tumors with intact IFN response[76]. Aside from its immunomodulatory effects, IFNs are involved in cellular antiviral functions. While type I IFNs play a more predominant role in the viral defense, type II IFNγ can also exert antiviral properties, as it regulates an overlapping set of genes, and even induce type I IFNs expression in an amplification loop[77].

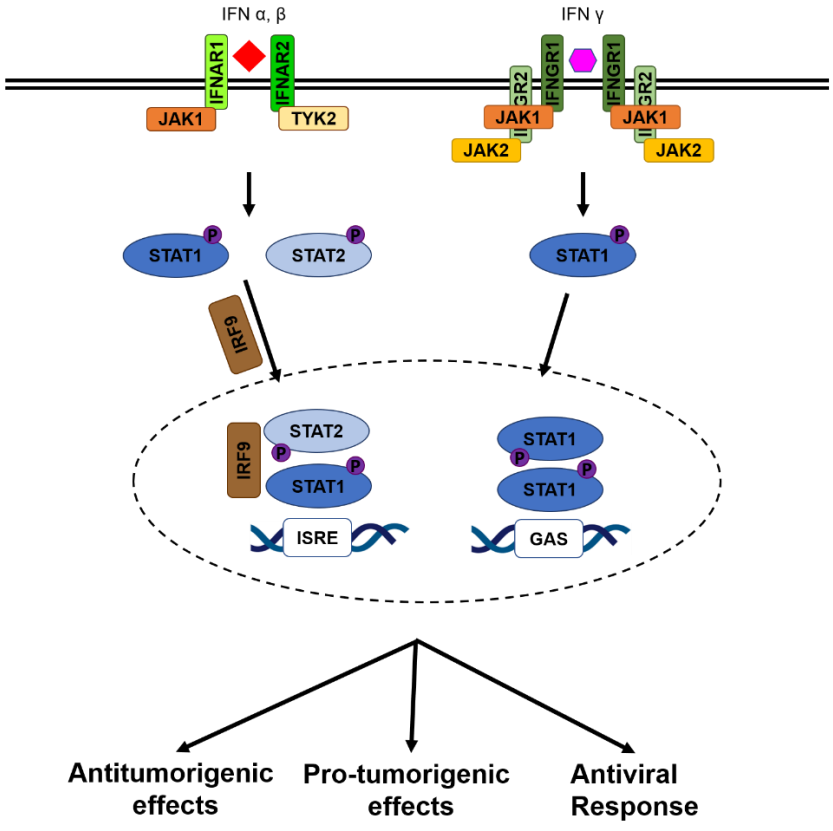


Figure 1. The interferons signaling pathway

Defects in the IFN response is one of the most well-characterized and best-established mechanism of resistance to ICIs. For example, exome sequencing analysis of patient samples from patients treated with ICI discovered defects in the IFNγ pathway in 9 out of 12 non-responders to anti CTLA-4[78]. In support, various CRISPR-Cas9 screens corroborate these findings. For example, functional *in vivo* CRISPR screen in B16 melanomas carrying guide RNAs into either normal mice or TCR-deficient mice treated with anti PD-1 and GVAX vaccine revealed members of the JAK-STAT signal transduction pathway (JAK1/2, STAT1, IFNGR1/2) and their negative regulator, PTPN2, promoted anti-PD-1 resistance[79]. Furthermore, a CRISPR-Cas9 screen using gRNA-carrying B16 cells co-cultured with T cells identified the IFN response pathway as enriched as well[80]. Importantly, sequencing analysis from patients with matched biopsies of pre-treatment and resistant melanoma samples revealed *JAK1* and *JAK2* mutations in resistant lesions[81].

6.4. Oncolytic viruses for the treatment of cancers

Oncolytic virus (OV) is another form of immunotherapy that makes use of natural or genetically altered viral strains to preferentially target cancer cells[82]. Interest in the tumor killing potential of OV dates back to the early 20th century when, occasionally, cancer patients who contracted viral infection went into remission[83]. By the mid 20th century, some natural OVs were explored as treatment options for cancer, including adenovirus, Coxsackievirus, poliovirus, amongst others. Due to limitations of OV therapy, which include increased morbidity, high viral clearance, and low efficacy caused near-abandonment of this strategy in the mid 1970s[84]. However, there has been a renewed interest in OVs over the last two decades due to progress in genetic engineering that allows modifications to overcome these limitations. In 2015, Talimogene laherparepvec (T-vec), a modified strain of the herpes simplex virus 1 (HSV1), became the first FDA-approved OV for the treatment of melanoma[85].

6.5. Mechanism of action of Oncolytic Viruses (OVs)

OVs utilize a dual mechanism to target cancer tumors. First, OVs preferentially replicate in cancer cells resulting in tumor lysis. Second, these viruses induce a host antitumor immune response.

6.5.1. Cancer targeting effects of OV:

Many OVs are effective as an anti-cancer therapy due to their ability to recognize cell surface receptors that are upregulated in certain cancers[86]. For T-vec and other HSV1 strains, entry into tumors rely on interactions with nectin-1/2 and herpesvirus entry mediator (HVEM) that are overexpressed in squamous cell carcinoma and colorectal cancer[87, 88]. In the case of Coxsackievirus, intercellular adhesion molecule (ICAM-1) mediates entry into host cells, and ICAM-1 is upregulated in melanoma, prostate, and breast cancers[89-91].

The host's antiviral mechanism revolves around the protein kinase R (PKR)[92]. When PKR is activated upon viral RNA binding, it phosphorylates the eukaryotic initiation factor (eIF)2α. Phosphorylated eIF2α acts as its own inhibitor against its unphosphorylated state by sequestering the Guanine nucleotide exchange factor eIF2β. As eIF2α can no longer return to its active GTP-bound state, translation initiation of viral proteins is abrogated[93]. PKR is also involved in cellular stress signaling, and thus is often inactivated in cancers by oncogenic RAS[94]. In many cancers, during tumor progression IFN signaling is disrupted as it plays in important role in mediating stress-induced cell cycle arrest, which may also render cancer cells more susceptible to infections[95].

6.5.2. OV-mediated activation of the immune response:

Once infected, tumors undergo immunogenic cell death (ICD), characterized by cell surface exposure of damage-associated molecular patterns such as heat shock proteins, ATP, and

DNA[96]. ICD activates DCs, which then migrate to lymph nodes and activate T-cells, shaping the adaptive anti-tumor immune response. Cancer cell lysis by OVs also leads to the release of tumor associated antigens (TAAs) which are taken up by neighboring cells to prime naïve CD8+ T-cells via cross-presentation, thus extending oncolysis effect to uninfected tumor cells[97].

6.6. Prototypical Oncolytic viruses (OV)

6.6.1. Herpes Simplex Virus 1

HSV1, a highly common pathogen, is a linear double-stranded DNA virus that causes cold sore and keratitis in the eyes[98]. HSV1 initially binds to host cells via interactions between viral proteins gB and/or gC with heparan sulfate proteoglycans (HSPGs) present on the F-actins cytoplasmic projections of the host-cell, called filopodia[99]. Subsequently, pH-independent membrane fusion between viral envelope and cellular membrane occurs, which is mediated by a complex consisting of viral gD and cellular HVEM and nectin-1/2[98]. Due to its large genome (130-250 kbps) consisting of mostly nonessential genes, HSV1 is easy to genetically manipulate and allow insertion of transgenes to further improve its safety and effectiveness as an anticancer therapy.

