Activation and Subversion of MDA5-Dependent Immune Responses by the Engineered

Oncolytic Poliovirus PVSRIPO

by

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Jack Keene

Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor
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ABSTRACT

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Abstract

Cancer-specific cytopathogenicity of oncolytic viruses is often defined by viral sensitivity to innate antiviral immune responses, e.g. type I Interferons (IFNs), limiting cytotoxicity to cells lacking these responses. However, recent work suggests some cancer cells inhibit IFN-sensitive oncolytic viruses, preventing efficacy. IFNs are also anti-proliferative in cancer and activate anti-tumor immunity.

In this work I show that the recombinant poliovirus PVSRIPO, currently in clinical trial as a treatment for glioblastoma, induces and evades IFN-β signaling in cancer cell lines infected at low doses. Likewise, IFN-α treatment of cancer cells inhibited PVSRIPO less than on the related encephalomyocarditis virus (EMCV). Antibody blockade of the IFN-α/β receptor had no effect on either virus in IFN-secreting melanoma cell lines. Depletion of the pattern recognition receptor MDA5 or inhibition of TBK1/IKKε eliminated IFN responses to PVSRIPO or EMCV and promoted EMCV, but not PVSRIPO, replication. The Toll-like receptor 3 (TLR3) agonist poly(I:C) suppressed EMCV (semi-independently of type I IFN signaling) but not PVSRIPO. Thus, MDA5 and TLR3 provoke type I IFN-dependent and -independent antiviral effects, likely involving upregulation of genes downstream of TBK1/IKKε. PVSRIPO subverts anti-viral immunity in cancer cells at low doses and activates type I IFNs through MDA5, supporting its oncolytic and immunotherapeutic use even in IFN-competent cancers.

Dedication

For Dad.

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1. Introduction

1.1 Oncolytic Viruses as Cancer Immunotherapy

Viruses were proposed as cancer therapeutics more than 40 years ago (Bluming & Ziegler, 1971; Hansen & Libnoch, 1978). Technological advances of the last 20 years in virology, genetics, and rodent cancer models furthered the preclinical development of oncolytic viruses (OVs) as effective therapeutics. Conceptually, OVs replicate within and lyse cancer cells while leaving normal cells unharmed.

Early work on OVs focused on *in vitro* cytotoxicity and human tumor cell xenografts in immunocompromised mouse models (Prestwich et al., 2009). Many of the cancer cell lines from these early experiments lacked type 1 Interferon (IFN) signaling (Stojdl et al., 2000). Therefore, OVs were engineered to remove virulence factors enabling viral evasion or disruption of the innate immune system, including IFN signaling. Tumors also immunosuppress their microenvironments in humans and immunocompetent mouse models, which should promote OV replication and allow viral spread throughout the tumor mass (Qian & Pollard, 2010). These observations lead to a series of assumptions: 1) OVs would replicate and spread through patient tumors as in mouse models; 2) engineered IFN sensitivity would restrict OV cytotoxicity to cancer cells; and 3) immunosuppressive tumor microenvironments would allow extensive

infiltration and infection of tumor cells by OVs without inhibition by the patient's immune system. However, much evidence contradicts these assumptions.

Many cancer cell lines produce, secrete, and respond to IFNs. Given the inherent heterogeneity of tumors, and the high number of innate immune cells commonly present in the tumor stroma, the presence of innate antiviral IFN competency in neoplastic lesions has to be assumed (Li et al., 2007). Therefore, IFN signaling becomes an important consideration for OV treatment. Several engineered IFN-sensitive OVs are profoundly inhibited in cancer cells *in vivo* and *in vitro*, including measles virus (Allagui et al., 2017), oncolytic herpes simplex virus (Jackson et al., 2016), Semliki Forest virus (Ruotsalainen et al., 2015), and vesicular stomatitis virus (Westcott et al., 2015). PVSRIPO has shown success in immunocompetent mouse models and humans, but treatment did not correlate with widespread proliferation, as one would expect if treatment relied solely on viral cytotoxicity (Brown et al., 2017; Desjardins et al., 2018). Taken together, this evidence suggests that the interaction of OVs with the host innate immune system is far more complicated than initially realized.

Treatment of tumors with Newcastle disease virus in immunocompetent mouse models provoked IFN-dependent anti-tumor adaptive immunity (Zamarin et al., 2014). PVSRIPO elicits tumor antigen specific anti-tumor immune effector responses in immunocompetent mouse tumor models (Brown et al., 2017). Cytotoxicity, therefore, is

not the only mechanism of OV therapy, and may not even be its main component. Therefore, the question of the relative contribution of innate antiviral immunity to OV therapy is of eminent importance. Indeed, engineered type IFN sensitivity as a means of tumor specificity (the main mechanism of tumor selectively of most proposed OV platforms) may preclude clinical efficacy. Thus, additional criteria in assessing the clinical potential of prospective OVs should be considered, e.g. the potential for productive replication in the presence of active type 1 IFN signaling or the role of the antiviral inflammatory immune system in the clinical response to OVs.

1.1.1 PVSRIPO in Glioblastoma

PVSRIPO is an engineered rhino:poliovirus chimera with the internal ribosome entry site (IRES) of human rhinovirus type 2 (HRV2) and the coding region of the live-attenuated vaccine strain of poliovirus type 1 (PV1, Sabin), both picornaviruses of the enterovirus genus (Fig. 1) (Gromeier et al., 1996). A 61-patient phase-I clinical trial showed intratumoral infusion of PVSRIPO in recurrent World Health Organization (WHO) stage IV glioblastoma (GBM) produced a high incidence of long-term survivors 36 months post PVSRIPO infusion, with patients alive >6 yrs after treatment (Desjardins et al., 2018). GBM is the most aggressive form of brain cancer with only a 15-month median overall survival with standard of care at the time of diagnosis. Currently, standard treatment for GBM is gross resection of the tumor followed by a combination

of radiotherapy and treatment with the alkylating cytostatic drug temozolomide (Stupp et al., 2005). These abysmal survival figures motivated the use of PVSRIPO as an experimental treatment.

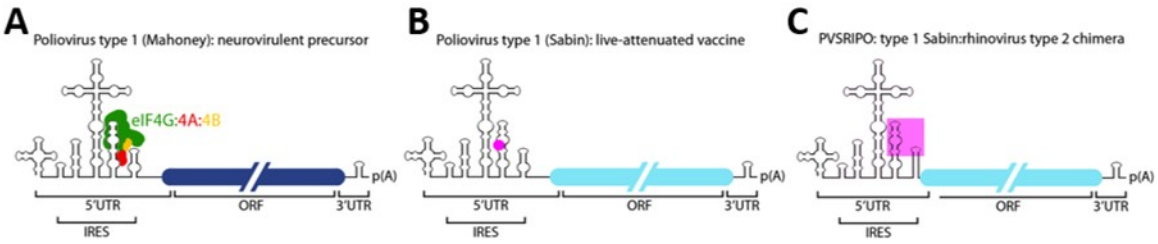

Figure 1: Genetic structure of PV Type 1 (Mahoney), (Sabin), and PVSRIPO
(A) PV1 (Mahoney) is the prototype neurovirulent strain. The eukaryotic initiation factor 4G (eIF4G):4A:4B translation initiation helicase complex binds in domains V and VI of the IRES. (B) PV1 (Sabin) is the live-attenuated vaccine strain with key attenuating mutations in domain V of the IRES (pink circle) and in the open reading frame (ORF, light blue). (C) PVSRIPO is a chimera of the HRV2 IRES and PV1 (Sabin) ORF and 3'UTR; there are significant sequence differences between domains V and VI of PV1(S) IRES and HRV2 IRES (pink box). Untranslated region (UTR), polyadenylate tract [p(A)]. Adapted from (Brown et al., 2014c); used with permission (see Appendix A).

1.1.1.1 Mechanisms of PVSRIPO cancer-specific replication

PVSRIPO tissue tropism is determined by expression of the PV receptor CD155, also called PVR. This surface protein is broadly expressed on neoplastic cells of solid tumors including GBM (Chandramohan et al., 2017), melanoma (Bevelacqua et al., 2012), and breast cancer (Ochiai et al., 2006). CD155 is also expressed in certain sites in the gastrointestinal tract and motor neurons, sites of replication for PV (Mendelsohn et al., 1989). Monocyte-derived cells, e.g. macrophages and dendritic cells (DCs), also express CD155, and are priority targets for PV replication, at least in the gastrointestinal tract

(Shen et al., 2017). Intriguingly, PVSRIPO-infected human DCs resist cytotoxicity, but exhibit prolonged type I IFN-dominant inflammatory stimulation (Brown et al., 2017).

High-dose intracerebral injection of PVSRIPO is non-pathogenic and does not induce viral shedding in non-human primates (Dobrikova et al., 2012) or humans (Desjardins et al., 2018). Multiple mechanisms cooperate to attenuate PVSRIPO in neurons relative to its pathogenic precursor, but all of them are based on inclusion of the HRV2 IRES (Gromeier et al., 2000a).

The HRV2 IRES associates with the double-stranded RNA binding protein 76 (DRBP76, also called NF90) in neurons to form a ribonucleoprotein (RNP) complex with deficient ribosome recruitment and reduced translation initiation (Merrill et al., 2006). DRBP76 localizes to both the cytoplasm and nucleus in neurons, but is restricted to the nucleus in malignant glioma cells. PVSRIPO translation occurs at a site in the endoplasmic reticulum; this difference in DRBP76 localization may partially explain neuro-attenuation of PVSRIPO (Merrill & Gromeier, 2006).

An additional mechanism mediating specificity of PVSRIPO replication is the sensitivity of HRV2 IRES-mediated translation to mitogenic cell signaling pathways. Unhinged Ras-Raf-ERK1/2-MNK signaling is a defining property of malignant cells and linked to a bewildering variety of cell functions including proliferation, cell-cycle, terminal differentiation, and survival (Davies et al., 2002). Work from our lab showed

cap-independent translation initiation of viral IRESs benefits from this dysregulation (Brown et al., 2014a; Brown et al., 2014b). Thus, limitation of PVSRIPO replication competency and cytotoxicity to cancer cells relies on multiple complementary mechanisms involving both translation initiation and CD155 expression.

1.2 Picornaviruses

The *Picornaviridae* family consists of small, non-enveloped viruses with positive (+) single-stranded RNA (ssRNA) genomes of 7-8kb and icosahedral capsids. There are at least 30 genera of picornavirus and more than 70 species, which cause a diverse range of pathologies in many vertebrate species (Zell et al., 2017). Genera of disease-causing viruses in mammalian hosts include *Enterovirus*, *Cardiovirus*, *Aphthovirus*, *Hepatovirus*, *Parechovirus*, and *Teschovirus* (summarized in Table 1, adapted from Bedard & Semler, 2004; used with permission, see Appendix A).

Table 1: Major Picornavirus genera and clinical diseases

Genus	Representative species	Clinical diseases
Enterovirus	*Poliovirus*	Poliomyelitis; hand, foot and mouth disease; myocarditis; ocular conjunctivitis
	Coxsackievirus	
	Human rhinovirus	Common cold
Cardiovirus	Encephalomyocarditis virus (EMCV)	Mouse encephalomyelitis and human cardioviral disease
	Theiler's virus	
Aphthovirus	Foot and mouth disease virus (FMDV)	Foot and mouth disease in livestock
Hepatovirus	Hepatitis A virus	Liver disease
Parechovirus	Human parechovirus	Chronic meningoencephalitis; neonatal carditis
Teschovirus	Porcine teschovirus	Teschen-Talfan neurological disease

1.2.1 Enterovirus and Cardiovirus Diseases

Enteroviruses remain important human pathogenic viruses. Poliovirus, the causative agent of paralytic poliomyelitis, and rhinoviruses, the causative agents of the common cold, fall within the *Enterovirus* genus. Poliomyelitis was one of the most feared diseases of the 20th century, until discovery of the PV vaccine, and the common cold is a significant drain on the economic well-being of the world. These two viruses are the origins of the genetic components of PVSRIPO (Gromeier et al., 1996). Coxsackieviruses also exhibit human-specific tropism, causing diseases like foot-and-mouth syndrome in young children (Bedard & Semler, 2004). The host range of poliovirus and rhinoviruses

is limited to humans. Cardioviruses, including the prototypical EMCV, have a comparatively broad host range, causing disease in rodents, livestock, and humans (Carocci & Bakkali-Kassimi, 2012). EMCV causes myocarditis and encephalitis in infected animals and is highly contagious.

1.2.2 Genomic Structure of Picornaviruses

Picornaviruses lack the 7-methyl guanosine cap present in eukaryotic mRNA, canonically required for translation initiated by the cap-binding protein eukaryotic initiation factor 4E (eIF4E). Rather, a viral protein, VPg, is covalently linked to the 5′ end of the genomic RNA (Fig. 2) (Lee et al., 1977; Nomoto et al., 1976). VPg serves as a primer for synthesis of (+)-strand genomes and (-)-strand template RNA (Nomoto et al., 1977; Paul et al., 1998). Picornavirus genomes serve as templates for protein translation but, as they lack a 5′ cap, require an alternative, cap-independent mechanism to initiate translation. The structured 5′ untranslated regions (UTRs) of picornaviruses contain IRESs that recruit eIFs for ribosome recruitment and translation initiation (Fig. 2) (Jang et al., 1988; Pelletier & Sonenberg, 1988). Picornavirus genomes encode 3′ poly(A) tracts (Fig. 2) which, besides participating in the priming of (-)-strand RNA synthesis (Paul et al., 1998), is required for proper translation initiation at their IRESs (Bradrick et al., 2007).

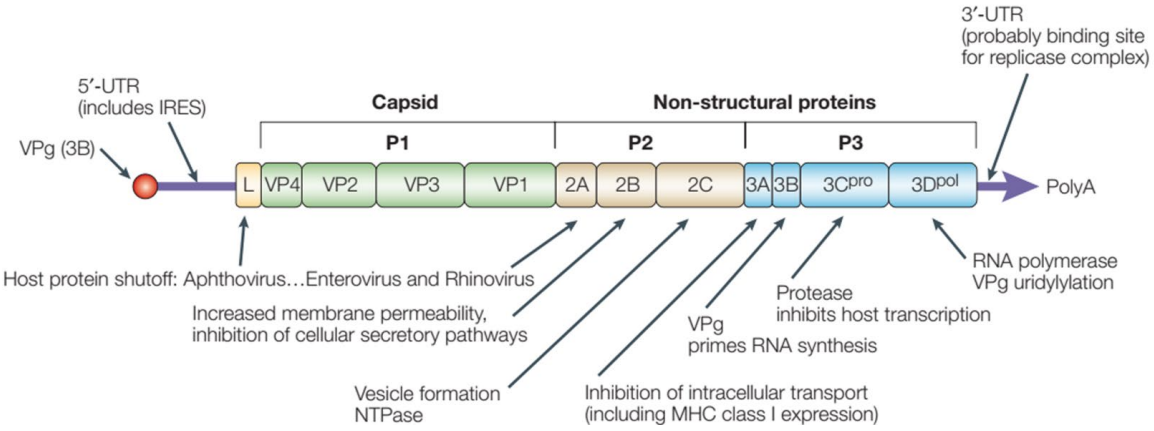

Figure 2: Picornavirus genome, polyprotein products, and their main functions Adapted from (Whitton et al., 2005), see text for details. Used with permission (see Appendix A).