As HSV are neurotropic and establish latent infections in the peripheral nervous system, modifications have been made to address safety issues. Deletion of both copies of the gene encoding Infected cell protein (ICP) 34.5 has been a strategy employed in several strains of engineered HSV1 (T-vec, G207, HSV1716). ICP34.5 can complex with protein phosphatase 1 alpha to dephosphorylate the eIF2α, thus dampening the antiviral effect of PKR[100]. In addition, *ICP6*, a viral gene encoding a large subunit of ribonucleotide reductase needed for viral DNA synthesis, is also often inactivated by insertion of *LacZ* genes (G47, G207)[101]. Aside from improving safety, efforts have been made to increase the host antitumor response mediated by

HSV. For example, ICP47 blocks antigen presentation by MHC class I and thus are targeted for deletion (T-vec, G47)[102]. T-vec is also armed with two copies of human granulocyte-macrophage colony-stimulating factor (*GM-CSF*)[103]. An immunostimulatory cytokine, GM-CSF leads to recruitment of DCs and macrophages to the tumor site, resulting in T-cell priming in the regional lymph nodes[104].

6.6.2. Vesicular Stomatitis Virus

Vesicular Stomatitis Virus (VSV) is a negative sense-RNA virus belonging to the *Rhabdoviridae* family. VSV can infect a wide-range of animals, but infection in humans is often asymptomatic and non-lethal[105]. However, VSV has increased infection rates in researchers and agricultural workers who are in contact with VSV-infected animals[106]. The viral G protein is important for viral entry, as it binds to members of the low-density lipoprotein (LDL) receptors on the host cells[107]. Upon attachment, VSV enters via endocytosis. Endosomal acidification then mediates fusion of the viral envelope with the endosomal membrane, releasing the viral ribonucleoprotein[107]. Research on VSV has also intensified due the fact that VSVs are used as expression vectors in the development of HIV and Ebola vaccines[108, 109].

Despite its safety profile, VSV can be neurotoxic in rodents and in some non-human primates[110]. As a result, strategies to attenuate the neurotropism characteristics of VSV have been employed to maximize its clinical utility. This has been done via mutations in the G protein important for viral entry[107]. This has been accomplished by introducing point mutations (VSV-G6R, VSV-G5R) or by truncating the cytoplasmic tail of the G protein from 29 amino acids to 9 (VSV-CT9) or 1 (VSV-CT1)[105]. Targeting the Viral M protein has also proved to ameliorate the safety and oncoselectity of VSV. M protein interrupts host protein expression and suppresses cellular response to pathogens by binding to the Rae1-Nup98 complex to inhibit mRNA nuclear

export[111]. Most strategies to target the M protein have included either deleting or mutating the methionine residue at position 51 (VSV-M51R, VSV-d51)[105].

Conversely, genetic engineering approaches to improve the tumor-specific immune response mediated by VSV has been achieved by inserting genes encoding for immunostimulatory cytokines such as IL-4, IL-12, and IL-23[112, 113]. Interestingly, addition of some cytokines not only aid in increasing the immune response but also improve safety. IL-23 activates Natural killer (NK) cells and CD4+ T-cells to release nitric oxide in the central nervous system (CNS), thus enhancing viral clearance[114].

7. Hypothesis and summary of aims:

As mutations in the IFNγ response pathway are a bona fide ICI resistance mechanism, which would also simultaneously lead to deregulation of the normal cellular antiviral response, we hypothesize that ICI-resistant melanomas are more susceptible to OV treatment. To address our hypothesis, we first examined OV susceptibility in matched cell lines generated from a pre-treatment biopsy and an anti-PD-1-resistant progressing lesion that possessed a *JAK2* LoF mutation. Second, we formally tested whether JAK1 or JAK2 silencing leads to OV susceptibility. Third, we restored JAK2 expression in JAK2-null lines and evaluated OV sensitivity. Finally, we tested the effects of JAK1/2 pharmacological inhibition in combination with OV in melanomas with intact IFN functions.

8. Results

8.1. JAK2 loss-of-function (LoF) mutation in tumors of previously treated patient sensitizes tumors to oncolysis by oncolytic viruses (OVs)

While ICIs have shown unprecedented results in the treatment of melanoma, durable response has only been achieved in a subset of patients. According to a recent study, in addition to patients that exhibited *de novo* resistance to ICIs, approximately one third of patients who had initial response to anti-PD-1 and anti-CTLA-4, relapsed[115]. To dissect the mechanism of resistance, Dr. Ribas and colleagues generated matched cell lines from a pre-treatment biopsy (M420) and an anti-PD-1-resistant progressing lesion (M464) with inactivating JAK2 mutation. M464 possessed a *JAK2* F547 LoF splice site mutation with loss of heterozygosity (LOH)[81]. Dr. Ribas kindly provided these cell lines, and we confirmed by immunoblot analysis that M420 responds to both type I and II IFNs stimulation, as seen by the phosphorylation of STAT1 and downstream induction of PD-L1 expression **(Fig. 2A)**. In contrast, M464 only responded to type I IFNs, due to the absence of JAK2 expression **(Fig. 2A)**. To assess the impact of a *JAK2* LoF mutation on the potency of OVs, we performed a dose-response cytotoxicity assay using Vesicular Stomatitis Virus (GFP-expressing VSV-d51), which is sensitive to the antiviral effects of IFNs[116]. Briefly, cells were infected with VSV-d51 at multiple multiplicities of infection (MOI) for 72 hours and cell viability was measured. As these experiments are performed *in vitro*, we performed cytotoxicity in the absence or presence of 200IU/ml of IFNγ pre-treatment to better mimic conditions in the TME.

We observed no significant difference in viral sensitivity between the pair of cells lines **(supp. Fig. 4A)**. However, following pre-treatment with IFNγ at 200IU/ml, M464 was

significantly more sensitive to the cytotoxicity effect of VSV-d51, as seen by a 22-fold decrease in ED_{50} when JAK2 is lost in M464 (p = 0.0197) **(Fig. 2B)**. We repeated the same experiment with an additionally modified OV strain, Herpes Simplex Virus 1 (HSV1-dIPC0), which is the same OV strain as T-VEC, currently approved for the treatment of melanomas. The M464 *JAK2* LoF mutant cell line had a 7-fold decrease in ED_{50} compared to the JAK2 wild-type M420 line (p = 0.0201, n = 3) **(Supp Fig. 2).** Taken together, these results suggest JAK2 loss sensitizes the cells against the oncolysis effects of OVs.