The ORF of picornaviruses is composed of a single polyprotein proteolytically processed by virus-encoded proteases. This polyprotein is cleaved into three major precursors: P1, P2, and P3 (Fig. 2). P1 encompasses structural proteins required for assembly of the viral capsid, VP1-4. P2 contains non-structural proteins involved in membrane rearrangement (2B and 2C), viral RNA replication (2C), and, in the case of enteroviruses, 2A protease ($2A^{pro}$), which auto-proteolytically cleaves from the synthesizing polyprotein and shuts down host cell cap-dependent translation. P3 contains viral proteins which inhibit intracellular transport (3A), the genome-linked protein VPg (3B), the RNA-dependent RNA polymerase (RdRp) $3D^{pol}$, and $3C^{pro}$, a protease required for processing the polyprotein into its active components (Bedard & Semler, 2004). Cardioviruses and aphthoviruses also contain a leader (L) protein between the IRES and P1 (Fig. 2). The aphthovirus L protein is a protease which cleaves

eIF4G (Devaney et al., 1988). EMCV L protein is not catalytically active, but rather inhibits nucleocytoplasmic transport through binding to Ran-GTPase (Porter et al., 2006).

1.2.3 Picornavirus Infectious Cycle

The precise mechanism of cellular entry by picornaviruses is incompletely understood, but evidence from structural and biochemical studies allows for a plausible model of infection for enteroviruses like poliovirus.

1.2.3.1 Receptor for Poliovirus and EMCV

Cellular infection by picornaviruses requires binding of the virus to its receptor. The receptor for PVs, including PVSRIPO is CD155, also called poliovirus receptor (PVR) and nectin-like molecule 5 (Necl-5) (Mendelsohn et al., 1989). CD155 is the high-affinity ligand for T cell immunoreceptor with Ig and ITIM domains (TIGIT), present on T cells and NK cells (Joller et al., 2011), and for DNAX accessory molecule-1 (DNAM-1, also called CD226), expressed on NK cells (Castriconi et al., 2009). It has physiologic roles as an immune checkpoint determinant (Joller et al., 2011) and a cell adhesion molecule (Gromeier et al., 2000b). While both humans and mice express CD155, the murine protein does not allow poliovirus infection. Human CD155-transgenic (tg) mice are permissive of poliovirus and are susceptible to poliomyelitis (Koike et al., 1991). Vascular cell adhesion molecule 1 (VCAM-1) is the receptor for EMCV on murine

vascular epithelial cells (Huber, 1994). The receptor for EMCV on human cells remains incompletely characterized, but a 70 kDa sialoglycoprotein has been proposed as the receptor on HeLa and K562 cells (Jin et al., 1994).

1.2.3.2 Receptor-mediated viral entry

Binding of the PV virion to its receptor at physiological temperatures induces a conformational change in the virion, converting it from a 160S particle to a 135S particle which frequently elutes from the cell surface, thought to result in an abortive infection (Fenwick & Cooper, 1962). Infection at lower temperatures allows virion:receptor binding, but not efficient conversion to the 135S particle (Huang et al., 2000). The 135S particle has an externalized VP4 and VP1 N-terminus, which may form a pore in the cell membrane, as it does in liposome membranes (Fricks & Hogle, 1990). Pore formation requires CD155 binding (Strauss et al., 2015). This pore facilitates entry of the viral RNA to the cell without the capsid proteins. The empty capsid then disassociates from the receptor and reverses any conformational changes that caused pore formation, leading to the accumulation of a symmetrical 80S particle (Belnap et al., 2000). Many enteroviruses appear to share this entry mechanism with poliovirus, but it is unclear if EMCV utilizes this method of cellular entry.

1.2.3.3 Mechanisms of cell and picornavirus protein translation and translation shutdown

Soon after picornavirus genome entry, the genome is translated in a cap-independent mechanism and shuts down host cell cap-dependent translation. Host cap-dependent translation initiates when the eIF4E component of the cap-binding complex (eIF4F) binds to the 5′ cap of the mRNA (Step 1, Fig. 3A). In addition to eIF4E, eIF4F also consists of eIF4G, a scaffolding protein, eIF4A, a helicase that unwinds secondary RNA structures on the mRNA, and eIF4B. Binding of eIF4E to the cap recruits the 40S ribosomal subunit in complex with eIF2-Met-tRNA-GTP, eIF3, and eIF1A (Levin et al., 1973). This complex, combined with eIF1, forms the 43S pre-initiation complex (PIC; Step 2, Fig. 3A). Once recruited, the 43S PIC scans along the mRNA until an authentic start codon (usually AUG) is reached (Step 3, Fig. 3A). The 60S large ribosomal subunit is recruited, initiation factors are released, and elongation of the nascent polypeptide begins (Jackson et al., 2010).

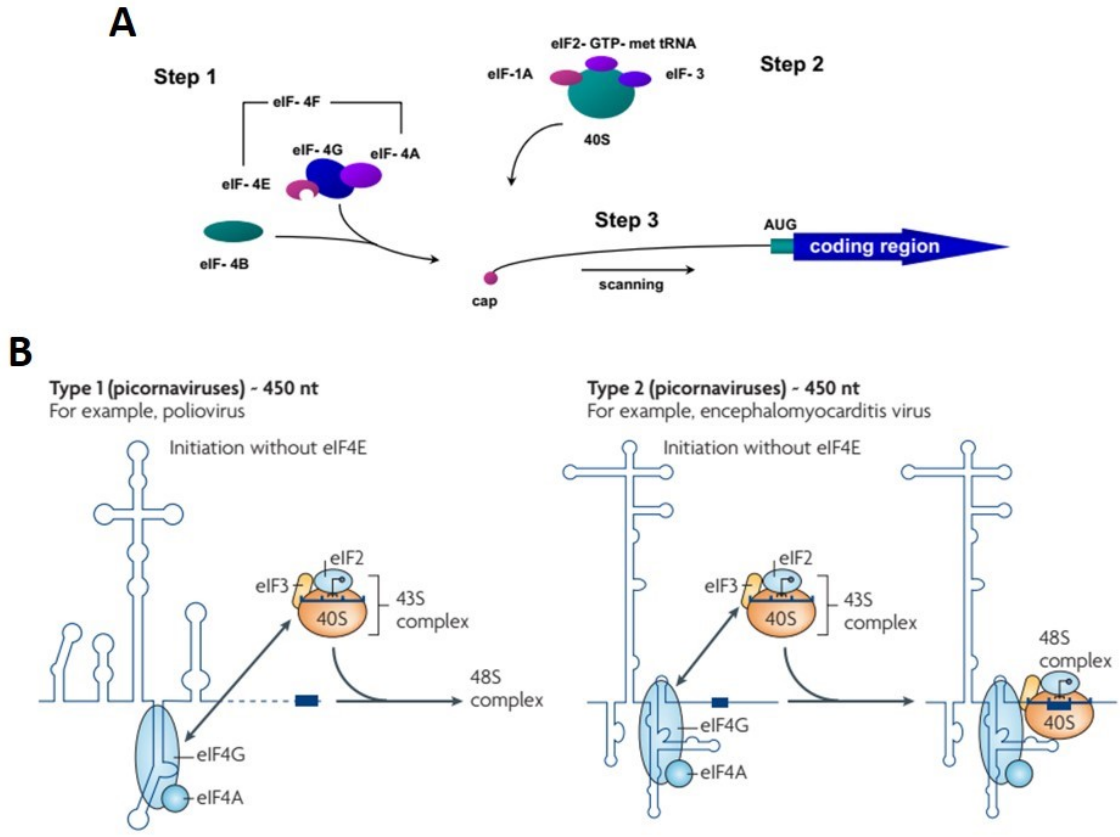

Figure 3: Mechanisms of cap-dependent and –independent translation initiation
(A) Steps in canonical cap-dependent translation initiation, summarized in text; adapted from (Bedard & Semler, 2004). (B) Steps in alternative, cap-independent translation initiation of picornavirus Type 1 IRESs, e.g. poliovirus IRES, and Type 2 IRESs, e.g. EMCV IRES; adapted from (Jackson et al., 2010). Used with permission (see Appendix A).

Picornaviruses use an alternative, cap-independent, mechanism to initiate translation. While picornavirus IRES sequences are highly divergent, they are classified into types based on similar secondary structure. Enteroviruses, like PVSRIPO, have a Type 1 IRES, while cardioviruses like EMCV have Type 2 (Fig. 3B). Both Type 1 and 2 IRESs recruit eIF4G, eIF4A, and the 40S ribosomal subunit without eIF4E involvement. It

is also thought that other proteins, termed IRES trans-activating factors (ITAFs), may be required for translation of picornaviruses, but few of these ITAFs have been conclusively identified (Sweeney et al., 2014). Once the polyprotein is synthesized it can be cleaved into functional subunits by viral proteases, described in Section 1.2.2.

The enterovirus $2A^{pro}$ cleaves eIF4G1 between the eIF4E and eIF4A binding domains soon after infection, causing rapid shutdown of host cell cap-dependent translation. The C-terminal moiety of eIF4G1 retains binding to the PV IRES and ribosome recruitment, thereby allowing viral translation (Krausslich et al., 1987; Lloyd et al., 1987). As cardiovirus 2A lacks protease activity, the mechanism of EMCV-mediated shutdown of host protein synthesis is less well understood. It may involve activation of a translational repressor protein, eIF4E-binding protein 1 (eIF4E-BP1), which inhibits cap-dependent translation by association with eIF4E and intercepting eIF4G binding (Gingras et al., 1996). Shutdown of host cell cap-dependent translation by EMCV is far less efficient and expeditious than shutdown by poliovirus (Jen et al., 1980).

1.2.3.4 Replication of picornavirus genomes

All picornaviruses share a common mechanism for the replication of their genomes. The first step of this process is the uridylylation of VPg to VPgpUpU with a cis-replicating RNA element (cre) as a template (Paul et al., 2000). Location and sequence of this cre varies between picornavirus species, but typically it is a stem-loop structure

14-23 nucleotides (nts) long with two adenine nts that serve as the template (Paul & Wimmer, 2015). *In vitro* this uridylylation requires VPg, the cre, viral $3CD^{pro}$, and $3D^{pol}$ (Paul et al., 2000).

VPgpUpU serves as a primer for synthesis of a complementary negative-sense RNA strand by the viral RdRp $3D^{pol}$ (Paul et al., 1998). This results in the fully duplexed dsRNA replicative form (RF) of the virus (Fig. 4). Next, the 5′ end of the RF is disrupted by cellular and viral proteins, and $3D^{pol}$ synthesizes a new (+)-strand using the minus strand as a template and additional VPgpUpU as primers (Paul et al., 1998). Multiple (+)-strand genomes are simultaneously made from a single (-)-strand forming a replicative intermediate (RI) that consists of a single minus strand and 6-8 nascent plus strands (Fig. 4) (Bishop et al., 1969; Richards et al., 1984). These structures are most well-described in PV infections, but similar RI and RF structures have been found during EMCV replication (Thach et al., 1974). The newly formed RNA genomes are encapsidated by viral structural proteins recruited by viral protein 2C to the site of replication to form viral progeny (Vance et al., 1997; Wang et al., 2012)

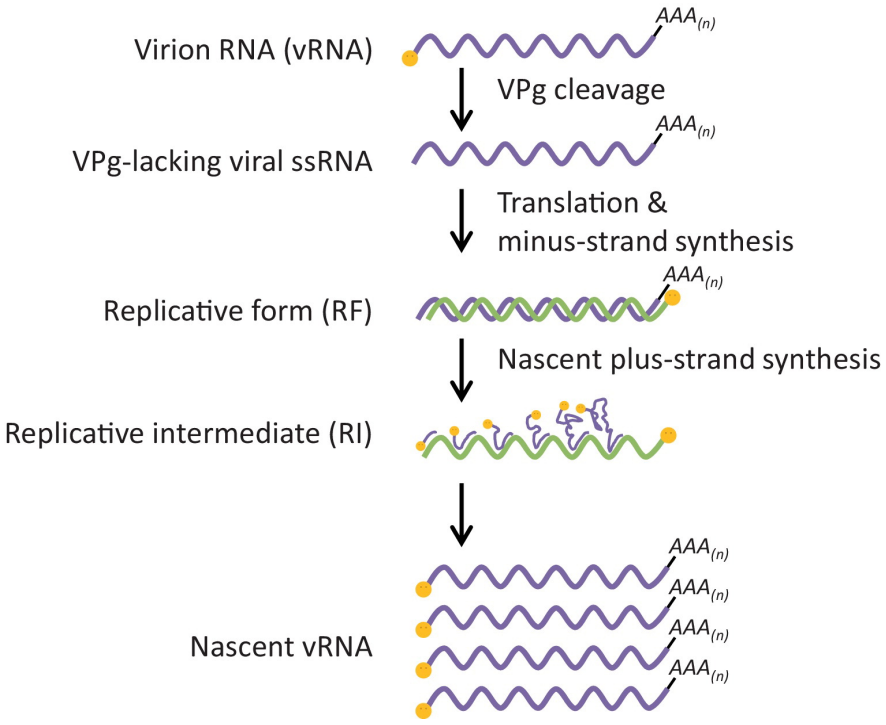

Figure 4: Steps of picornavirus genome replication

VPg is cleaved from the viral RNA (vRNA), then minus-strand synthesis by the viral RdRp takes place, primed by VPg. The completed dsRNA RF dissolves and additional plus-strand synthesis of multiple RNAs simultaneously from the same minus-strand templates primed by viral VPg and synthesized by the viral RdRp. The nascent vRNA is packaged in virions. Purple line, viral positive-strand RNAs. Green line, viral negative-strand RNAs. Orange circle, VPg/3B. Adapted from (Feng et al., 2014b); used with permission (see Appendix A).

1.2.3.5 Picornavirus virion assembly and release

Structural proteins contained in the P1 protein are cleaved from the viral polyprotein in *cis* by $2A^{pro}$ during its auto-catalytic cleavage (Kitamura et al., 1981). P1 is then further processed by $3CD^{pro}$ into 3 subunits: VP1, VP3, and VP0. This cleavage is associated with the assembly of the three proteins into capsid pentamers, which

spontaneously assemble into empty capsids of 12 pentamers (Hogle et al., 1985). In infected cells the pentamers and empty capsids are in dynamic equilibrium. Encapsidation concludes when a provirion is assembled from the pentamers and viral RNA and autocatalytic processing converts VP0 into the final structural proteins VP2 and VP4 (Hindiyeh et al., 1999). The virion is then released from the cell in a lytic or non-lytic manner (Bird & Kirkegaard, 2015).

1.3 Mechanisms of Antiviral Immunity

Vertebrate species are protected from pathogens by two complementary systems: the innate and adaptive immune systems. Major cells of the adaptive immune system include T and B lymphocytes (Iwasaki & Medzhitov, 2004). These lymphocytes recognize antigens previously encountered by the host to establish long-lived protective memory. B cells produce antibodies against target antigens to neutralize or destroy invading microbes while T cells recognize target antigens on cell surfaces. Most antigens recognized by the adaptive immune system are from pathogens, but it can recognize self-antigens as well, often causing auto-immune diseases including type 1 diabetes (Horwitz et al., 1998). Cytotoxic T lymphocytes (CTLs) are of significant interest for cancer immunotherapy as they recognize tumor-associated antigens (TAAs) and lyse TAA-positive cells. PVSRIPO treatment induces an anti-TAA CTL response *in vitro* and appears to facilitate intratumoral CTL infiltration *in vivo* (Brown et al., 2017).

The innate immune system provides a more general mechanism to establish short-term pro-inflammatory and antimicrobial responses; these mechanisms are the first line of defense against microbial invasion. Key cells of this system are DCs, macrophages, neutrophils, basophils, and eosinophils. These innate immune cells promote and regulate antimicrobial responses, present antigens to adaptive immune cells, and aid in tissue repair. However, almost all cells have some level of involvement in innate immune system responses (Platanias, 2005). Typically, an infected cell recognizes a microbial infection and upregulates intracellular antimicrobial proteins including cytokines, e.g. IFN. Upon binding to autocrine or paracrine receptors at cell surfaces, cytokines activate a diverse range of pro- and anti-inflammatory effects to regulate all aspects of the innate and adaptive immune response.