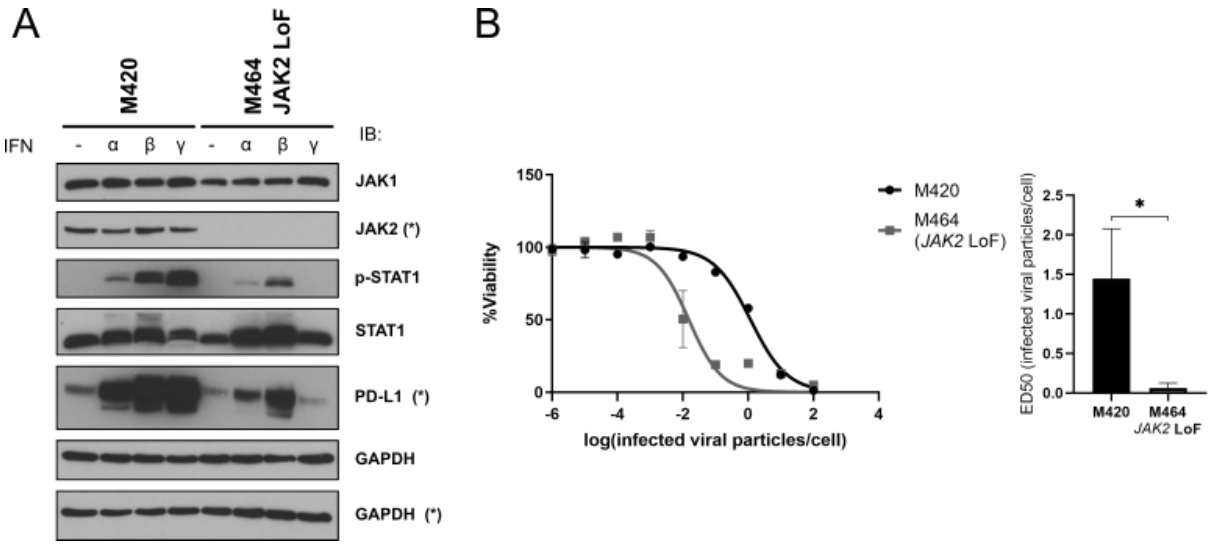


Figure 2. JAK2 loss in relapsing patient sample confers sensitivity to VSV-d51 oncolysis. **A)** Immunoblot analysis of M420 and M464 melanoma lines upon type I and II IFNs exposure at 500 IU/ml for 48 hours. **B)** Log-transformed dose response curve for M420 and M464 upon VSV-d51 infection following IFNγ pre-treatment at 200 IU/ml for 24 hours. Cell viability was measured 72 hours after infection, and % viability was obtained by normalizing against mock-infected control (left panel). Unpaired t-test of comparing ED_{50} of M420 and M464 in 3 independent experiments (* $p<0.05$, n = 3, error bars represented as S.E.M) (right panel).

8.2. JAK1 or JAK2 knockdown sensitizes melanoma to Vesicular Stomatitis Virus (VSV)

To genetically validate our findings, we used RNA interference to individually target *JAK1* or *JAK2* in the melanoma HMVII cell line with a functional IFNs response. Knockdowns using 2 independent siRNAs targeting either *JAK1* or *JAK2* abrogate STAT1 phosphorylation and induction of the ISG, PD-L1 **(Fig. 3A, B)**. JAK1 knockdown significantly sensitized HMVII to VSV-d51 lysis resulting in approximately 240-fold and 80-fold decrease in ED_{50} for 2 independent siRNAs ($p = 0.0400$, $p = 0.0410$ respectively) **(Fig. 3D)**. Furthermore, JAK2 knockdown increased HMVII sensitivity to VSV-d51, resulting in 149-fold and 65-fold decrease in ED_{50} for 2 independent siRNAs; however, we observed more experimentally variability between our replicate experiments ($p = 0.1030$, $p = 0.1050$, respectively) **(Fig. 3C)**. These results demonstrate loss of either JAK1 or JAK2 expression sensitize melanomas to OV-mediated lysis.

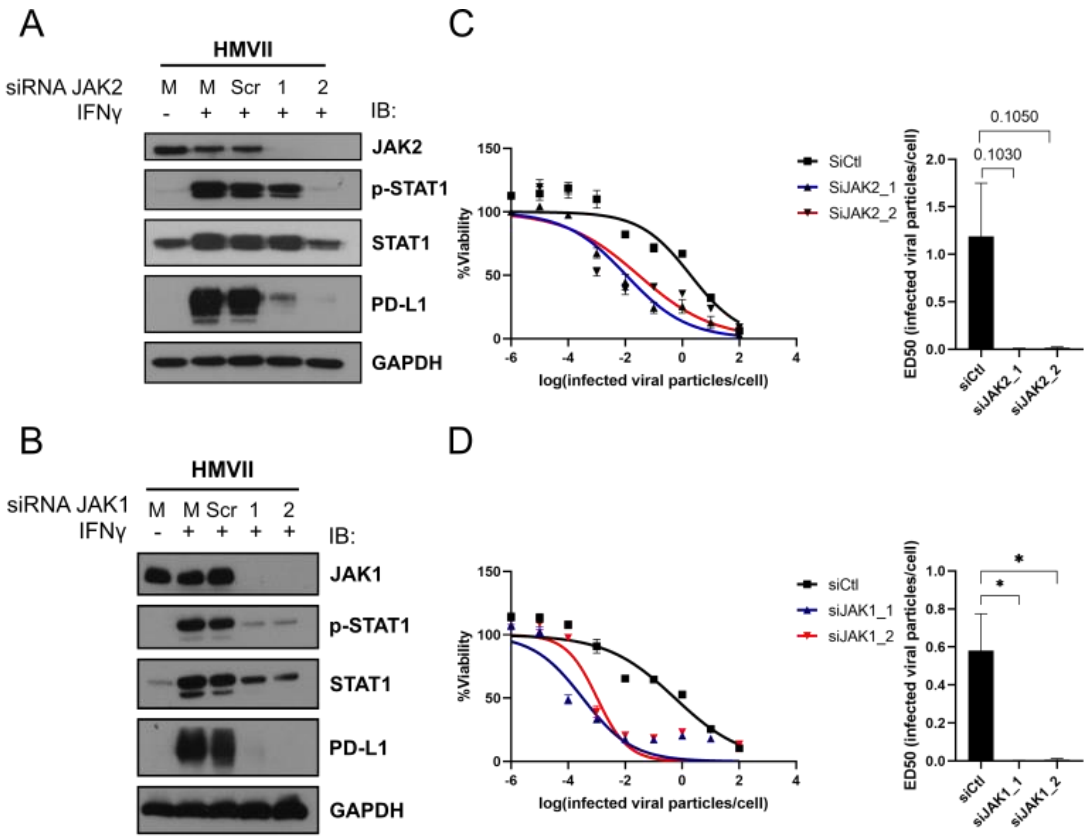


Figure 3. SiRNA knockdown of JAK1 and JAK2 increase oncolysis effect of VSV-d51. Immunoblot showing **A)** JAK2 and **B)** JAK1 knockdown using siRNA. 24 hours after siRNA transfection, media was removed, and cells were treated with 500IU/ml IFNγ for 48h before cells were lysed for immunoblotting. Log-transformed dose response curve for HMVII transfected with siRNAs targeting **C)** JAK2 and **D)** JAK1 for 24h, then IFNγ pre-treatment at 200IU/ml for 24h before VSV-d51 infection for 72h. Cell viability was measured, and % viability was obtained by normalizing against mock-infected control (left panel). Unpaired t-test comparing ED_{50} of JAK1 and JAK2 knockdown in HMVII cell line in 3 independent experiments (* $p<0.05$, n = 3, error bars represented as S.E.M) (right panel)

8.3. JAK2 rescue in a *JAK2* loss melanoma line confers resistance to oncolytic viruses (OVs)

The high mutational burden and resulting high expression of neoantigens in melanomas is thought to induce a T-cell anti-tumor immune response[117] . Thus, for melanomas to progress, they must escape immunosurveillance. We sought to screen a library of melanoma cell lines for mutations in the IFN response pathways that may be important to bypass immunosurveillance. Most cell lines investigated had a functional JAK-STAT signaling pathway, as seen phosphorylation of STAT1 upon type I or type II IFNs stimulation and expression of an interferon-stimulate gene (ISG), PD-L1 **(Fig. 4A) (supp Fig. 1)**. Out of 18 cell lines screened, only Colo857 did not respond to IFNγ due to loss of JAK2 expression **(Fig. 4B)**. We rescued JAK2 expression and studied how the rescue affects the potency of VSV-d51 cytotoxicity. JAK2 reintroduction restored phosphorylation of STAT1 and induced expression of the ISG, HLA class I **(Fig. 4C)**. Cytotoxic assay showed JAK2 overexpression led to a 112-fold increase in ED_{50} following virus treatment in the presence of exogenous IFNγ ($p = 0.0414$) **(Fig. 4D**), but no significant differences without IFNγ protection **(supp Fig. 4B)**. A similar trend was observed when we infected Colo857 with HSV1-dIPC0, where JAK2 overexpression resulted in a 2-fold increase in ED_{50} ($p = 0.0321$) **(supp. Fig 3)**.

A

Cell lines	Driver Mutations	IFN response
HMVII	NRAS	Yes
WM3629	NRAS	Yes
Meljuso	NRAS	Yes
GAK	NRAS	Yes
WM983B	BRAF	Yes
451 Lu	BRAF	Yes
1205 Lu	BRAF	Yes
IGR1	BRAF	Yes
Malme-3M	BRAF	Yes
A375	BRAF	Yes
UACC 62	BRAF	Yes
WM 266-4	BRAF	Yes
WM 9	BRAF	Yes
Colo 857	BRAF	Non-responsive to IFNγ
WM 3918F	NF1	Yes
MEWO	NF1	Yes
CHL-1	Triple wild-type	Yes
WM3211	Triple wild-type	Yes

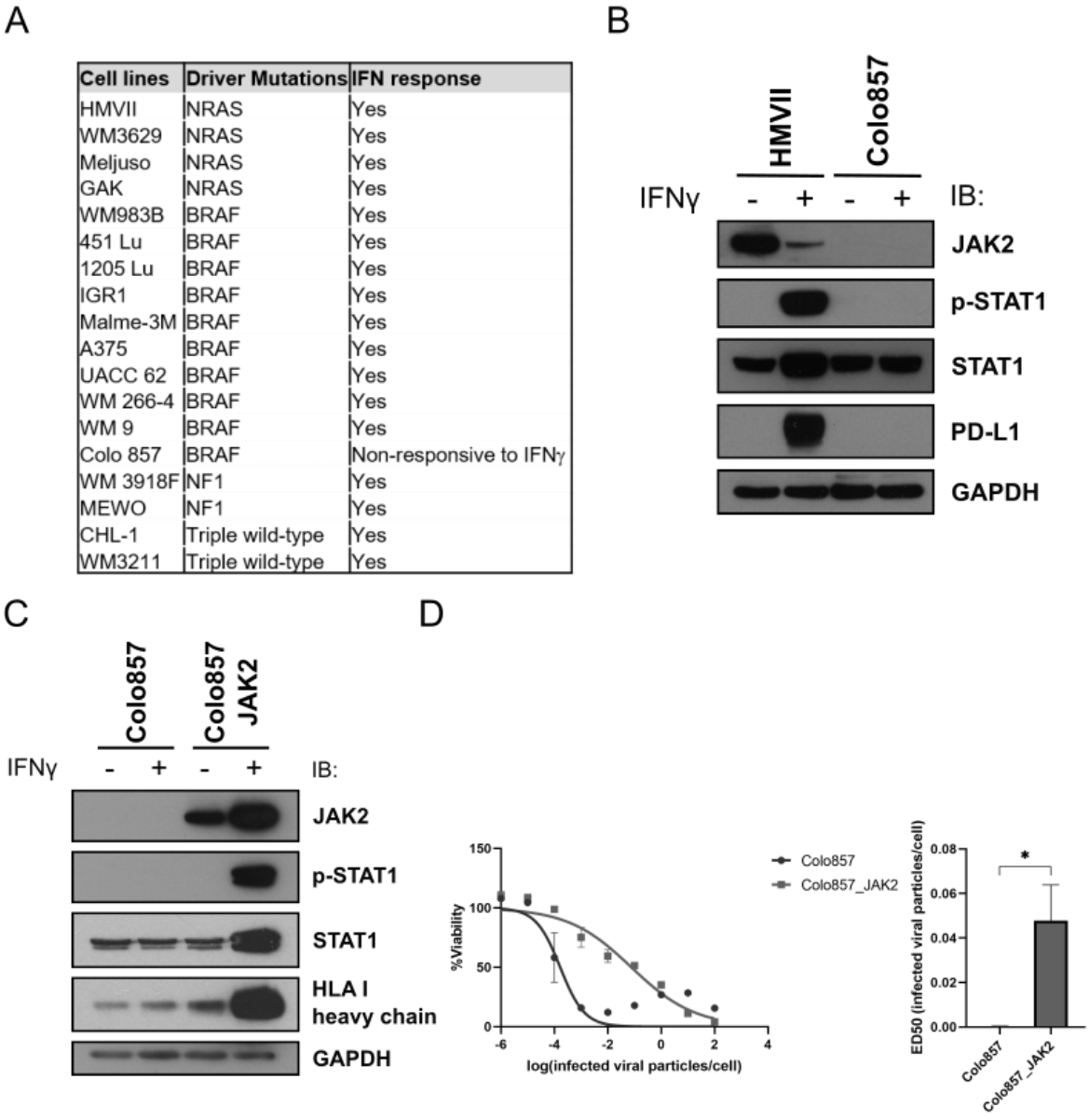


Figure 4. JAK2 rescue in melanoma cell line Colo 857 confers resistance against VSV-d51 A) Summary of results for IFN responsiveness in a panel of melanoma cell lines. **B)** Immunoblot analysis of melanoma cell lines, Colo857 with JAK2 loss and HMVII upon IFNγ induction. **C)** Immunoblot showing JAK2 rescue restores functional IFN response. **D)** Log-transformed dose response curve for Colo857 with JAK2 rescue upon VSV-d51 infection following IFNγ pre-treatment at 200 IU/ml for 24 hours. Cell viability was measured 72 hours after infection, and % viability was obtained by normalizing against mock-infected control (left panel). Unpaired t-test of comparing ED_{50} of JAK2 rescue in Colo857 in 3 independent experiments (* $p<0.05$, n = 3, error bars represented as S.E.M) (right panel).