1.3.1 Detection of Intracellular Pathogens

Mammalian cells express many pattern recognition receptors (PRRs) to detect specific molecules expressed by invading microbes called pathogen-associated molecular patterns (PAMPs). Two well-characterized families of PRRs implicated in antiviral innate immunity are the Retinoic acid-inducible gene-I (RIG-I)-like Receptors (RLRs) and the Toll-like Receptors (TLRs) (Kawai & Akira, 2008).

1.3.1.1 RIG-I-like receptors (RLRs)

Melanoma differentiation-associated gene-5 (MDA5) is a member of the cytosolic RLR family of PRRs along with RIG-I and Laboratory of Genetics and Physiology 2 (LGP2) (Fig. 5) (Kang et al., 2002; Yoneyama et al., 2005). RLRs contain an internal DExD/H-box RNA helicase domain with ATPase activity and a C-terminal regulatory domain (Cui et al., 2008). MDA5 and RIG-I, but not LGP2, also contain two N-terminal Caspase activation and recruitment domains (CARDs) (Loo & Gale, 2011). RIG-I and MDA5 both bind to double-stranded RNA (dsRNA), but RIG-I binds short dsRNA with 5′ tri-phosphate groups, while MDA5 binds long dsRNA ≥2kbp (Hornung et al., 2006; Kato et al., 2008; Pichlmair et al., 2006). Thus, RIG-I and MDA5 recognize different virus families. RIG-I recognizes (+)- and (-)-strand RNA viruses including Hepatitis C virus (Foy et al., 2005), Sendai virus (Kato et al., 2006), vesicular stomatitis virus (Kato et al., 2005), and influenza A virus (Loo et al., 2008). MDA5 primarily senses picornaviruses (Gitlin et al., 2006). The role of LGP2 in innate immunity is somewhat mysterious, but it appears to regulate activation of the other two RLRs (Satoh et al., 2010).

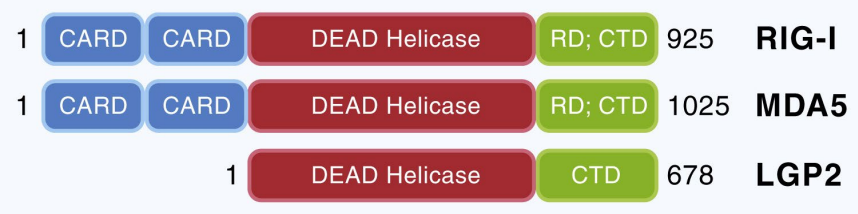

Figure 5: Structural representation of RLRs

RIG-I and MDA5 contain two tandem N-terminal CARDs (blue boxes), an ATPase containing internal DExD/H-box RNA helicase domain (DEAD Helicase, red box), and a C-terminus domain (CTD, green box), also called a regulatory or repressor domain (RD). LGP2 lacks the tandem N-terminus CARDs. Adapted from (Loo & Gale, 2011); used with permission (see Appendix A).

MDA5 recognizes the dsRNA RF of picornaviruses (Feng et al., 2012; Triantafilou et al., 2012). The helicase domain binds to the dsRNA dependent on ATP and multimerizes, forming a filamentous RNP complex (Peisley et al., 2011). Under unusual conditions MDA5 may bind to cellular RNAs, but normally potential target RNAs are modified by Double-stranded RNA-specific adenosine deaminase (ADAR) to prevent recognition (Liddicoat et al., 2015). MDA5-dsRNA RNPs then translocate to the mitochondria where they interact with Mitochondrial antiviral signaling protein (MAVS), also called VISA, IPS-1, and Cardif, through their respective CARDs (Kawai et al., 2005).

MAVS binding to RLRs activates Tumor-necrosis factor (TNF)-receptor-associated factor (TRAF)-family-member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1) and Inhibitor of NF-κB (IκB) kinase (IKK) ε (Paz et al., 2006; Seth et al., 2005). These two serine/threonine kinases phosphorylate IFN regulatory factor (IRF) 3

and IRF7 which then hetero- and homo-dimerize to form active transcription factors (Fitzgerald et al., 2003). IRF3/7 transcribe type 1 IFNs and other proinflammatory, antiviral genes. MAVS also activates the transcription factor NF-κB by phosphorylation of IκB by a complex of IKKα/β/γ and subsequent disassociation. NF-κB upregulates a variety of genes including pro-inflammatory cytokines (Alexopoulou et al., 2001).

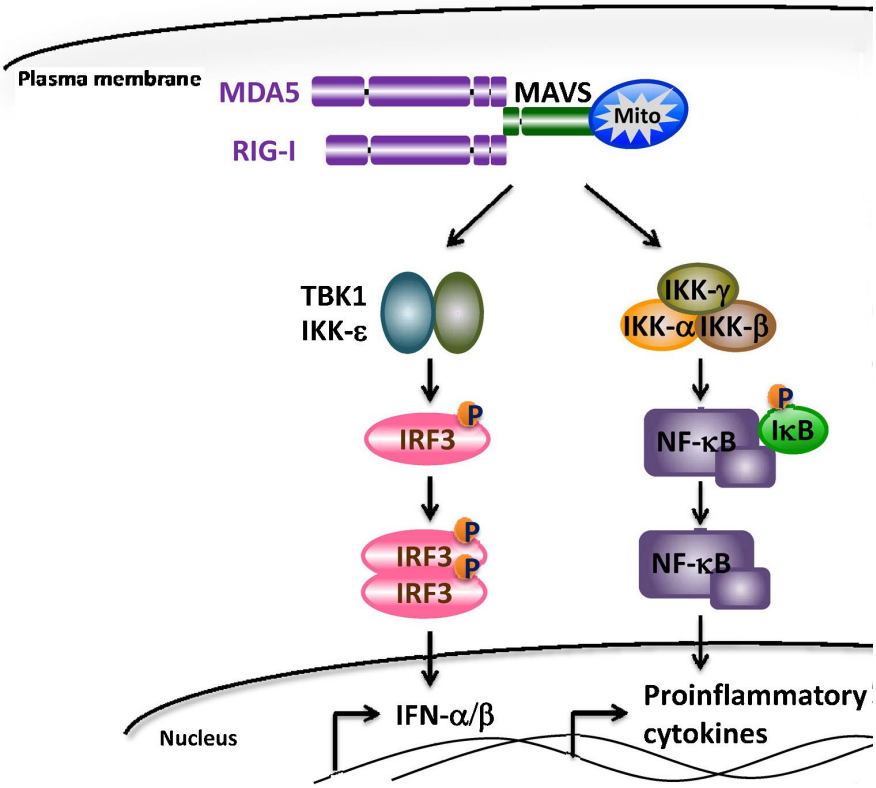

Figure 6: MDA5 and RIG-I signal transduction
Adapted from (Feng et al., 2014b) and described in depth in text. Used with permission (see Appendix A).

1.3.1.2 Toll-Like receptors (TLRs)

TLRs are a family of membrane-bound PRRs with 13 identified members in mammals that recognize many viral and bacterial PAMPs. All TLRs contain an extracellular or endosomal leucine-rich repeat domain for binding target PAMPs, a transmembrane domain, and a cytosolic Toll-Interleukin-1 (IL-1) receptor (TIR) homology domain which is required for intracellular signaling (Kawai & Akira, 2006). Once bound to the target PAMP, TLRs hetero- or homo-dimerize and interact with adapter molecules through the TIR domain (O'Neill et al., 2013).

One subfamily of TLRs, consisting of TLR3, 7, 8, and 9, localizes to intracellular membranes, e.g. endosomes, and specialize in the detection of nucleic acids (Iwasaki & Medzhitov, 2004). TLR3 senses viral dsRNA and the dsRNA analog polyinosinic-polycytidylic acid [poly(I:C)] (Alexopoulou et al., 2001), TLR 7 and 8 sense viral single-stranded (ss) RNA (Diebold et al., 2004; Heil et al., 2004; Lund et al., 2004), and TLR9 senses bacterial and viral DNA (Hemmi et al., 2000; Lund et al., 2003). A key component of this subfamily's discrimination between cellular and foreign nucleic acids lies in the localization of the TLRs to the endosome and the placement of the leucine-rich domain within the endosome. Thus, only nucleic acids contained within the endosome are recognized and activate downstream signaling (Fig. 7).

A second subfamily of TLRs localize to the cell surface and detect a variety of bacterial PAMPs. The prototypical example of this subfamily is TLR4, which recognizes lipopolysaccharide (LPS), a component of Gram-negative bacterial cell walls (Poltorak et al., 1998). Other members of this subfamily include TLR1, 2, 5, and 6. TLR2 forms a heterodimer with TLR1 to detect triacylated lipopeptides or with TLR6 to recognize diacylated lipopeptides (Jin et al., 2007; Kang et al., 2009). TLR5 detects flagellin, a component of bacterial flagella (Hayashi et al., 2001).

Downstream signaling of TLRs requires a complex sequence of protein-protein interactions involving IL-1 receptor (IL-1R)-associated kinases (IRAKs) and the adaptor TRAFs (Fig. 7). Generally, TLRs require one of two adapter molecules: Myeloid differentiation primary-response protein 88 (MyD88) or TIR domain-containing adaptor protein inducing IFN-β (TRIF; also known as TICAM1). Both MyD88 and TRIF contain TIR domains which interact with the TIR domains of TLRs (Fig. 7). Most TLRs interact with MyD88 to induce signal transduction through protein complexes that involve several other cofactors and kinases (O'Neill et al., 2013).

Activation through MyD88 causes activation of the NF-κB transcription factor along with activation of the mitogen-activated protein kinases (MAPKs) p38 and JUN N-terminal kinase (JNK), which activate and upregulate of a variety of anti-pathogenic pathways and proteins (Muzio et al., 1997). MyD88 signal transduction also activates

IRF7, which serves as a transcription factor for type 1 IFNs (Kawai et al., 2004). TLR3 and TLR4 are notable in that they interact with TRIF independently of MyD88 for signal transduction (Oshiumi et al., 2003; Yamamoto et al., 2003). This activates IRF3 transcription factor via phosphorylation by TBK1/IKKε in the same manner as the RLRs.

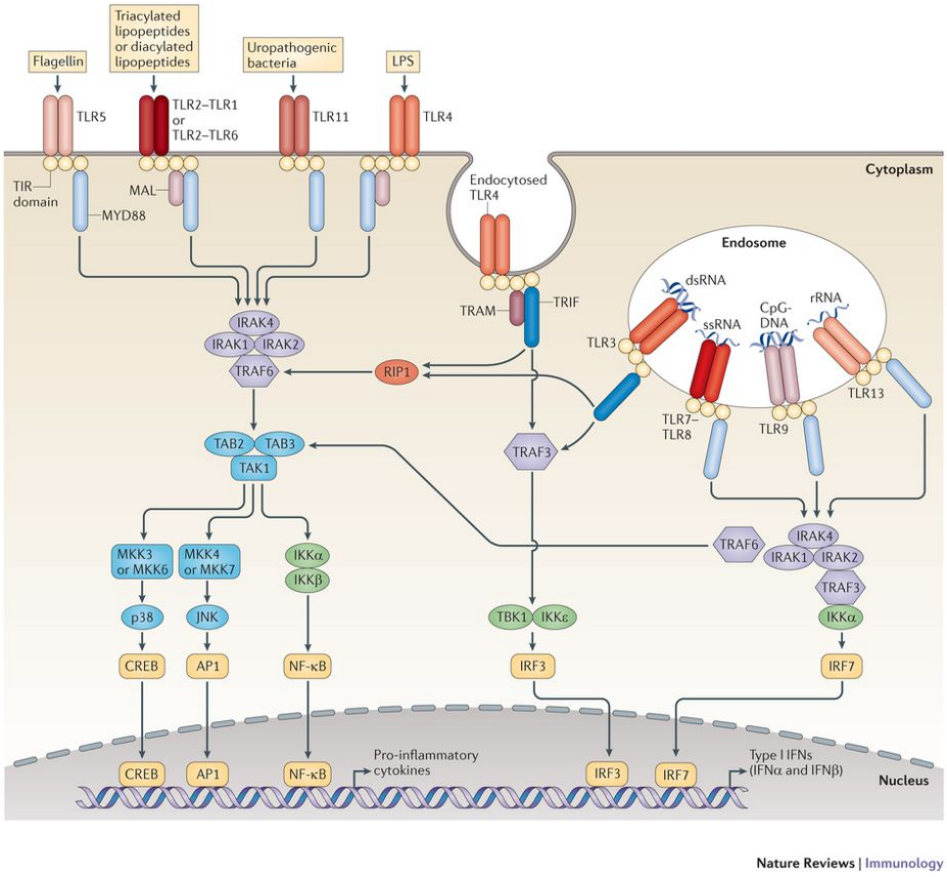

Figure 7: Toll-like Receptor (TLR) signaling pathways

TLR5 and heterodimers of TLR2–TLR1 or TLR2–TLR6 localize to the plasma membrane, whereas TLR3, TLR7–TLR8, and TLR9 localize to the endosomes. TLR4 localizes at both the plasma membrane and endosomes. Following ligand-induced dimerization, most TLRs signal through MyD88 and/or MyD88-adaptor-like protein (MAL). This enables downstream signal transduction through kinase complexes to transcription factors CREB, AP1, NF-κB, and IRF7. It also enables activation of the MAPKs p38 and JNK. TLR3 and endosomal TLR4 signal through TRIF, not MyD88. Activated TRIF uses TRAF3 to activate TBK1/IKKε to phosphorylate and activate the transcription factor IRF3, which induces type I IFNs. TLR signaling induces pro-inflammatory cytokines and other antimicrobial pathways. Adapted from (O'Neill et al., 2013); used with permission (see Appendix A).

1.3.2 The Interferon Response

IFNs were one of the first class of cytokines identified when chicken chorio-allontoic membrane were infected with heat-inactivated influenza virus, the membranes washed, then fluid from the membranes used to "interfere" with influenza infection of fresh membrane (Isaacs & Lindenmann, 1957). Since then enormous amounts of research have been done to identify the regulation and effects of IFNs. Three classes of IFNs have now been identified, each with a distinct receptor: types I, II, and III. These receptors are expressed in a cell-type dependent manner, and each IFN has distinct immune modulatory effects. However, all three utilize canonical Janus kinase/Signal transducer and activator of transcription (JAK/STAT) pathways for signal transduction.

1.3.2.1 Type I Interferons

There are many type I IFNs with substantial sequence and structural homology and a single shared receptor. In humans, this includes IFN-α (which has 13 different subtypes), IFN-β, IFN-ε, IFN-κ and IFN-ω (Platanias, 2005). Of these, IFN-α and IFN-β are the most well described and serve as the prototypical examples. The shared receptor for the type I IFNs is a heterodimeric receptor composed of the two ubiquitously expressed transmembrane protein subunits, IFN-α/β receptors 1 and 2 (IFNAR1/2) (Cleary et al., 1994; Novick et al., 1994).

IFN-α/β were among the earliest cytokines found to signal through the JAK/STAT pathway (Shuai et al., 1993). Transduction of type I IFN signaling requires two members of the JAK family, JAK1 and Tyk2, and two members of the STAT family, STAT1 and STAT2 (Gauzzi et al., 1996). JAK1 and Tyk2 associate with the IFNAR intracellular domains (Fig. 8). Following activation by binding of type I IFNs to IFNAR, JAK1/Tyk2 phosphorylate STAT1/2 at tyrosines 701 and 660, respectively. Phosphorylated STAT1 homodimers or STAT1/STAT2 heterodimers activate transcription of genes with Gamma-activated sequences (GAS) in their promotors. STAT1/STAT2 heterodimers also bind IRF9 to form a complex known as IFN-stimulated gene factor 3 (ISGF3) which activates transcription of genes with promotors containing IFN-stimulated response elements (ISREs) (Ramana et al., 2000). These transcription factor complexes have some specificity for subsets of IFN stimulated genes (ISGs), but there is significant overlap as well.