8.4. Ruxolitinib dampens interferons (IFNs) response and improves oncolysis effects of Vesicular Stomatitis Virus (VSV)

We next asked whether pharmacological treatment of a FDA-approved JAK inhibitor, Ruxolitinib (Rux), could replicate our siRNA results sensitizing melanomas to OV. Rux is a JAK1/2 inhibitor that is used to treat rheumatoid arthritis and myelofibrosis[118]. In the HMVII cell line with intact IFN signaling, treatment with Rux at 5μM for 24 hours was able to inhibit STAT1 phosphorylation and PD-L1 induction in the presence of IFNγ at 500 IU/ml **(Fig. 5A)**. Furthermore, Rux treatment did not affect the viability of HMVII cells, even at extremely high concentrations of 100uM **(Fig. 5B)**. Rux itself did not significantly affect sensitivity to VSV-d51 **(supp Fig. 4E)**. However, upon combination pre-treatment of both Rux (or DMSO) and IFNγ for 24 hours, we observed that this combination enhances the oncolysis effect by 59-fold ($p = 0.0472$) compared to DMSO control **(Fig 5C)**. Thus, addition of Rux blunted the IFN response, sensitizing the HMVII cell line to VSV oncolysis.

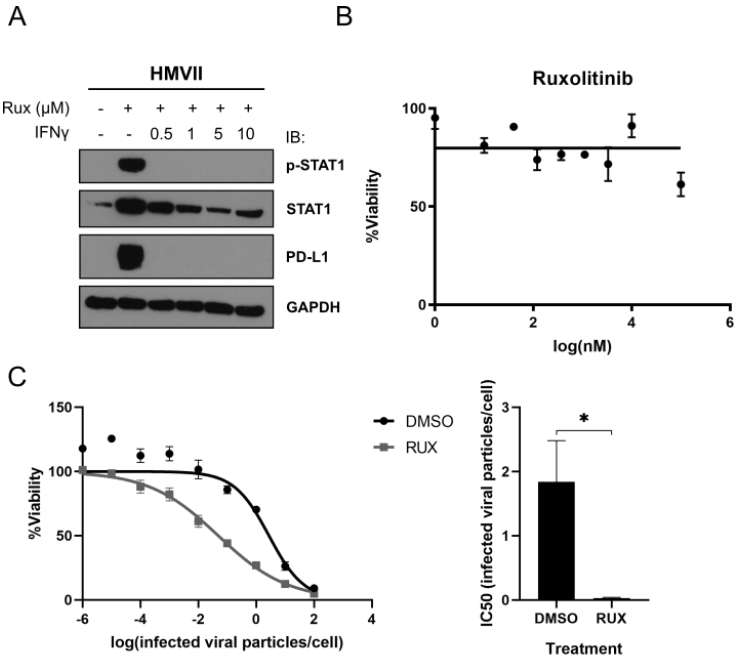


Figure 3. Ruxolitnib co-treatment with IFNγ dampened IFN response, increasing melanoma's sensitivity against VSV-d51. **A)** Immunoblot showing the effect of pre-treating Rux at various dosage and IFNγ at 500IU/ml for 24h on IFNγ response. **B)** Log-transformed dose response curve for HMVII treated with increasing dosage of Rux for 24h. **C)** Log-transformed dose response curve for HMVII pre-treated with either Rux at 5 uM or DMSO control and IFNγ at 200 IU/ml before VSV-d51 infection. Cell viability was measured 72 hours after infection, and % viability was obtained by normalizing against mock-infected control (left panel). Unpaired t-test comparing ED_{50} in HMVII cell line treated with Rux or DMSO in 3 independent experiments (* $p<0.05$, n = 3, error bars represented as S.E.M).

9. Discussion

9.1. Summary of results and future directions

In this study, we examined whether mutations in the IFNγ response pathway linked to ICI resistance resulted in increased melanoma susceptibility to OVs. Using matched cell lines generated from a pre-treatment biopsy and an anti-PD-1-resistant progressing lesion, we discovered that cell lines derived from the resistant lesion possessing homozygous *JAK2* loss had a significant increase in susceptibility to VSV and HSV. Second, we formally determined that JAK1 and JAK2 loss sensitize melanomas to OV in siRNA experiments. Third, by restoring JAK2 expression in *JAK2*-null lines we reduced the oncolysis effect of OV. Finally, we determined that using a FDA-approved JAK1/2 inhibitor sensitized melanoma to OV. We should note that we did not observe increased sensitivity in the absence of IFNγ; however, in conditions mimicking the TME with IFNγ, we observed 2-240-fold increase in sensitivity to both HSV1 and VSV in melanoma with defects in IFNs response.

As future directions, we will confirm our results in additional *in vitro* models, and employ *in vivo* mouse models. For example, we will test OV efficacy in immunocompetent genetically engineered mouse (GEM) models of melanoma, as well as human melanoma cell lines with JAK1/2 loss in immunodeficient NOD Scid mice. We will also work to generate human patient-derived xenograft models from surgical cases obtained from pre-treatment and ICI-resistant melanomas with mutations in the IFN response pathway for OV studies.

9.2. Frequency of genetic alterations in the interferons (IFN) signaling pathways in melanoma

Activation of oncogenic pathways, such as MAPK pathway that trigger early proliferation also induces DNA damage response and senescence through production of type I IFNs[119]. Studies have shown that, to overcome cell-autonomous and oncogene-induced senescence, melanomas suppress type I and II IFNs signaling to escape anti-tumor immune response[120]. As a result, response to type I and II IFNs is thought to be commonly dysregulated in melanoma[121]. For example, a recent study identified genetic alterations in the IFN pathway (including single-nucleotide variations, indels, homozygous deletions) in 12.6% (36 of 287) of treatment-naïve cutaneous melanoma samples in The Cancer Genome Atlas cutaneous melanoma data, and 22% (11 of 49) of melanoma lines in the Cancer Cell Line Encyclopedia[122]. Early sequencing studies from patients treated with ICI, reported that up to 75% of non-responders to ICIs (9 out of 12) have alterations in IFNγ signaling in their patient cohort[78]. A subsequent analysis of a larger patient cohort, reported that approximately 15% of non-responders (19 out of 123) carry these alterations[123]. In our study, out of the 18 melanoma cell lines examined for IFN response, only one cell line possessed a defect in the JAK-STAT transduction pathways leading to IFNγ resistance. It is likely that our method used to test IFNγ responsiveness that included treatment with high levels of IFN, was not able to appropriately separate cell lines with more variable degree of IFN response. Future studies using various concentrations and a more limited amount of IFN treatment may allow us better define cell lines with more subtle resistance to IFN treatment, and possible more sensitivity to OV. In conclusion, even though the defects in IFN pathway in melanomas are not as high as 75% as originally reported, there is still a considerable portion of melanoma defects in IFNs signaling. Furthermore, these defects may be more common in cases of

acquired resistance, where tumors evolved with genetic defects to escape ICIs treatment. However, there are only a limited number of sequencing studies that have reported genetic analysis of pre-treatment and resistance biopsies. To our knowledge, there has been only published analysis that performed next-generation sequencing on matched pre- and post- melanoma biopsies from ICI resistance patients[81]. Interestingly, of the 4 anti-PD-1 acquired resistance cases, half had either a *JAK1* or *JAK2* mutation [81].

9.3. Combining oncolytic viruses (OVs) with immune checkpoint inhibitors (ICIs) and beyond

Given the complexity of treatment resistance due tumor heterogeneity and the TME, many clinical trials focus on combining existing therapies to elicit a more effective response[124]. Although OVs have great potential, there may be a limit to what they can achieve as a monotherapy. Pre-existing neutralizing antibodies due to early infections or immunization and rapid viral clearance limits the spread and efficacy of OVs treatment[125]. Thus, in numerous cancers, combination therapies are being tested to further enhance the effectiveness of OVs[126].