1.3.2.2 Type II Interferon

IFN-γ is the only known type II IFN and is a dimeric cytokine structurally unrelated to the type I IFNs (Gray & Goeddel, 1982). Unlike the type I IFNs, IFN-γ is expressed predominately by immune cells, and is more immune-modulatory than immune-stimulatory. T cells, NK cells, DCs, and macrophages all produce IFN-γ (Fultz et al., 1993; Ohteki et al., 1999; Young, 1996). Type I IFNs are typically secreted in

response to microbial infection, but IFN-γ is upregulated in an autocrine and paracrine manner in response to other cytokines including IL-12 (Young, 1996).

Type II IFN has a unique IFN-γ receptor (IFNGR) comprised of two IFNGR1 and two IFNGR2 subunits in two heterodimers (Fig. 8) (Schroder et al., 2004). IFNGR1 is constitutively expressed in the classic IFN-γ responsive T, B, and myeloid cells, but IFNGR2 expression is significantly lower and represents the limiting factor of responsiveness to the cytokine. IFNGR2 expression is regulated, at least in part, by cellular differentiation (Bernabei et al., 2001). IFNGR1/2 associate with JAK1/2, respectively, through IFNGR1/2 intracellular domains (Kaplan et al., 1996; Kotenko et al., 1995). Binding of IFN-γ to its receptor leads to activation of these two kinases, which in turn phosphorylate STAT1, but not STAT2 (Shuai et al., 1993). Thus, IFN-γ does not stimulate the formation of ISGF3, but only the STAT1 homodimer transcription factor and upregulation of genes with GAS elements.

1.3.2.3 Type III Interferons

Type III IFNs are the most recently discovered IFNs with three known members in humans, IFN-λ1, -λ2, and –λ3 (also known as IL-29, IL-28A, and IL-28B, respectively) (Kotenko et al., 2003; Sheppard et al., 2003). Like the type I IFNs, type III IFNs have potent antiviral effects and are secreted in response to viral infections. *In vitro* studies indicate recombinant IFN-λ2 has antiviral effects on EMCV, but recombinant type III

IFNs did not affect EMCV *in vivo* (Ank et al., 2006). Type III IFNs also have a more limited set of responsive cells, thought to be a result of expression of the receptor on a smaller subset of cells (Zhou et al., 2007).

Type III IFNs are closely related to the type I IFNs and share many signaling characteristics. Type III IFNs have a heterodimeric receptor made up of IFN-λ receptor 1 (IFNGR1, also known as IL-28RA) and IL-10 receptor 2 (IL-10R2). IFNGR1 is unique to the type III IFN receptor while IL-10R2 is shared with receptor complexes for the cytokines IL-10, IL-22, and IL-26 (Fig. 8) (Donnelly & Kotenko, 2010). Functional downstream signaling of type I and type III IFNs are remarkably similar; they are stimulated by viral infection or activation of PRRs by synthetic agonists (Ank et al., 2008), activate the JAK1 and Tyk2 kinases after binding to their respective receptors, and they induce phosphorylation of STAT1/2 and formation of the STAT1 homodimer and ISGF3 transcription factors (Kotenko et al., 2003). It is also notable that, like the type I IFNs, type III IFNs are being investigated for their potential in augmenting cancer immunotherapy, and these studies have shown some promise in mouse models (Lasfar et al., 2006).

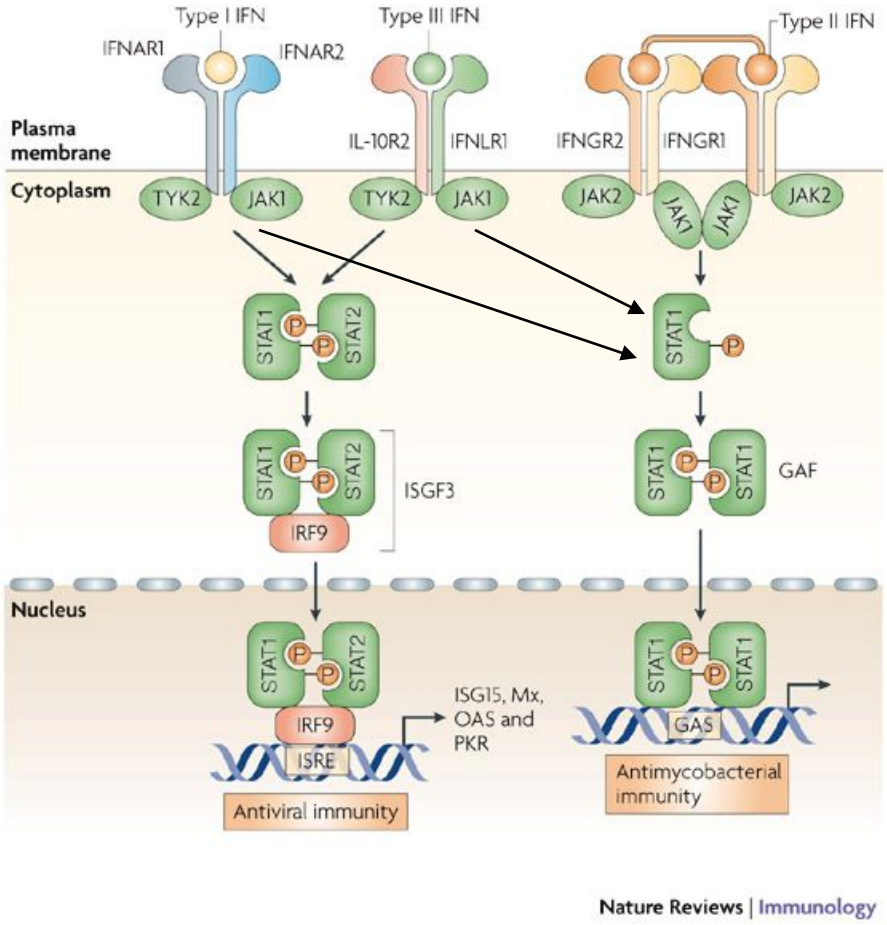

Figure 8: Interferon receptor signaling

Type I, II, and III IFNs use different receptors at the cell surface. IFNAR1/2 heterodimers form the receptor for type I IFNs, IL-10R2/IFNLR1 heterodimers form the Type III IFN receptor, and two IFNGR1/2 heterodimers form the Type II IFN receptor. Type I and III IFNs signal through JAK1 and TYK2 to phosphorylate STAT1/2. Type II IFNs activate JAK1/2 to phosphorylate STAT1. Phosphorylated STAT1 homodimerizes to form the IFN-γ activating factor (GAF) or heterodimerizes with phosphorylated STAT2 to form IFN-stimulated gene factor 3 (ISGF3) along with IRF9. ISGF3 binds to the ISRE and activates transcription while GAF binds to GAS. Adapted from (Sadler & Williams, 2008); used with permission (see Appendix A).

1.3.2.4 Antiviral mechanisms of IFN-stimulated genes

According to Interferome v2.0, a database of IFN regulated genes, there are over 1000 genes transcriptionally upregulated by one or more of the IFNs (Rusinova et al., 2013). Included in the list are the RLRs and TLRs, IFN signal transduction molecules including kinases and transcription factors, and all three types of IFNs. Many ISGs have binding sites for some or all of the transcription factors previously discussed. In addition, most ISGs are regulated by transcriptional, translational, and post-translational mechanisms (Platanias, 2005). Thus, it is difficult to determine how a specific ISG of interest is regulated. However, a great deal is known on the antiviral mechanisms of specific ISGs, and they serve as useful markers for cellular activation of antiviral and pro-inflammatory states.

2'-5'-Oligoadenylate synthetase 1 (OAS1) is upregulated by type I, II, and III IFNs and contains an ISRE, GAS, IRF3-binding site, and NF-κB binding site in its promoter region (Rusinova et al., 2013). Overexpression of this enzyme inhibits EMCV (Chebath et al., 1987). OAS1 synthesizes 2',5'-oligoadenylates from ATP which activate the endoribonuclease RNaseL (Dong & Silverman, 1995). Activated RNaseL degrades viral and cellular RNAs to create PAMPs and further stimulate IFN production via the RLRs. Degradation of viral RNAs also reduces viral propagation (Malathi et al., 2007).

IFN-induced protein with tetratricopeptide repeats 1 (IFIT1, also called ISG56) is upregulated by type I, II, and III IFNs. It is also upregulated by IRF3 independently of type I IFN signaling (Grandvaux et al., 2002). Much like RIG-I, IFIT1 binds to viral 5′-triphosphate RNA, but does not activate downstream antiviral signaling. Rather, it forms a complex with other members of the IFIT protein family to sequester bound RNA, inhibiting viral translation. IFIT1 does not inhibit EMCV, which has the viral protein VPg at the 5′ end of its RNA genome rather than a triphosphate group. IFIT1 does inhibit VSV and Hepatitis C virus (Pichlmair et al., 2011; Raychoudhuri et al., 2011).

1.3.3 Strategies of Viral Evasion of Intracellular Immune Responses

Viral pathogens utilize numerous mechanisms to either replicate in cells in an antiviral state or prevent innate immune responses from initiating. One mechanism to prevent innate immune initiation is to mask viral PAMPs so they are not recognized by PRRs. PV remodels endoplasmic reticulum membranes to create membrane-enclosed organelles, similar to vesicles, that act as sites of virus translation and transcription, and may be required for efficient viral RNA synthesis (Suhy et al., 2000). Similar replication organelles are produced during EMCV infection, but the structure and formation of the organelles is significantly different (Melia et al., 2018). A beneficial side effect of these viral organelles, from the virus's perspective, may be to sequester viral dsRNA in a compartment inaccessible by MDA5. This mechanism is not very effective, particularly

for EMCV, however. MDA5 -/- mice showed significant reduction in type I IFN production during EMCV infection compared to their MDA5 +/- littermates, and this loss of type I IFN signaling caused more robust virus replication and toxicity (Kato et al., 2006).

Viruses also evade immune surveillance by protease-mediated cleavage of key components of innate immune signaling pathways. This can be done either by viral proteases or by virus-mediated activation of cellular proteases, including caspases. Some research indicates enteroviruses utilize this strategy, but the methods and conclusions of these studies are controversial. Two groups have claimed enterovirus $3C^{pro}$ cleaves RIG-I during infection of HeLa cells with Coxsackievirus B3 (CV-B3), PV, HRV, and echovirus at 10 multiplicity of infection (MOI) (Barral et al., 2009; Feng et al., 2014a). RIG-I does not recognize enteroviral PAMPs, as such RIG-I cleavage is unlikely to inhibit the innate immune response; more likely it is an off-target effect of viral cytopathogenicity (Gitlin et al., 2006).

Enteroviruses allegedly inhibit IFN responses through proteolytic cleavage of MDA5 and/or MAVS, but there is disagreement on the mechanism. Barral et al. determined MDA5 cleavage during PV infection of HeLa cells at 10 MOI is caspase-dependent; MDA5 is also cleaved by caspases during chemical-induced apoptosis in 293T cells (Barral et al., 2007; Kovacsovics et al., 2002). Feng et al. claimed PV and CV-B3

2A^{pro} cleave MDA5 independent of caspases in HeLa cells infected with an MOI of 50 (Feng et al., 2014a). Putative MAVS cleavage during enterovirus infection was linked to caspases (Rebsamen et al., 2008), 2A^{pro} (Feng et al., 2014a), or 3C^{pro} (Mukherjee et al., 2011).

A final means of innate immune evasion conserved by multiple picornaviruses is host cell translation shutdown. Cleavage of PRRs and their signal transduction partners may prevent or delay IFN signaling, but this mechanism allows replication of picornaviruses in cells after induction of an antiviral state by preventing translation of antiviral proteins. PV and EMCV both inhibit cap-dependent translation, albeit by different mechanisms and efficacy (Gingras et al., 1996; Jen et al., 1980; Krausslich et al., 1987). Shutdown of cap-dependent translation is highly cytotoxic and promotes viral translation; 2A^{pro} is essential for PV replication in type I IFN-treated cells (Morrison & Racaniello, 2009). However, IFN responses also inhibit cell translation while promoting translation of ISGs, so ISG mRNAs likely circumvent translation shutdown (Der & Lau, 1995). Thus, PVs and EMCV have secondary mechanisms to inhibit translation and transcription of host cell genes.

PVs and EMCV interfere with bidirectional nucleocytoplasmic trafficking. Most cellular processes, including innate immune responses, rely on shuttling of transcription factors from the cytoplasm to the nucleus, i.e. IRFs and STATs, or shuttling of mRNA

from the nucleus to the cytoplasm. Thus, nucleocytoplasmic trafficking disruption is a potent mechanism for viruses to prevent IFN responses and co-opt cellular processes for viral replication. PV $2A^{pro}$ cleaves nucleoporins during infection, which results in bidirectional loss of selective macromolecule trafficking across the nuclear envelope (Belov et al., 2004; Castello et al., 2009). EMCV likewise disrupts nucleocytoplasmic trafficking, not through protein cleavage but via interaction of the viral L protein and Ran-GTPase (Porter et al., 2006).

1.4 Introduction to the Dissertation Chapters

By focusing on attenuation of OVs by eliminating IFN-suppressive virulence factors, and on IFN-incompetent cancer cell models, the field had neglected the clearly important interaction of OVs with the innate immune system. It is also unknown how important picornavirus IFN evasion strategies are in a cancer cell context. PVSRIPO has demonstrated efficacy and safety in the clinic; by exploring the unique relationship of PVSRIPO with the host innate immune system, this work seeks to further understanding the potential of OVs, e.g. PVSRIPO, as cancer immunotherapy adjuvants. The chapters of this work address fundamental questions of IFN responses in cancer cells, their effects on PVSRIPO and EMCV at exceptionally low MOIs, and surprising discoveries of MDA5-mediated antiviral intracellular immune responses in cancer.

Chapter 2 summarizes the materials and methods employed to complete this research.

Chapter 3 has three sections and presents results adapted from a publication *in press* in *Journal of Virology* (Walton et al., 2018).

Section 3.1 investigates the endogenous IFN response of melanoma and breast cancer cell lines during low MOI infection with PVSRIPO and the effects on virus replication and cytopathogenicity. It also examines claims that PV inhibits innate antiviral immune responses through cleavage of MDA5 and/or MAVS.

Section 3.2 determines the effects of exogenous IFNs on PVSRIPO and EMCV replication in IFN-competent and –incompetent melanoma cells identified in section 3.1. It also investigates the effects of exogenous IFNs on PVSRIPO in glioma cells and concludes with a surprising discovery that cancer cells may have IFN-independent endogenous antiviral immune responses.

Continuing from the discovery in the previous section, Section 3.3 finds MDA5 is required for IFN responses to PVSRIPO and EMCV in melanoma cells and determines the extent to which MDA5-dependent and IFN-independent signal transduction inhibits the two viruses.

Chapter 4 concludes the discussion of the results and proposes future experiments to expand this work into the broader fields of cancer immunotherapy and innate immunology.

2. Materials and Methods

2.1 Cell Lines and Viruses

DM6, DM440, and DM443 melanoma cell lines were generously gifted by D. Tyler (UTMB, Galveston, TX). DU54 glioma cells were described previously (Gromeier et al., 1996). SUM149 cells were a gift from S. Nair (Duke University, Durham, NC). Melanomas, glioma, and HeLa [American Type Culture Collection (ATCC)] cell lines were propagated in growth medium consisting of Dulbecco's Modified Essential Medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS), 1x Antibiotic-Antimycotic (Anti-anti; Invitrogen), and 1x Non-Essential Amino Acids (NEAA; Invitrogen). 293FT (Thermo Fisher) cell lines were cultivated in above growth medium with 0.5 mg/mL Geneticin™ (Thermo Fisher). SUM149 cells were grown in 1:1 DMEM: Nutrient Mix F-12 (Ham) (Invitrogen), 10% FBS, 1x Anti-Anti, 1x NEAA. MDA5-depleted cell lines were maintained in growth media containing 1.5 μg/mL puromycin (Invitrogen). THP-1 (ATCC) cells were maintained in RPMI-1640 medium (Invitrogen) with 10% FBS, 1x Anti-Anti, 1x NEAA and differentiated to macrophages by incubation with 100nM 12-O-tetra-decanoylphorbol-13-acetate (TPA) added to growth medium for 48 hours followed by 48 hours recovery in normal growth medium. Mouse BMDCs and human DCs were prepared as described previously (Brown et al., 2017)

EMCV [VR-129B; (ATCC)] and PVSRIPO were propagated in HeLa cells (ATCC) (Brown et al., 2014a). Viruses were sterilized by filtration through a 0.45 μm syringe filter and concentrated with a 100-kDa centrifugal filter (Millipore). Viral titers were determined by plaque assay in HeLa cells as described previously (Gromeier et al., 1996).

2.2 Generation of Stable MDA5-depleted Cell Lines

Lentiviral shRNA vectors were produced from MISSION® shRNA pLKO.1 with shRNA sequence 5′-ccggcccatgacacagaatgaacaactcgagttgttcattctgtgtcatgggtttttg (Sigma-Aldrich) with ViraPower Lentiviral Packaging Mix in 293FT cells (Invitrogen) per manufacturer's instructions. Stable MDA5 knock-down cells were created by lentiviral transduction in growth medium plus 8 μg/mL Polybrene (Millipore), followed by selection in growth media containing 1.5 μg/mL puromycin (Invitrogen), colony picking, and expansion of monoclonal populations. Cells from at least 5 colonies were screened for loss of MDA5 after transfection with Lipofectamine® 2000 (Thermo Fisher) and 10 μg/mL high molecular weight (HMW) poly(I:C) (Invivogen) or Lipofectamine® 2000 alone for 24 hours.

2.3 Viral Treatment of Cells

Virus infections were done on 80-90% confluent cells that had been incubated for at least 24 hours in growth medium at 37°C, 5% CO_2. Cells from two plates in each

experiment were trypsinized, Trypan blue stained, and life/dead cells counted by Countess II automated cell counter (Invitrogen) per manufacturer's protocol. Cells were infected at indicated MOI by aspirating media and replacing it with 1 mL of pre-warmed growth medium containing virus or mock-infected (MI) with media alone and incubated at 37°C, 5% CO_2.

2.4 IFN Pretreatment

Cells were plated at a density of 4 x 10^5 cells in 2mL of growth media in 35 mm tissue culture plates (Greiner Bio-One). Following 24 hours incubation at 37°C and 5% CO_2, media was replaced with 1 mL growth media containing 100 U/mL recombinant human IFN-α2a (PBL Assay Science) and incubated for 24 hours at 37°C and 5% CO_2. Cells were then infected as described above.

2.5 IFNAR2 Blocking Antibody and Poly(I:C) Treatment

Either 2 x 10^4 or 4 x 10^5 cells were plated to 24- or 6- well tissue culture plates, respectively, with growth media containing either 1 μg/mL mouse anti-human IFNAR2 antibody (clone MMHAR-2, PBL Assay Science), 1 μg/mL mouse IgG2A (R&D Systems) isotype control, or no antibody (Tsugawa et al., 2014) and incubated overnight at 37°C and 5% CO_2. Cells were treated with virus at indicated MOI by replacing media with pre-warmed growth medium containing virus and 1 μg/mL antibody, where indicated, then incubated at 37°C and 5% CO_2. Alternatively, after antibody pre-treatment, 25

μg/mL poly(I:C) (Sigma-Aldrich) was added to cells for 6 hours, where indicated, and cells were treated with growth media containing virus and 1 μg/mL antibody.

2.6 TBK1/IKKε Inhibition

8×10^5 cells were plated in 35mm tissue culture plates and incubated for 24 hours at 37°C and 5% CO_2. 10μM BX795 (Invivogen) in dimethyl sulfoxide (DMSO) or equal volume carrier was then added to media for 1 hour, then cells were infected as described above.

2.7 Cell Lysis and Immunoblotting

At the indicated time, cells were lysed as described previously (Dobrikov et al., 2011) in polysomal lysis buffer [PLB; 20mM Tris (pH 7.4), 100 mM NaCl, 5 mM $MgCl_2$, 0.5% NP-40 (Sigma-Aldrich), 1X Halt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Scientific)]. Cell lysates were subjected to Bradford assay (Bio-Rad Protein Assay, Bio-Rad) and diluted in lysis buffer to equalize protein concentration. Cell lysates were then prepared for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by addition of 4X LDS buffer containing 20% β-mercaptoethanol (Sigma-Aldrich). All LDS containing samples were subjected to SDS-PAGE and Western blotting as described previously (Brown et al., 2017) using primary antibodies at 1:1000 dilution, unless otherwise noted, against PV 2C/2BC/P2 (Goetz et al., 2010), eIF4G1, p-STAT1(Y701), STAT1 (1:2000), PARP, GAPDH (1:10000), OAS1, eIF4A, IFIT1, MAVS

(Cell Signaling Technologies), MDA5 (1:500) (Enzo Life Sciences), Mengovirus 3Dpol (1:500, Santa Cruz Biotechnology), and tubulin (1:10000, Sigma Aldrich). Total protein stain was done with REVERT™ Total Protein Stain (LI-COR Biosciences) according to manufacturer's protocol. Secondary detection was done with horseradish peroxidase (HRP)-conjugated mouse or rabbit antibodies (1:10000, Cell Signaling Technologies), or with infrared 800CW fluorescent-conjugated mouse antibody (1:25000, LI-COR Biosciences). HRP was detected using WesternBright™ ECL chemiluminescent reagent (Advansta) according to manufacturer's protocol. An Odyssey Fc imager (LI-COR Biosciences) was used to image and quantify immunoblots. Protein quantification was calculated with LI-COR Image Studio software relative to loading controls. Cellular protein levels were standardized to expression in mock treated controls and depicted as fold-increase above mock while viral protein expression was standardized relative to maximum detected viral protein in each interval.

2.8 ELISA

Media was collected from cells at indicated time points, centrifuged at 500xg for 5 minutes, and supernatant frozen at -80°C. IFN-β ELISA was performed on supernatants from infected and MI cells at indicated hpi in parallel and duplicate from at least 4 independent experiments with the VeriKine-HS Human Interferon Beta Serum ELISA Kit or VeriKine Human Interferon Beta Serum ELISA Kit (PBL Biosciences)

according to manufacturer's protocol. IL-12 ELISA was performed with manufacturer's recommendations (R&D Biosystems).

2.9 Lactose Dehydrogenase Release Cytotoxicity Assay

Media was collected from cells at indicated time points, centrifuged at 500xg for 5 minutes, and supernatant frozen at -80°C. Lactose dehydrogenase (LDH) release assay was performed on supernatants with LDH Cytotoxicity Kit (Thermo Fisher) as in (Brown et al., 2017) per manufacturer's protocol using supernatant from freeze-thawed cells as a 100% cell lysis control and supernatant from MI cells at 0 hpi as a 0% lysis control.

2.10 Multi-step Growth Curves

Cells were incubated with virus containing media for 1 hour at 37°C, 5% CO_2, washed once in cold PBS, and 1 mL pre-warmed virus free growth medium was added to cells. Cells were incubated at 37°C, 5% CO_2 for the indicated time then subjected to 2 freeze-thaw cycles. Viral titers were determined by plaque assay, and pfu/cell calculated based on number of cells at 0 hpi.

2.11 Statistical Analysis

All data are shown as mean ± standard error of mean (SEM). Statistical significance was determined by one- or two-way analysis of variance (ANOVA)

followed by appropriate *post-hoc* Tukey's or Sidak's multiple comparison test; *p*-values of <0.05, indicated by *, were considered statistically significant.

3. Results

3.1 PVSRIPO is not inhibited by endogenous IFN Responses from cancer cell lines

3.1.1 Introduction

Loss of type 1 IFN responses was considered a hallmark of cancer and an effective method to limit OV cytotoxicity to cancer cells (Stojdl et al., 2000). However, some cancer cells inhibit IFN-sensitive OVs *in vitro* and *in vivo* including VSV, oHSV, SFV, and measles vaccine virus (Berchtold et al., 2013; Jackson et al., 2016; Ruotsalainen et al., 2015; Westcott et al., 2015). In addition, OVs may require type 1 IFNs to trigger anti-tumor immunity after treatment (Zamarin et al., 2014). Thus, determining the relationship of IFNs to PVSRIPO, and identifying IFN responses in PVSRIPO-treated cancer cells, may provide valuable insight on the treatment mechanism.

As part of this work, we assessed the induction of type I IFNs in melanoma and breast cancer cell lines after low dose PVSRIPO infection and the effects of these responses on the virus. We assayed innate immune signaling, viral translation and replication, and cytotoxicity over time. To reduce experimental bias all melanoma cell lines in our panel, i.e. DM440, -443 and -6, were positive for the BRAF(V600E) mutation commonly associated with melanoma (Davies et al., 2002; Zipfel et al., 2010). We found PVSRIPO treatment induces IFN responses in some cancer cell lines and PVSRIPO replication is not significantly inhibited by endogenous innate immune responses.

3.1.2 Results

3.1.2.1 Melanoma cells have intact IFN responses after PVSRIPO infection

All three of the tested melanoma cell lines (DM440, DM6, and DM443) showed productive infection of PVSRIPO when treated with an MOI of 0.1, as shown by visible cleavage of eIF4G1 by 18 hpi and total loss of intact eIF4G1 by 36 hpi (Fig. 9A). This coincided with expression of viral 2C, detected in all cell lines 18 hpi and at maximum levels by 24-36 hpi, depending on the cell line (Fig. 9A). While detection of viral protein and eIF4G1 cleavage is significantly slower than has been previously reported, this can be explained by the low initial MOI and inefficient viral entry inherent to polioviruses (Strauss et al., 2015). Combined, this results in an actual infection rate of less than 1% of cells.

While viral translation does not vary significantly between tested melanoma lines, IFN responses do. DM440 and DM6 cells both showed increased phosphorylated STAT1 (Y701) by immunoblot after 0.1 MOI PVSRIPO infection, which is an indicator of IFN production and autocrine/paracrine responses (Fig. 9A). Quantification of p-STAT1 (Y701) showed statistically significant increases 24 hpi in DM440 cells and 36 hpi in DM6 cells (Fig. 9B). DM440 and DM6 also had detectable IFN-β by ELISA at 36 and 48 hpi with 0.1 MOI PVSRIPO, respectively (Fig. 9C). DM443 cells, on the other hand, showed no such upregulation of either p-STAT1 (Y701) or IFN-β (Fig. 9B, C). This indicates

DM440 and DM6 cell lines have intact type I IFN response mechanisms, contrary classical dogma that cancer cells lack such signaling capacity (Wong et al., 1997).

Loss of total protein is a common occurrence during lytic cell death from poliovirus infection (Brown et al., 2017). While it is not the most quantifiable method of assessing cytotoxicity, it can be detected as loss of loading control signal in immunoblot. DM440 and DM443 cells both show significant loss of the α-tubulin loading control at 36 hpi, while DM6 cells showed similar loss by 48 hpi (Fig. 9A). This indicates all cells underwent lytic cell death from PVSRIPO infection.

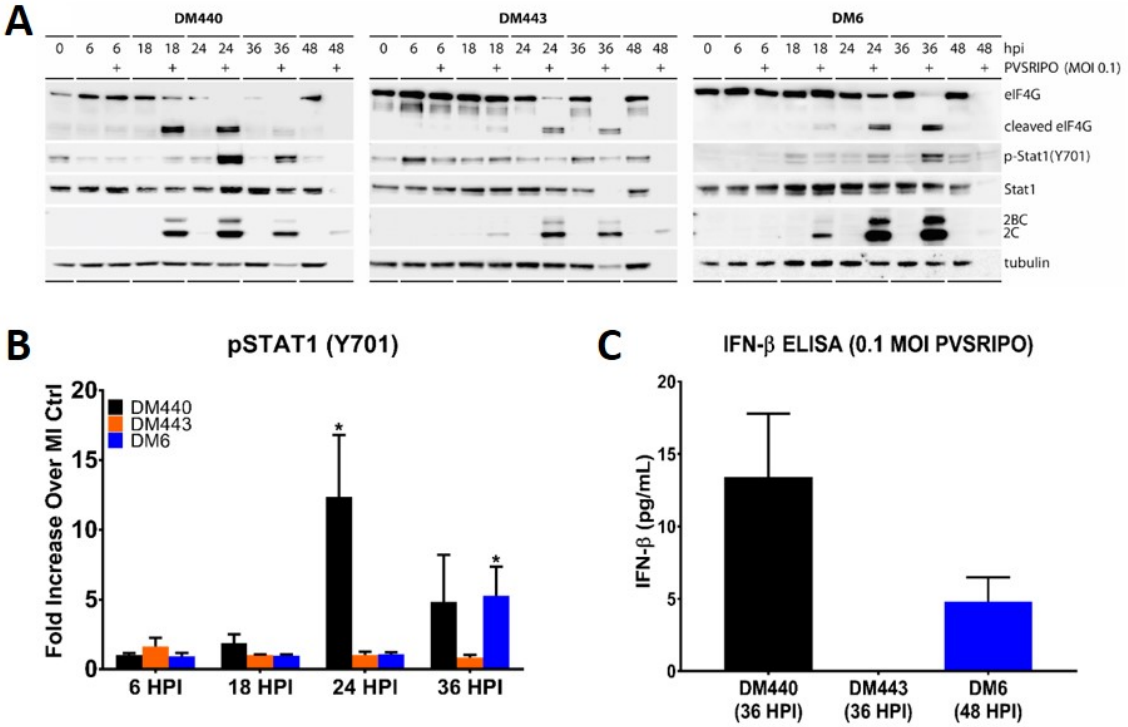

Figure 9: PVSRIPO stimulates IFN-β and STAT1 phosphorylation (Y701) in melanoma Melanoma cell lines (DM440, DM443, DM6) were infected with 0.1 MOI PVSRIPO for indicated time. (A) Immunoblots of lysates probed for viral translation (2BC/2C), cytotoxicity (eIF4GI cleavage) and type I IFN response [p-STAT1(Y701)]; representative blots of 4 independent series. (B) Fold-change in p-STAT1(Y701) from immunoblots in (A) relative to MI controls; mean ± SEM (n=4), $p < 0.05$ (*) infected vs. MI. (C) IFN-β released by ELISA; mean ± SEM (n=4).

3.1.2.2 PVSRIPO replication and cytotoxicity are not affected by intrinsic innate immune signaling in melanoma

Since DM443 cells do not produce type I IFNs, we hypothesized this melanoma cell line would be more permissive of PVSRIPO replication than IFN-producing DM440 and DM6 melanoma cell lines. Contrary to this expectation, DM440 cells were the most permissive for both viral replication and cytopathogenicity of the three tested melanoma

cell lines. PVSRIPO propagation was logarithmic in all tested cell lines, as shown by multi-step growth curve with an initial MOI of 0.1, with DM440 exhibiting the most prolific growth (Fig. 10A). Recovered virus from all three lines was approximately equal 8 hpi, but 16 and 24 hpi viral titers were significantly higher in DM440 cells relative to the other two cell lines (Fig 10A). All three equalized again 36 hpi (Fig. 10A). This indicates replication competence of PVSRIPO is not affected by cell intrinsic IFN responses but may be influenced by other cell-specific conditions.

Lytic cell death after PVSRIPO infection was quantified by detection of LDH released into the media by infected cells, which showed significant cytotoxicity indicating 100% cell killing by 24 hpi (DM440, DM443) or 48 hpi (DM6; Fig. 10B). It is possible DM6 cells were slightly more resilient to PVSRIPO infection than DM440 or DM443, as they showed maximum cell lysis later after infection, but the LDH release assay did not show statistically significant differences between the cell lines, and therefore is inconclusive (Fig. 10B). Quantification of eIF4G1 cleavage, however, showed significant differences between the three cell lines (Fig. 10C). DM440 showed eIF4G1 cleavage, a major indicator of poliovirus cytotoxicity (Etchison et al., 1982), earlier than the other two cell lines, which agrees with viral replication data (Fig. 10C). These results indicate that PVSRIPO exhibits full cytopathogenic and replication potential in

melanoma cells at extremely low MOIs, regardless of the presence of an active IFN response.