Across all cancers, combination of ICIs and OVs appear to be the commonly studied immunotherapy combination being employed in the clinic[127]. In addition to directly lysing cancer cells, OVs also mediate killing of cancer cells via their interactions with the immune system, and this indirect immunogenic effect alone can reduce tumors[128]. For example, a heat-inactivated, non-replicating strain of vaccinia virus was shown to eradicate tumors by increasing DCs cross-priming and CD8+ T cells infiltration[129]. Furthermore, another study showed UV-inactivated reovirus was immunogenic and showed antitumor properties in mice[130]. Therefore, in addition to patients that have defects in the IFNs signaling pathway, OVs can potentially benefit other patients that failed ICI therapy due to low CD8+ T cell infiltration, by turning immunologically “cold” tumors

"hot"[131]. Moreover, some strains like HSV1 and vaccinia virus were shown to induce PD-L1 expression on tumors upon infection, making anti-PD-1 therapy all the more effective in these cases[132, 133]. Therefore, the immune activation effect of OVs rationalizes the potential combination between OVs and ICIs.

Our results have a number of important clinical implications. First, we have provided a precision-medicine strategy to target cancers with genetic defects in the IFN response pathway. Second, we have provided an ICI salvage therapy strategy that may be effective in many acquired ICI resistant patients. However, a number of questions remain moving forward. Currently, OVs are primarily used in melanoma "to turn cold tumors hot". Our results suggest that, in addition to priming the melanoma TME, the direct oncolysis effect of OVs will contribute to the success of this ICI/OV combination therapy by targeting subsets ICI-resistant melanoma clones from expanding clonally leading to relapse. Future clinical studies are needed to evaluate how to best implement the use of OVs in the clinic, as either a salvage therapy to target tumors that have evolved to resist ICI blockade through defects in the IFN response, or as priming agents prior to ICI blockade to activate the immune system.

There have also been efforts to find combination partners to potentiate the oncolysis effects of OVs[134]. Early studies have focused on the use of chemotherapy in combination with OVs[135]. Cyclophosphamide was combined with HSV1 due to its immunosuppressive ability, which increased and prolonged viral replication[136]. Conversely, cisplatin was shown to upregulate Growth Arrest and DNA Damage-inducible protein 34 (GADD34) that is involved in cellular stress response[137]. GADD34 acted as a functional homolog for the viral protein ICP34.5, thus enhancing replication of ICP34.5-deficient HSV1 in cancer cells[138]. Our finding that Rux treatment can potentiate the effects of OVs against melanoma supports another rational combination to

enhance OVs' efficacy. FDA-approved JAK inhibitors are used to treat inflammatory and myeloproliferative diseases, and are also being examined in the treatment of solid tumors[139-141]. While IFN signaling is critical for antitumor immunity, prolonged IFN exposure can lead to adaptive resistance mechanisms that are independent of PD-L1 expression mentioned above, leading to T cell exhaustion[142]. Therefore, currently there is one active clinical trial testing the combination of JAK inhibitors with anti PD-1 therapy, pembrolizumab, across multiple tumors[12, 143]. However, consistent with reports that IFN defects results in ICI resistance, initial results have not been promising[12, 143]. Our results would suggest that use of JAK inhibitors and OVs could be a potential superior strategy. JAK inhibitors will function not only to alleviate the challenge of T cell exhaustion due to chronic inflammation, but also potentiate the oncolysis effect of OV. Importantly, this strategy could expand the patient population that may benefit from this combination, and not just patients who have genomic defects in IFNs response.

In summary, we showed, through patient-derived models, genetic alterations, and pharmacological inhibition, that mutations in the IFN response pathway associated with resistance to ICI also sensitize melanomas to OVs. Our results suggested OVs as a form of precision medicine for melanoma patients with defects in IFN response, or as salvage therapy for ICI-resistant patients due to defects in IFN signaling.

10. Materials and Methods:

Cell Culture:

HMVII, WM3629, Meljuso, GAK, WM983B, 451Lu, 1205Lu, Malme-3M, A375, UACC62, WM266-4, WM9, WM3918F, MEWO, IGR1, WM3211, CHL-1, and Col857 were maintained in RPMI1640 medium (Wisent Bioproducts) supplemented with 5% heat-inactivated fetal bovine serum (Gibco, Life Technologies) and 1% Penicillin/Streptomycin. Patient-derived M420, M464, HEK293FT, and Vero cell lines were maintained in DMEM medium (Wisent Bioproducts) supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin**.** All cell lines were kept at $37^{O}C$ in humidified 5% CO_2 incubator. M420 and M464 was a gift from Dr Antoni Ribas. Vero line was a gift from Dr Martin Richer.

Plasmids and siRNA:

pDONR223-JAK2 was a gift from William Hahn and David Root (Addgene plasmid #23915). JAK2 ORFs were subcloned into plenti6.3 expression plasmids (InVitrogen) according to Thermo Fisher Scientific's Gateway cloning protocol.

For lentiviral transduction, HEK293FT was transfected with 2µg of plenti6.3-JAK2, 1.5µg of psPAX2, 0.5µg of pMD2g using 15µl each of Lipofectamine 3000 Transfection Reagent and P3000 Enhancer Reagent (Thermo Fisher Scientifics) in Opti-MEM reduced serum media (Thermo Fisher Scientifics). Viral media was collected at 48h and 72h after transfection and filtered through 0.45µM filter. Viral media was concentrated using Polyethylene glycol (Sigma Aldrich) and stored at -80^{o}C until use. Colo857 was incubated with viral media and polybrene at 6µg/ml for 24h. Infected cells were selected using Blasticidin S HCl (Invitrogen) for 7 days.

siRNAs targeting JAK1/2 and Scramble negative control were purchased from Dharmacon. siRNA transfection was performed according to manufacturer's protocol

siRNA Scramble negative control: UAGCGACUAAACACAUCAA

siRNA JAK1_1: CCACAUAGCUGAUCUGAAA

siRNA JAK1_2: UGAAAUCACUCACAUUGUA

siRNA JAK2_1: GAGCAAAGAUCCAAGACUA

siRNA JAK2_2: GCCAGAAACUUGAAACUUA

Oncolytic viruses and propagation:

VSV-d51 and HSV-dICP0 are gifts from Dr Nahum Sonenberg. GFP-expressing VSV-d51 contains a deletion at the amino acid 51 of the M protein, while GFP-expressing HSV-dICP0 has the infected cell protein 0 gene deleted. Vero cells were infected with either VSV-d51 or HSV-dICP0 at low MOI of 0.01 for 1-2 hour. Afterwards, viral media was removed and replaced with normal media. Plates were incubated for 1-2 days until cytopathic effects were observed in 80% of cell population. Cells and supernatants were harvested and underwent 3 cycles of freeze-thaw. Supernatants were clarified using low-speed centrifugation and filtration through 0.45μm filter. Supernatants were then ultracentrifuged at 23000 RPM using a 36% sucrose cushion, and the pelleted viruses were resuspended in small amount of media.

Standard plaque assays were performed to measure viral titer. Vero cells seeded in monolayers the night before were infected with increasing MOI for 3 hours. Viral media was then replaced, and wells were overlaid with 2.5% agarose-containing RPMI1640 media solution. Plates were kept in the 37^{o}C incubator for 3-5 days until plaques were seen under light microscope.