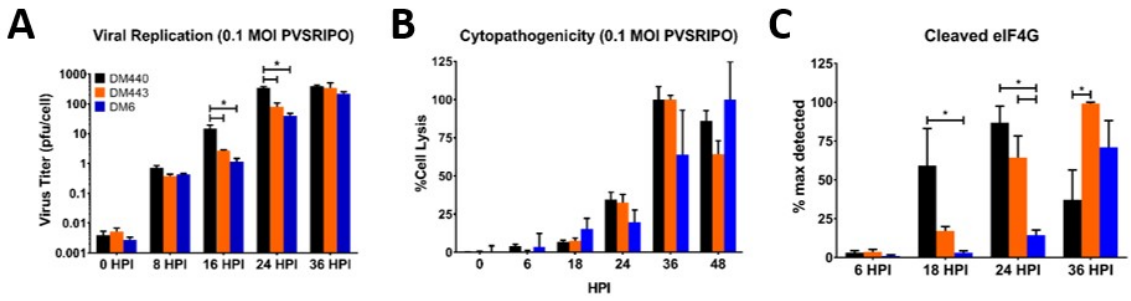

Figure 10: Melanoma-intrinsic IFN signaling does not inhibit PVSRIPO growth or cytopathogenicity

(A) Multi-step growth curves of PVSRIPO in DM440, DM443, and DM6 cells infected at an MOI of 0.1; mean ± SEM (n=3). (B) Percent cytotoxicity determined by LDH release into media after indicated time from DM440, DM443, and DM6 cells infected with 0.1 MOI PVSRIPO; MI cells as a 0% lysis control; mean ± SEM (n=4). (C) Cleaved eIF4G1 as percent maximum detected by immunoblot of DM440, DM443, and DM6 cells infected with 0.1 MOI PVSRIPO; mean ± SEM (n=4).

3.1.2.3 PVSRIPO replicates in IFN-competent melanoma cells at low MOIs without specific cleavage of MDA5 or its downstream effector MAVS.

Many viral pathogens intercept antiviral IFN responses to replicate in host cells with innate antiviral immunity (Chan & Gack, 2016). Some evidence suggests enteroviruses prevent type I IFN upregulation through proteolytic cleavage of MDA5, MAVS, or other innate antiviral response modifiers. The published record is contradictory on the mechanism of this proposed degradation. Putative MDA5 cleavage in enterovirus-infected cells has been reported to rely on the viral $2A^{pro}$ independent of caspases (Feng et al., 2014a). It has also been claimed to occur in a caspase-dependent

manner (Barral et al., 2007). Presumptive cleavage of MAVS has been linked either to $2A^{pro}$ or viral $3C^{pro}$ (Feng et al., 2014a; Mukherjee et al., 2011).

To examine the possible role of MDA5 or MAVS cleavage in IFN-subversion by PVSRIPO, IFN-competent DM440 cells were infected with PVSRIPO at 0.1 MOI; viral translation and eIF4G1 cleavage (by $2A^{pro}$) were compared to MDA5 and MAVS in immunoblots (Fig. 11A). We did not observe changes to MDA5 or MAVS abundance or integrity 18-24 hpi, but eIF4G1 cleavage and viral 2C was readily detectable in this interval (Fig. 11A). Levels of MDA5 and MAVS declined 36 hpi in parallel to loss of total immunoblot signal, probably the result of widespread lytic enterovirus cytotoxicity (Fig. 11A). The absence of MDA5 and MAVS degradation simultaneous to eIF4G1 cleavage is evidence that viral $2A^{pro}$ does not cleave MDA5 or MAVS. They may be affected by general proteolytic degradation by cellular proteases that occurs with enterovirus-induced cell lysis (Barral et al., 2007).

If PVSRIPO spread is not contained by antiviral type I IFN responses in IFN-competent cells, viral cytotoxicity and propagation should occur regardless of the MOI. To test this, we infected DM440 cells at three different MOIs (0.1, 0.01, and 0.001) and tracked viral cytopathogenicity and propagation (Fig. 11A, B). Infection of DM440 cells with PVSRIPO revealed significant viral translation and host cytotoxicity at all tested MOIs (Fig. 11A). Lower MOIs simply delayed the kinetics of these events. Peak viral

translation preceding complete cytolysis was observed at 24 (MOI of 0.1), 36 (MOI of 0.01) or 48 hpi (MOI of 0.001) (Fig. 11A). Lower MOIs delayed viral replication kinetics but did not change the characteristic logarithmic growth of PVSRIPO (Fig. 11B).

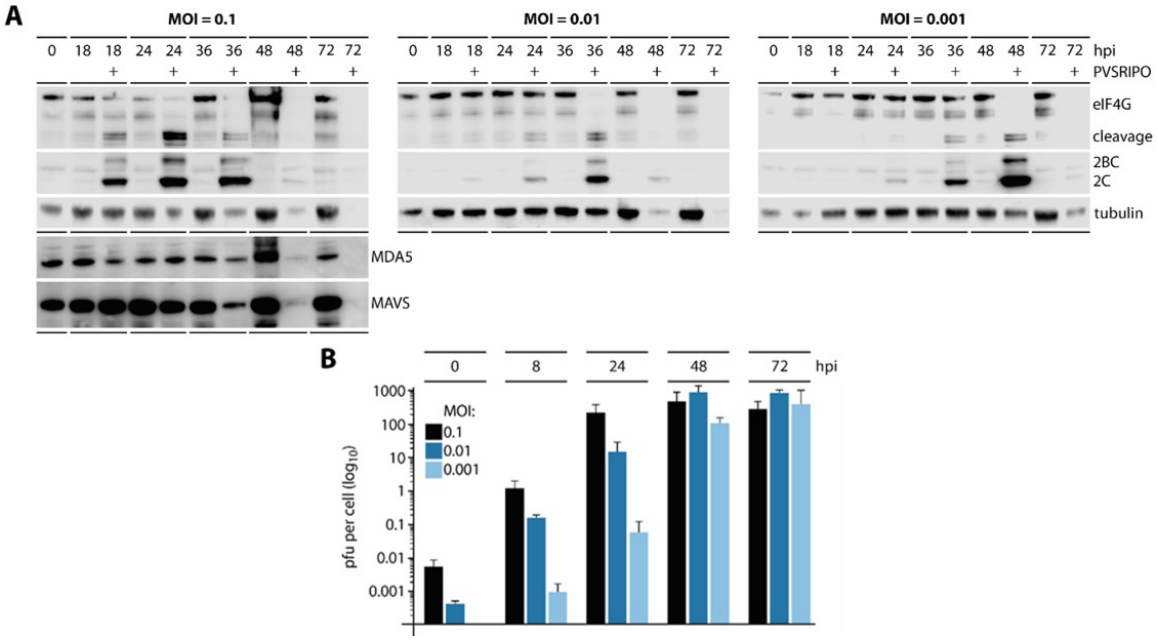

Figure 11: PVSRIPO replicates effectively in innate immune competent melanoma cell lines at low MOI without MDA5 or MAVS cleavage

DM440 cells were mock or PVRIPO-infected at 0.1, 0.01 or 0.001 MOI. (A) Immunoblot of cell lysates at indicated hpi were probed for viral translation (2BC/2C), cytotoxicity (eIF4GI cleavage), MDA5, and MAVS. Globally decreased signal at later hpi is due to gross sample loss from cytolysis. Immunoblots are representative of 2 independent experiments. (B) Multi-step growth curves of infected cells; mean ± SEM (n=3). Done in collaboration with Matthew T. Sacco.

3.1.2.4 PVSRIPO subverts intrinsic innate immune responses in breast cancer

PVSRIPO treatment of SUM149 breast cancer xenografts in athymic mice causes tumor regression without intratumoral viral persistence (Holl et al., 2016). In addition,

significant intratumoral innate immune cell infiltration and upregulation of pro-inflammatory gene products were detected post PVSRIPO treatment (Holl et al., 2016). To determine if SUM149 cells could be a source of pro-inflammatory signaling, we infected these cells *in vitro* with PVSRIPO and then assayed innate immune response.

SUM149 cells infected with 0.1 MOI PVSRIPO secreted detectable IFN-β 36 hpi (Fig. 12A). Immunoblots of infected SUM149 cells also showed upregulation of p-STAT1(Y701), MDA5, and STAT1 24 hpi (Fig. 12B). Based on the immunoblots, it is possible STAT1 and MDA5 upregulation preceded STAT1 (Y701) phosphorylation. We examine this possibility in more depth later in this work. Viral 2C and cleaved cellular eIF4G1 were detectable by immunoblot starting 24 hpi and continuing throughout the time course (Fig. 12B). This indicates PVSRIPO replicates productively in breast cancer cells despite active innate antiviral immune responses, as it does in melanoma cells (Fig. 9A). It is possible the robust immune response to PVSRIPO infection of SUM149 cells was a contributor to the infiltration of innate immune cells and upregulation of pro-inflammatory transcripts *in vivo*, but further work is necessary to test this hypothesis.

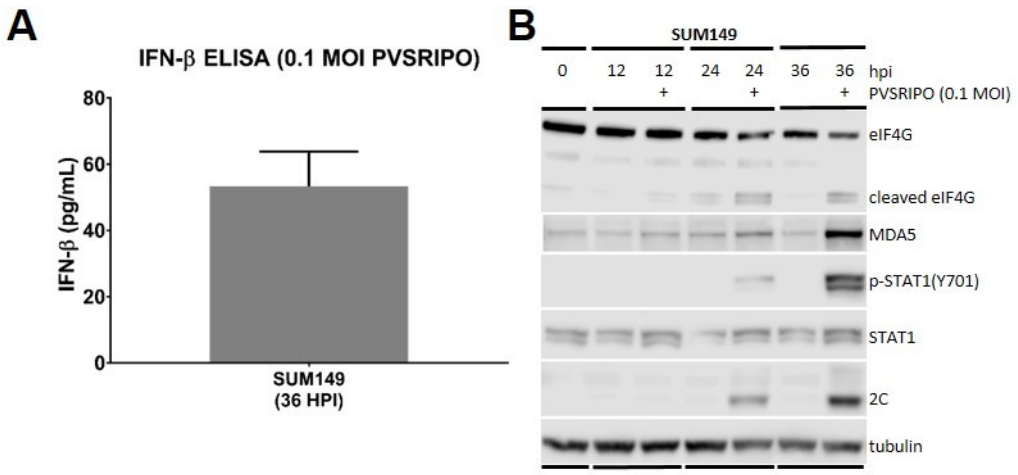

Figure 12: PVSRIPO replicates in innate immune competent breast cancer cells (A) IFN-β released from SUM149 cells infected with 0.1 MOI PVSRIPO by ELISA 36 hpi; mean ± SEM (n=4). (B) Immunoblots of lysates from SUM149 breast cancer cells infected with 0.1 MOI PVSRIP for indicated time probed for viral translation (2C), cytotoxicity (eIF4G1 cleavage) and type I IFN response [p-STAT1(Y701), MDA5, STAT1].

3.1.3 Discussion

Two of the three melanoma cell lines tested showed robust IFN secretion and STAT1 (Y701) phosphorylation during PVSRIPO infection (Fig. 9). Many OVs have been engineered to remove virulence factors that suppress or evade innate antiviral immune signaling (Russell et al., 2012). As such, some OVs exhibit compromised replication and cytotoxicity in IFN-competent cancer cells (Berchtold et al., 2013). PVSRIPO cancer tropism relies not on deletions of virulence factors, but dysregulated translation initiation (Merrill & Gromeier, 2006) and widespread expression of the poliovirus receptor CD155 (Chandramohan et al., 2017) in cancer cells. The kinetics of eIF4G1 cleavage, cell lysis, and PVSRIPO replication during infection did not vary significantly

between the three tested melanoma lines and the cell line most permissive for PVSRIPO was DM440, a robustly IFN competent melanoma line (Fig. 10). Infections of DM440 cells at MOIs of 0.01 and 0.001 showed delayed growth kinetics, but not a change in the general replication pattern, indicating no inhibition by the innate immune system (Fig. 11). This suggests PVSRIPO subverts or evades, but does not prevent, innate immune responses. It also suggests innate immune responses do not prevent PVSRIPO-mediated oncolysis. Secretion of type I IFNs from breast cancer cells infected by PVSRIPO suggests the possibility that previously observed immune cell infiltration into PVSRIPO-infused SUM149 xenograft tumors may be partially driven by cancer cell intrinsic innate immune responses (Fig. 12) (Holl et al., 2016). However, a great deal of future work would need to be done to test this hypothesis.

We deliberately used multiplicities of infection (MOI) of ≤ 0.1 throughout this study to capture the effect of innate antiviral responses on multiple successive rounds of virus propagation. Low MOIs of OVs *in vivo* have highly dynamic and complex spread which likely impacts clinical results (Rodriguez-Brenes et al., 2017). Since host cell entry of PV RNA is inefficient, due to CD155-mediated particle disintegration (Strauss et al., 2015) and 'sloughing' off of non-infectious virus particles (Fenwick & Cooper, 1962; Strauss et al., 2015), 'actual' MOI is much lower than the intended MOI. After a 60 min. attachment step, we recovered only ~0.004 pfu/cell in PVSRIPO-infected melanoma cells

infected at an intended MOI of 0.1 (Fig. 11). The trend of recovering only 1-4% of virus added initially continued for infections of 0.01 and 0.001 MOI (Fig. 11). Thus, initial infection of <1% of host cells was suitable to interrogate the effects of innate antiviral IFN response on multiple rounds of PVSRIPO replication and better represents clinical OV infusion.

MDA5 is a PRR for viral dsRNA and activates innate antiviral immune responses, including IFNs, through a signal cascade that includes its binding partner MAVS (Kawai et al., 2005). It has been suggested enteroviruses inhibit IFN production by cleavage of MDA5 and its adapter MAVS during infections with MOIs ≥10 , though there is disagreement on the mechanism of cleavage (Barral et al., 2007; Feng et al., 2014a; Mukherjee et al., 2011). Infection of immune competent cells with PVSRIPO showed eIF4G1 cleavage, a well-known target of proteolytic degradation by poliovirus (Etchison et al., 1982), precedes loss of MDA5 and MAVS, and that degradation of MDA5 and MAVS coincided with general loss of host protein late in infection (Fig. 11A). Thus, MDA5 and MAVS were likely affected by the gross, indiscriminate proteolytic degradation that occurs during enterovirus-induced cell lysis. Such degradation does not constitute a mechanism to intercept innate IFN responses; it represents a passive bystander effect of viral cytopathogenicity. We therefore cannot confirm any of the mutually contradictory claims of enterovirus interference with MDA5 or MAVS.

3.2 PVSRIPO, but not EMCV, Subverts IFN Responses in Cancer Cells

3.2.1 Introduction

Pathogenic PV replicates in cells pretreated with type 1 IFNs, and as already shown, PVSRIPO is highly cytopathogenic and replicates effectively in innate immune competent cells (Morrison & Racaniello, 2009). This suggests PVSRIPO may be relatively resistant to exogenous type I IFN signaling. This question is of interest, as a significant number of cells within a solid tumor are stromal innate immune cells which can produce antiviral, pro-inflammatory cytokines during treatment with oncolytic viruses, and may inhibit other OVs (Quail & Joyce, 2013; Westcott et al., 2015). As EMCV is an IFN-sensitive picornavirus related to PV, EMCV serves as a control for experiments to explore the relationship between PVSRIPO and the host cell innate immune response.