Agarose overlay was removed, and cells were fixed with formalin at room temperature for 30 minutes. Cells were dyed with 0.1% crystal violet solution, and number of plaques were counted. Viral titer was calculated using the equation: Plaque Forming Unit (PFU)/ml = (Number of plaques / (Dilution factor * Volume of viral media).

Interferons protection:

Interferons α-2b, β, and γ were purchased from PBL Assay Science. For signaling studies, IFNs were used at 500 IU/ml for 48 hours. For viability assay, cells were treated with IFNγ at 200 IU/ml for 24 hours.

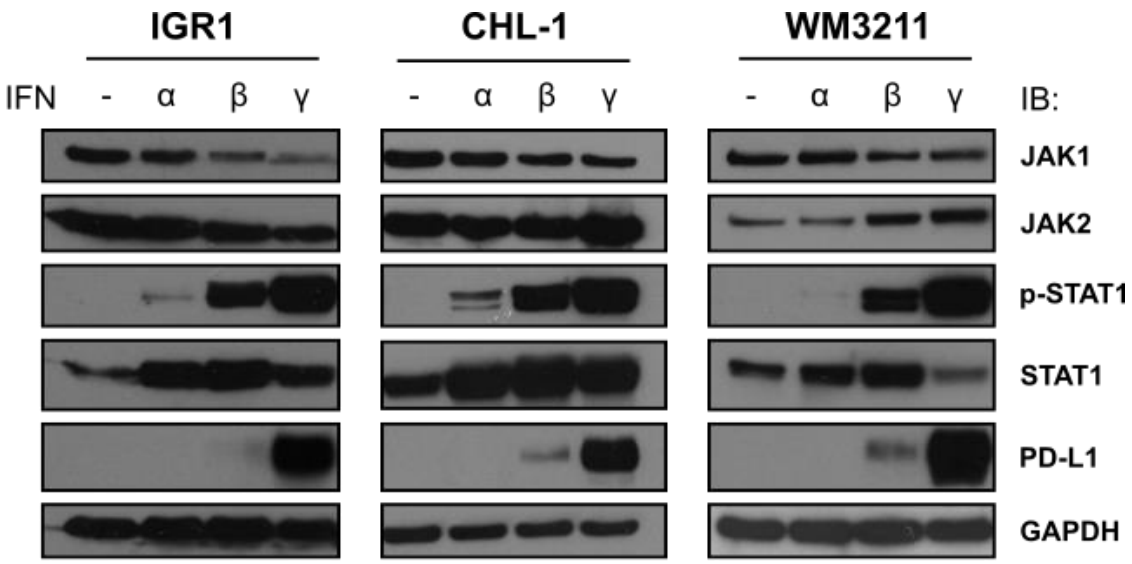
Immunoblot:

Cells were lysed in Pierce RIPA buffer (Thermo Fisher Scientifics) supplemented with Halt protease inhibitor cocktail (Thermo Fisher Scientifics). Cell lysates were cleared using centrifugation and protein concentrations were measured using DC Protein Assay (BioRad). Lysates were denatured in NuPAGE LDS sample buffer (Invitrogen) and run on 8% Tris-glycine SDS polyacrylamide gel. Proteins were transferred to nitrocellulose membrane using the Transplot Turbo Transfer System (BioRad) before immunoblotting. Antibodies against JAK1 (1:1000; #3344), JAK2 (1:1000; #3230), phospho-STAT1(Y701) (1:1000; #7649), STAT1 (1:1000; #9172), PD-L1 (1:3000; #13684), and GAPDH (1:3000; #2294) were purchased from Cell Signaling Technology. Antibodies against HLA class I heavy chain (1:10000; MUB2037P) was obtained from NordicMUBio. Anti-mouse (1:2000; #7076) and anti-rabbit (1:2000; #7074) IgG HRP-linked secondary antibodies were purchased from Cell Signaling Technology.

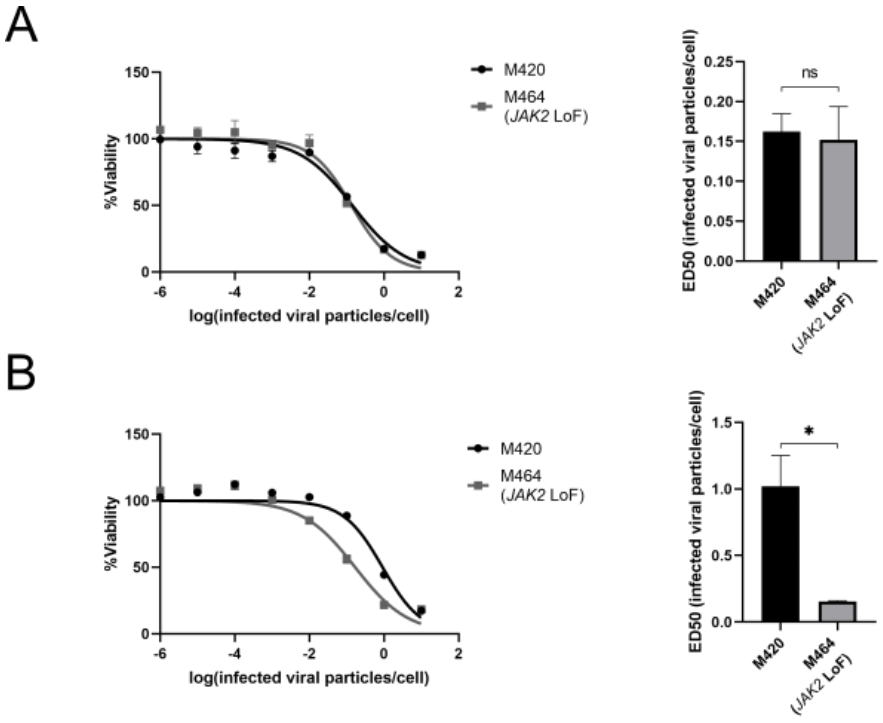
Viability assay:

5000 cells were seeded in 96-well plates in quadruplicates. After pre-treatment with IFNγ at 200IU/ml for 24 hours, cells were then infected with OVs at increasing MOI for 3 hours, before viral media was removed and replaced with normal media. Plates were incubated for 72 hours, and cell viability were measured using Cell Titer Glo Luminescent Assay (Promega). Luminescence was read using FLUOstar Omega Luminometer at gains of 2000. Experiments were performed in quadruplicates. Log-response curves were plotted using GraphPad to calculate the ED_{50}, setting the readings of mock-treated cells at 100% survival point, and the readings of medium only with reagents at 0% point. Grubbs' test was performed to identify outliers. Three independent experiments were performed, and unpaired student's t-test was performed on the resulting ED_{50}.