3.2.2 Results

3.2.2.1 PVSRIPO, but not EMCV, translates efficiently in innate immune competent melanoma cells with activate antiviral IFN signaling

To test the effects of IFN on PVSRIPO, IFN-competent DM440 cells were pre-treated with recombinant human IFN-α2 or vehicle in growth media. IFN was withdrawn and cells infected with PVSRIPO (Fig. 13A, B, and C) or EMCV (Fig. 13D, E, and F) with appropriate mock-infected controls. IFN-α2 pretreatment of DM440 cells

upregulated MDA5, OAS1, and STAT1 throughout the infection time course, showing cells mount IFN responses when infected with either PVSRIPO or EMCV (Fig. 13A, D).

STAT1 phosphorylation (Y701) was elevated in IFN pretreated cells relative to vehicle treated, MI controls during the entire infection time series, and elevated to a statistically significant level in vehicle treated, PVSRIPO-infected DM440 cells 36 hpi (Fig. 13B). Similarly, EMCV-infected IFN-α2 pretreated DM440 cells showed elevated STAT1 phosphorylation (Y701) at all time points and upregulated p-STAT1(Y701) without pretreatment 18 and 24 hpi (E). This indicates neither EMCV nor PVSRIPO completely suppresses innate antiviral immune signals in primed melanoma cells and provides further evidence DM440 cells mount antiviral responses during picornavirus infection.

IFN-α2 pretreatment delayed, but did not eliminate, eIF4G1 cleavage in PVSRIPO infected DM440 cells (Fig. 13A). Likewise, it inhibited or delayed viral 2C expression at all time points during infection (Fig. 13C). In contrast, immunoblots of EMCV infected cell lysates showed no detectable PARP cleavage, an indicator of apoptosis (Nicholson et al., 1995), nor loss of tubulin loading control 36 hpi, but infected, untreated cells showed significant cytopathogenicity at this time point (Fig. 13D). IFN-α2 pretreatment of DM440 cells abrogated detectable viral 3Dpol in EMCV infected cells, which was readily detectable starting at 18 hpi in vehicle treated cells (Fig. 13F). Based

on these results, PVSRIPO likely subverts antiviral immune signaling downstream of IFNs while EMCV does not.

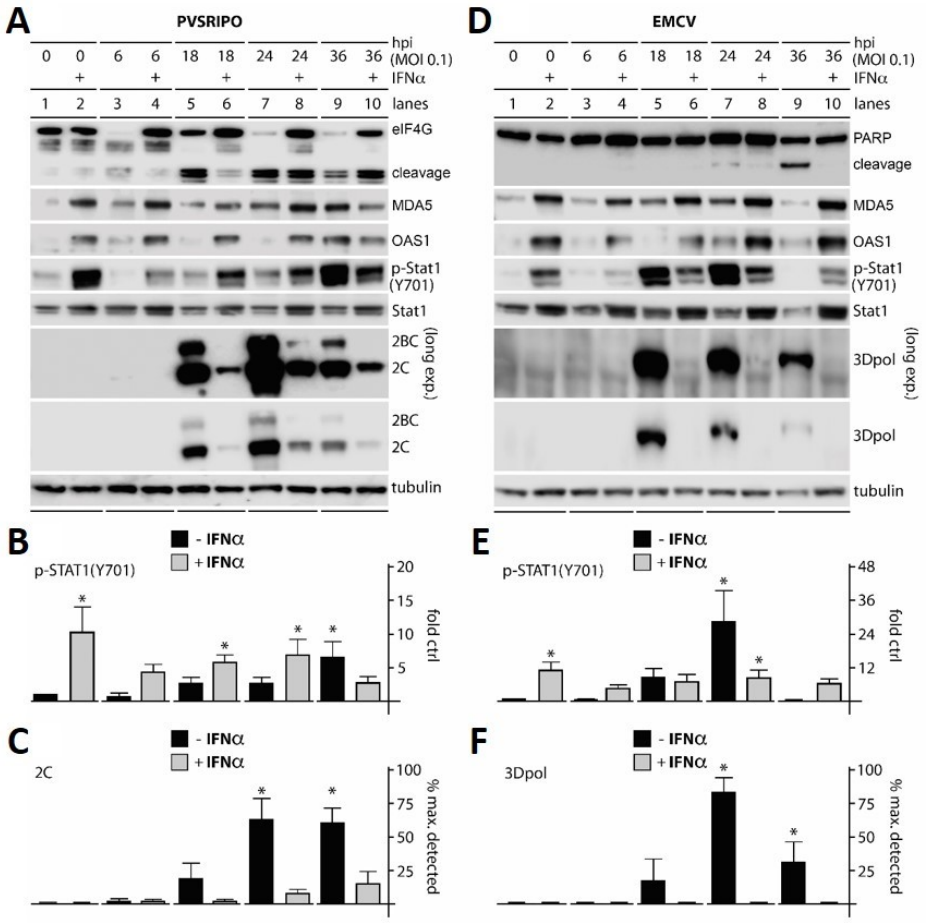

Figure 13: IFN pretreatment activates antiviral responses in DM440 cells and inhibits translation of EMCV more than PVSRIPO

DM440 cells were treated for 24h with 100 U/mL IFN-α2a (+) or vehicle (-), then MI (0h), or infected with 0.1 MOI PVSRIPO, or EMCV for indicated time. (A, D) Immunoblots of cell lysates infected with PVSRIPO (A) or EMCV (D) probed for viral translation [2BC/2C (A), 3Dpol (D)], cytopathogenicity [eIF4G1 (A), PARP (D) cleavage] and type I IFN response [p-STAT1(Y701), MDA5, OAS1]; blots are representative of 5 independent series. (B, E) Fold-change in p-STAT1(Y701) after PVSRIPO (B) or EMCV (E) infection relative to control; mean ± SEM (n=5), $p < 0.05$ (*) vs. MI control. (C, F) Viral protein expression after PVSRIPO (C) or EMCV (F) infection as percent of maximum detected viral protein; mean ± SEM (n=5), $p < 0.05$ (*), IFN-pretreated vs. untreated.

3.2.2.2 EMCV replication and cytopathogenicity are more inhibited by IFNs in innate immune competent melanoma cells than PVSRIPO

Comparison of viral yields between IFN-α2 pretreated and untreated DM440 cells showed PVSRIPO propagation is slightly inhibited but EMCV is substantially inhibited (Fig. 14A). IFN-α2 pretreatment reduced PVSRIPO yields a maximum of ~50-fold 24 hpi, but reduced EMCV yields ~12,000-fold at 24 hpi (Fig. 14A). Fold-reduction of EMCV between IFN-α2 pretreated and untreated cells dropped to ~1,400 48 hpi. EMCV may overcome inhibition by IFN-α2 pretreatment, or antiviral effects may be downregulated by a negative feedback loop. In support of the latter, STAT1 phosphorylation (Y701) was lower in infected, IFN pretreated cells compared to infected untreated cells, indicating the IFN response is suppressed at later time points, e.g. by the Suppressor of cytokine signaling (SOCS) proteins (Fig. 13A, lanes 9-10; D, lanes 5-6) (Yoshimura et al., 2007).

To determine the impact of exogenous IFN-α2 pretreatment on lytic cell death after viral infection, LDH in the media of infected cell cultures was determined at several time points. Cell lysis was detectable in all infected cultures, but IFN -α2 pretreatment protected cells from EMCV cytopathogenicity significantly more than PVSRIPO cytopathogenicity (Fig. 14B). LDH release data, combined with eIF4G1 and PARP cleavage data, indicate PVSRIPO is more IFN resistant than EMCV and cancer cells mount effective antiviral responses to exogenous IFNs.

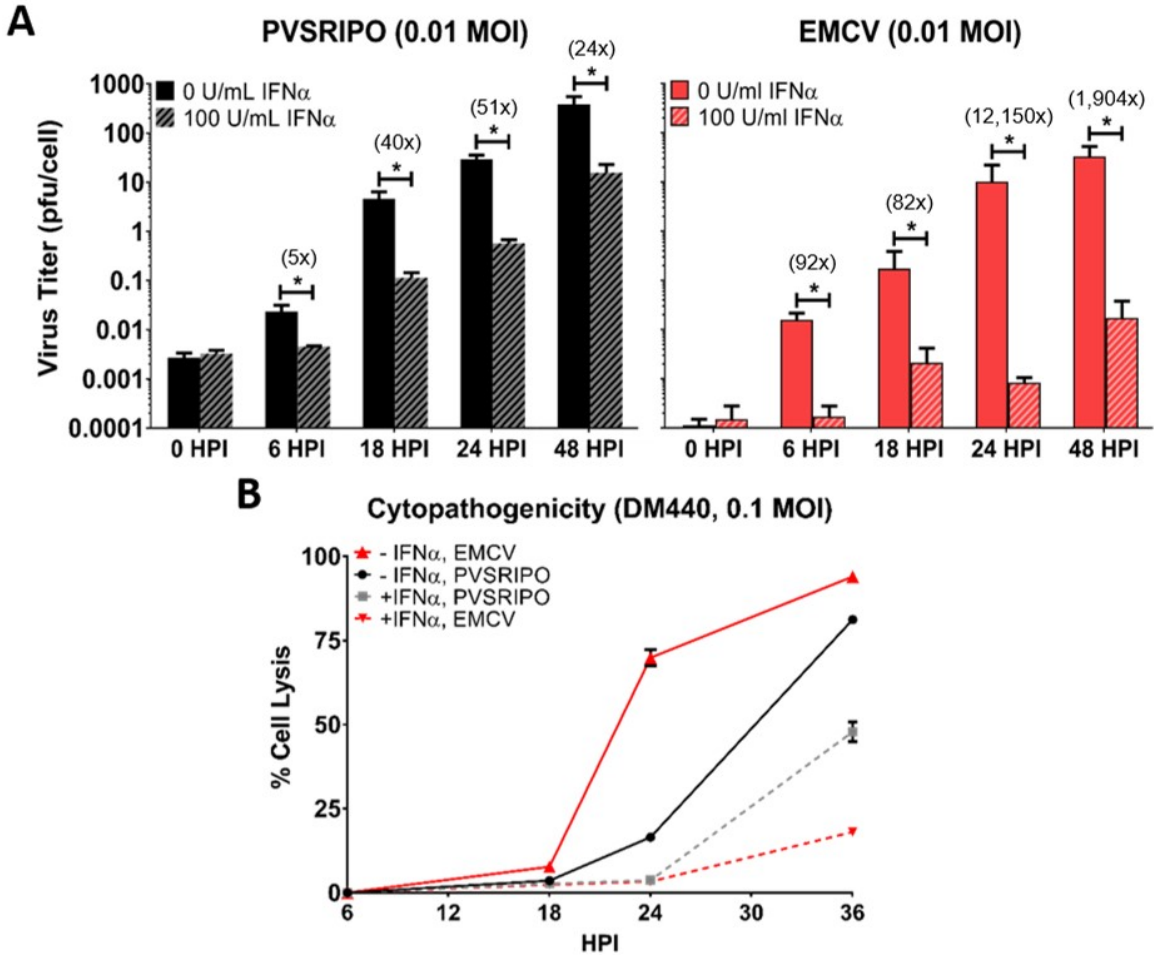

Figure 14: IFN pretreatment of DM440 cells inhibits EMCV replication and cytopathogenicity more than PVSRIPO replication and cytopathogenicity

DM440 cells were pretreated for 24h with IFN-α (+) or vehicle then infected with PVSRIPO, EMCV, or MI for indicated time. (A) Multi-step growth curve of IFN or vehicle pretreated DM440 cells infected with 0.01 MOI PVSRIPO (left) or EMCV (right); mean ± SEM (n=3), $p < 0.05$ (*) vehicle vs IFN-α pretreated. Parenthetical number refers to fold-difference between IFN-α pretreated and untreated cells. (B) Percent cytotoxicity determined by LDH released in media after indicated time by pretreated cells infected with 0.1 MOI; MI cells as a 0% lysis control, freeze-thawed cells as 100% lysis control; mean ± SEM (n=4), error bars excluded where smaller than symbol.

3.2.2.3 Innate immune incompetent melanoma cells mount antiviral responses to exogenous IFNs

DM443 melanoma cells do not upregulate IFNs or mount detectable antiviral immune responses to PVSRIPO infection (Fig. 9A). However, they may mount antiviral immune responses when stimulated with exogenous IFNs, which could inhibit oncolytic viruses even if the cancer cells do not produce IFNs.

To test if DM443 cells could mount antiviral immune responses, we treated the cells with IFN-α2 for 24 hours and infected them with PVSRIPO or EMCV (Fig. 15). IFN pretreatment increased STAT1 (Y701) phosphorylation and upregulated the ISGs MDA5, STAT1, and OAS1 relative to untreated (Fig. 15A, B). STAT1 remained elevated after infection with either PVSRIPO or EMCV (Fig. 15A, B). Expression of MDA5 and OAS1, on the other hand, fluctuated over time during PVSRIPO infection, which may be a result of differential regulation of these gene products (Fig. 15A) (Ourthiague et al., 2015).

IFN-α2 pretreatment abrogated EMCV 3Dpol protein expression (Fig. 15B), but only inhibited expression of PVSRIPO protein 2C (Fig. 15A). Cleavage of eIF4G1 by PVSRIPO was also significantly delayed in IFN-α2 pretreated DM443 cells, coinciding with lower 2C expression (Fig. 15A). This mirrors the results in the innate immune competent melanoma cell line DM440, which indicates that the capacity to release IFNs during viral infection is distinct from a cell's IFN responsiveness.

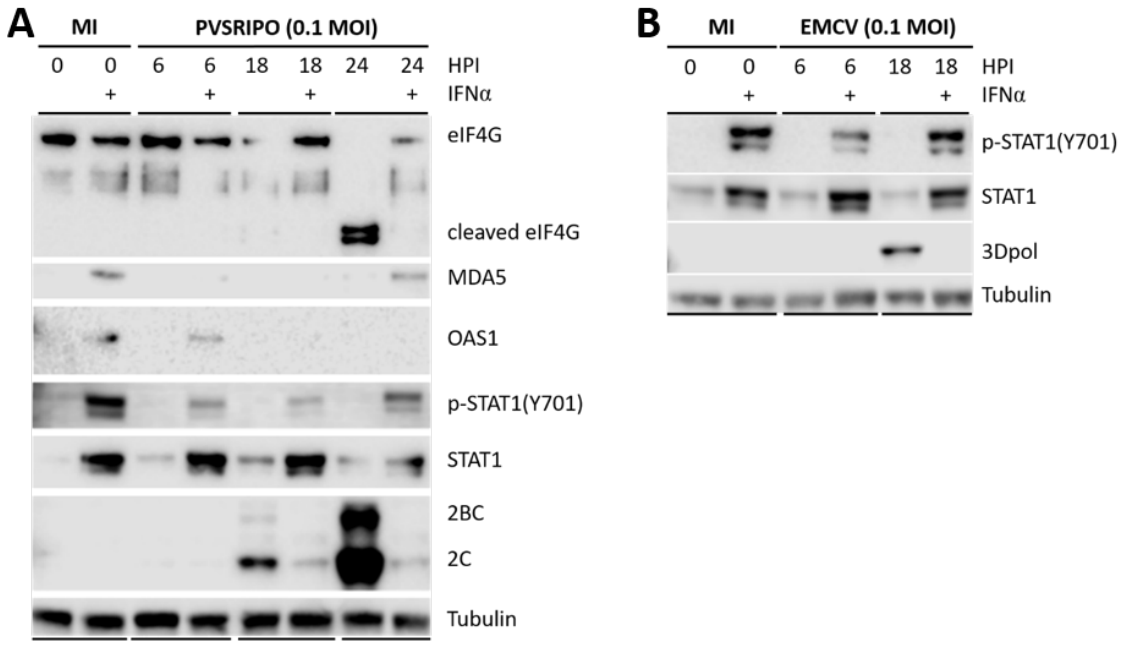

Figure 15: IFN pretreatment of DM443 cells stimulates ISGs and inhibits translation of EMCV more than PVSRIPO

DM443 cells were pretreated for 24h with 100 U/mL IFN-α (+) or vehicle, IFN was withdrawn, then cells mock infected (0h), or infected with 0.1 MOI PVSRIPO or EMCV. (A) Immunoblot of lysates from cells infected with PVSRIPO or MI for indicated time probed for viral translation (2BC/2C), cytopathogenicity (eIF4G1 cleavage) and type I IFN response [p-STAT1(Y701), MDA5, OAS1]. (B) Immunoblots of lysates from EMCV-infected or MI cells probed as in (A). Immunoblots are representative of 2 independent experiments.