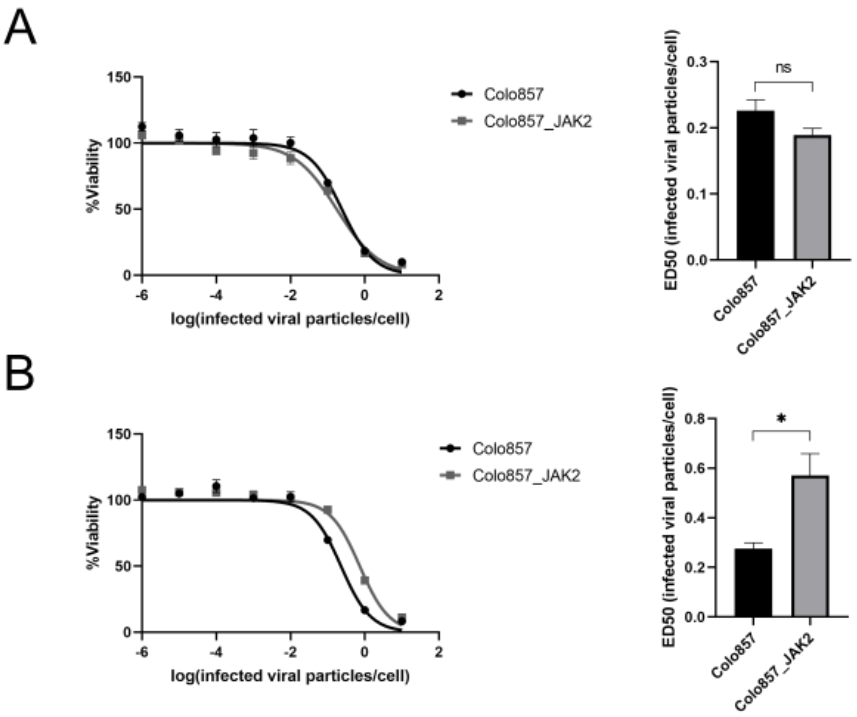
11. Supplemental Figures



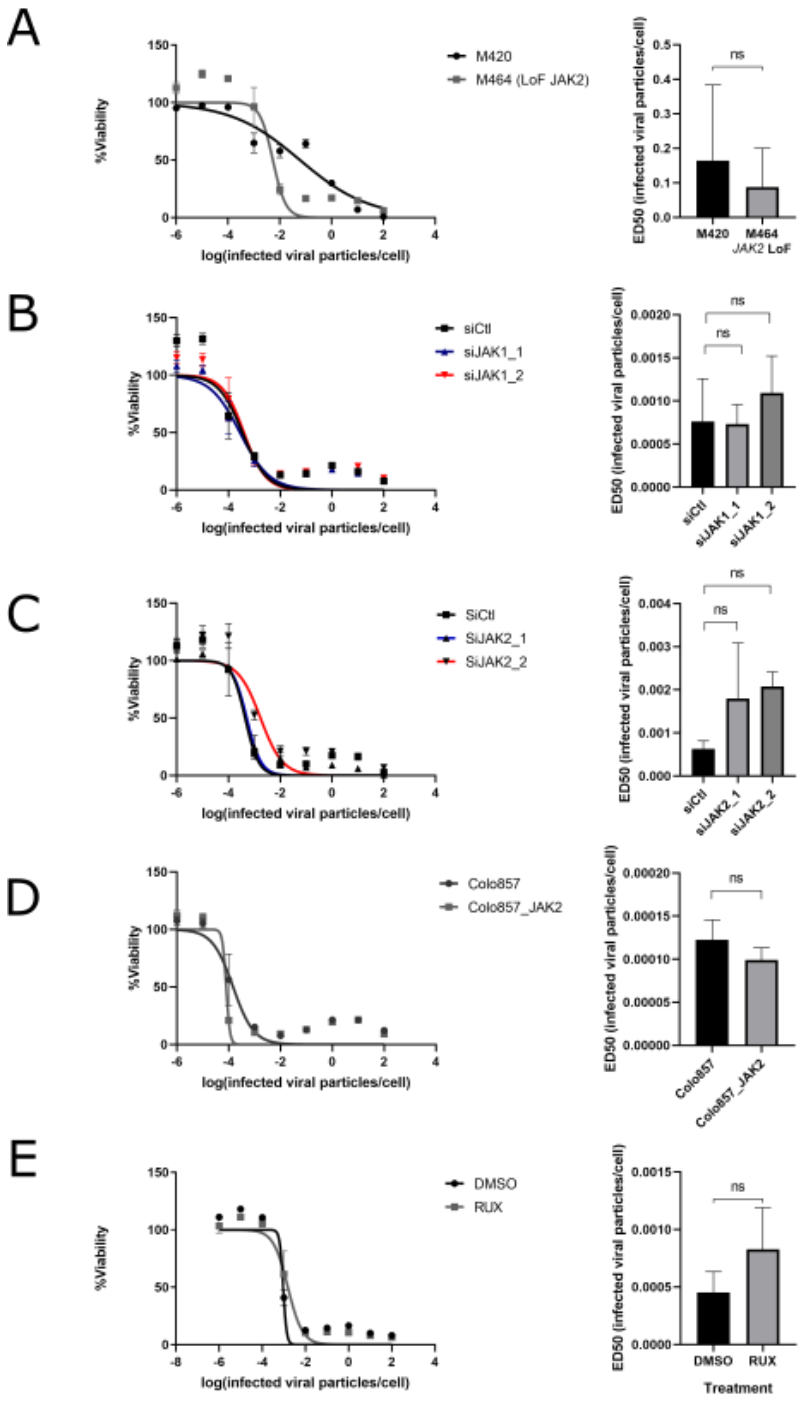
Supp Figure 1. Investigation of IFNs response in melanomas. Representative Immunoblots of panel of melanoma cell lines induced with type I and II IFNs to examine defects in JAK-STAT transduction pathway.



Supp Figure 2. *JAK2* loss enhances sensitivity to HSV1-dICP0. Log-transformed dose response curve for M420 and M464 upon HSV1-dICP0 infection. Cells are either **A)** left untreated or **B)** pre-treated with IFNγ at 200 IU/ml over 24h. Cell viability was measured 72 hours after infection, and % viability was obtained by normalizing against mock-infected control (left panel). Unpaired t-test comparing the ED_{50} of M420 and M464 in 3 independent experiments (* $p<0.05$, n = 3, error bars represented as S.E.M) (right panel).



Supp Figure 3. JAK2 overexpression in JAK2-loss Colo857 renders resistance against HSV1-dICP0. Log-transformed dose response curve for Colo857 and Colo857 overexpressing JAK2 upon HSV1-dICP0 infection. Cells are either **A)** left untreated or **B)** pre-treated with IFNγ at 200 IU/ml over 24h. Cell viability was measured 72 hours after infection, and % viability was obtained by normalizing against mock-infected control (left panel). Unpaired t-test comparing the ED_{50} of JAK2 rescue in Colo857 in 3 independent experiments (* $p<0.05$, n = 3, error bars represented as S.E.M) (right panel).



Supp Figure 4. Defects in JAK-STAT transduction pathway did not sensitize melanomas to VSV-d51 without IFNγ pre-treatment. Log-transformed dose response curve for **A)** M420 and M464 patient's sample, **B)** siRNA JAK1 and **C)** JAK2 knockdown of HMVII, **D)** Colo857 and Colo857 overexpressing JAK2, **E)** DMSO and Rux-treated HMVII upon VSV-d51 infection. Cell viability was measured 72 hours after infection, and % viability was obtained by normalizing against mock-infected control (left panel). Unpaired t-test comparing ED_{50} in the 3 independent experiments (ns $p > 0.05$, $n = 3$, error bars represented as S.E.M) (right panel).

11. References:

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