Multi-step growth curves of 0.01 MOI PVSRIPO and EMCV in IFN pretreated DM443 cells showed significant inhibition of EMCV in pretreated cells and far smaller inhibition of PVSRIPO. At 18 and 24 hpi, EMCV yields were reduced ~2000- and ~1500-fold, respectively, in IFN-α2 pretreated DM443 cells (Fig. 16B). Under the same conditions, PVSRIPO yield was reduced only 26- and 72-fold (Fig. 16A). These results, combined with the immunoblot results, suggest that DM443 cells mount antiviral

responses when stimulated with exogenous IFNs and inhibit EMCV more than PVSRIPO. However, this effect is smaller than in innate immune competent DM440 cells (Fig. 14A). Thus, DM440 cells likely have more consistent antiviral signaling during infection with both viruses, as they have intrinsic IFN signaling, which DM443 cells lack.

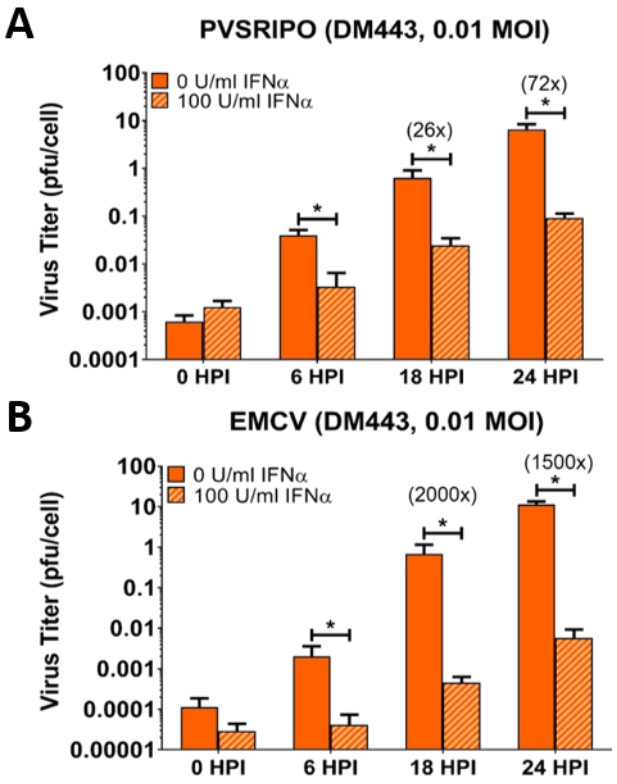

Figure 16: IFN pretreatment of DM443 cells inhibits propagation of EMCV more than PVSRIPO

DM443 cells were pretreated for 24h with 100 U/mL IFN-α (+) or vehicle, IFN was withdrawn, then infected with 0.01 MOI PVSRIPO or EMCV. (A, B) Multi-step growth curves of PVSRIPO (A) or EMCV (B); mean ± SEM (n=3), $p < 0.05$ (*) vehicle vs IFN-α pretreated.

3.2.2.4 Exogenous IFNs stimulate immune responses in glioma cells and slightly inhibit PVSRIPO replication

PVSRIPO is currently in clinical trial against recurrent glioblastoma, a cancer widely regarded as IFN-incompetent (Desjardins et al., 2018). Previous evidence regarding ISG upregulation and antiviral effects of exogenous type I IFN in IFN-incompetent melanoma cells suggests other cancer types may exhibit similar responses. To test the phenotype we pretreated DU54 cells, a previously characterized human glioma cell line, with IFN-α2, withdrew IFN-α2, and infected with 0.1 or 0.01 MOI PVSRIPO (Gromeier et al., 2000a). Similar to DM440 and DM443 cells, pretreatment of DU54 glioma cells with IFN-α induced STAT1 (Y701) phosphorylation and upregulated ISGs (Fig. 17A). Also like DM443 cells, DU54 cells did not have detectable intrinsic IFN responses to PVSRIPO infection, as shown by a lack of STAT1 (Y701) phosphorylation in the vehicle treated, PVSRIPO infected cells (Fig. 17A). IFN-α2 pretreatment of DU54 cells temporarily delayed eIF4G1 cleavage and 2C translation, but PVSRIPO overcame this inhibition by 30hpi (Fig. 17A, B). PVSRIPO propagation was reduced by only a maximum of ~10-fold at 6hpi in IFN-α2a-pretreated DU54 cells (Fig. 17C).

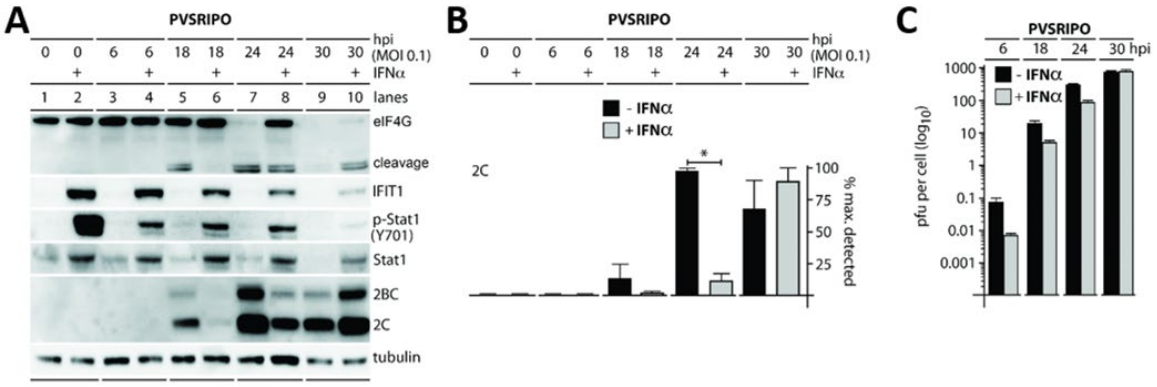

Figure 17: PVSRIPO overcomes antiviral immune responses in glioma cells after IFNα pretreatment

DU54 glioma cells were pretreated with 100 U/mL recombinant human IFN-α2a (+) or vehicle (24h). (A) Immunoblots of lysates from cells infected with 0.1 MOI PVSRIPO at indicated time probed for viral translation (2BC/2C), cytopathogenicity (eIF4G1 cleavage) and type I IFN response [p-STAT1(Y701), STAT1, and IFIT1]; immunoblot representative of 2 independent experiments. (B) Viral protein 2C expression at indicated time after 0.1 MOI PVSRIPO as percent of maximum detected viral protein; mean ± SEM (n=2), $p < 0.05$ (*), IFN-pretreated vs. untreated. (C) Multi-step growth curve of pretreated cells infected with 0.01 MOI PVSRIPO for indicated time; mean ± SEM (n=3).

3.2.2.5 Endogenous IFNs do not inhibit EMCV or PVSRIPO, but EMCV may be sensitive to other intrinsic innate immune responses

PVSRIPO replicates efficiently in IFN-competent and -incompetent cells, but EMCV is severely inhibited by IFN signaling. Therefore, we hypothesized EMCV replication and cytopathogenicity would be compromised in cells with intact innate antiviral IFN responses. To test this, we infected IFN-producing DM440 and IFN-nonproducing DM443 melanoma cells with 0.1 MOI EMCV and compared replication and lytic cell death by LDH release (Fig. 18). EMCV replication was significantly higher in the DM443 cells relative to DM440 cells at 24 and 48 hpi (Fig. 18A). Lytic cell death

was likewise higher in DM443 cells relative to DM440 cells at 16 and 24 hpi, but both cell lines showed 100% cytopathogenicity by 48 hpi (Fig. 18B).

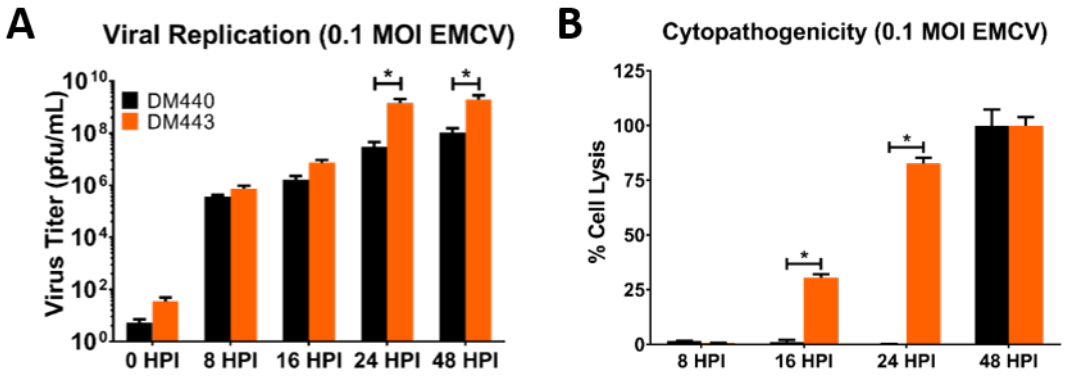

Figure 18: EMCV replication and cytopathogenicity are reduced in innate immune competent melanoma cells

DM440 and DM443 melanoma cells were infected with 0.1 MOI EMCV for indicated time (A) Multi-step growth curves; mean ± SEM (n=3), $p < 0.05$ (*), DM440 vs. DM443. (B) Percent cytotoxicity by LDH release; MI cells as a 0% lysis control, freeze-thawed cells as 100% lysis control; mean ± SEM (n=3), $p < 0.05$ (*), DM440 vs. DM443.

Since DM440 cells secrete type I IFNs and mount antiviral responses during PVSRIPO and EMCV infection, we hypothesized type I IFNs were the primary cause of antiviral signaling in infected cancer cells. However, type III IFNs also promote autocrine and paracrine STAT1 (Y701) phosphorylation, and were implicated in anti-tumor immune responses and picornavirus infection (Lasfar et al., 2011; Sheppard et al., 2003; Zhou et al., 2007). To determine if type I or type III IFNs caused innate immune responses during PVSRIPO and EMCV infection, we pre- and co-treated DM440 cells with IFNAR2 blocking antibody or appropriate controls and infected them with EMCV

or PVSRIPO. Antibody blockade of IFNAR2 prevents activation of the antiviral state by type I IFNs but not type III IFNs (Tsugawa et al., 2014).

Cells treated with anti-IFNAR2 showed little STAT1 (Y701) phosphorylation after infection with PVSRIPO or EMCV, compared to both no antibody and isotype antibody treated control cells (Fig. 19A, B). Anti-IFNAR2 cells also showed significant cytopathogenicity from PVSRIPO by loss of gross cellular protein signal and eIF4G1 cleavage (Fig. 19A). Surprisingly, blockade of IFNAR2 had no detectable effect on EMCV 3Dpol expression or cytotoxicity (Fig. 19B). Likewise, viral propagation was approximately equal for all antibody treatments with both EMCV and PVSRIPO (Fig. 19C). Thus, type I, not type III, IFNs activate phosphorylation of STAT1 (Y701) after EMCV and PVSRIPO infection. However, other innate antiviral immune responses may be necessary to suppress EMCV in melanoma.

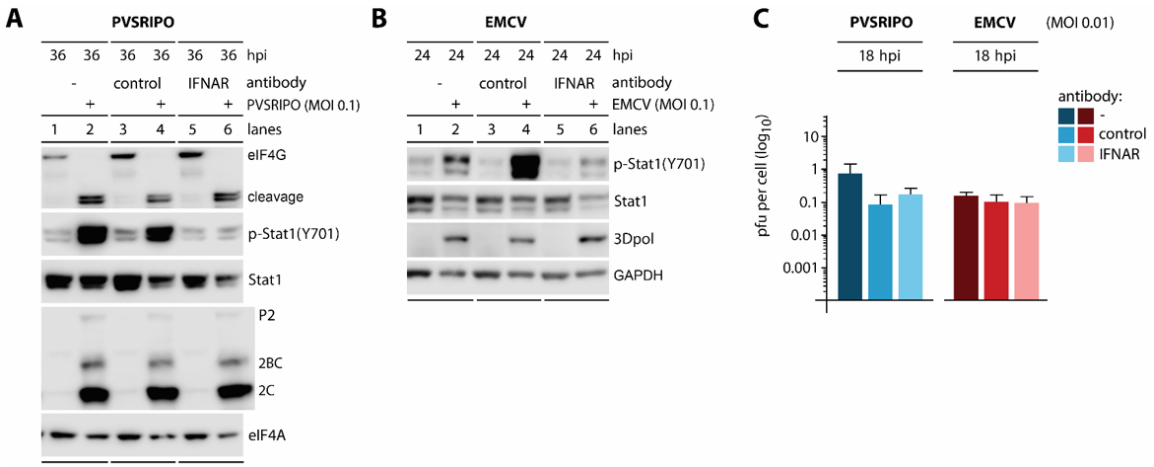

Figure 19: IFNAR2 blockade prevents STAT1 (Y701) phosphorylation but does not affect PVSRIPO or EMCV translation or replication

DM440 cells were pretreated with no antibody (-), isotype-matched control antibody (control) or IFNAR2 blocking antibody (IFNAR) for 24h, then co-treated with antibody and mock-, PVSRIPO-, or EMCV-infected. (A, B) Lysates from cells mock- and PVSRIPO- infected (A) or mock- and EMCV-infected (B) at MOIs of 0.1 were collected at the indicated hpi and analyzed by immunoblot with probes for viral translation (2BC/2C; 3Dpol), cytopathogenicity (eIF4G1 cleavage) and type I IFN response [p-STAT1(Y701) and STAT1]; immunoblots are representative of 2 independent experiments. (C) Virus yields of PVSRIPO/ EMCV-infected DM440 cells (MOI of 0.01) were determined at 18 hpi; mean ± SEM (n=3).

3.2.3 Discussion

Treatment of tumors with PVSRIPO induces significant upregulation of proinflammatory cytokines and chemokines including type I IFNs (Brown et al., 2017). As such, unraveling the interaction of host innate antiviral immune responses and PVSRIPO after exogenous IFN stimulation is of particular interest. IFN-α2 pretreatment of innate immune competent melanoma cells upregulates known ISGs and p-STAT1(Y701) (Fig. 13). Similarly, exogenous IFN-α2 stimulate ISGs and STAT1 (Y701)

phosphorylation in IFN-nonproducing cancer cell lines (Fig. 15, 17). Exogenous IFNs inhibit EMCV regardless of the cell's IFN production capacity (Fig. 14A, 16B). This suggests some cancer cells cannot activate antiviral immune responses during infection but mount antiviral immune responses downstream of IFNs and inhibit IFN-sensitive viruses like EMCV, and potentially other OVs. PVSRIPO, on the other hand, shows productive infection and cytopathogenicity in IFN-pretreated cells at the low MOIs used throughout this study. Expression of ISGs is maintained after IFN-α2 pretreatment of cancer cells during PVSRIPO infection (Fig. 13). Therefore, PVSRIPO likely evades antiviral immune signals downstream of IFNs, but does not completely inhibit them. This mimics the relationship of PVSRIPO and host cell innate immune responses in IFN competent cells without pretreatment.

Throughout this study we used STAT1 (Y701) phosphorylation as an indicator of type I IFN responses in treated cell lines (Shuai et al., 1993). However, multiple other cytokines utilize JAK/STAT signaling pathways to activate STAT1 including type III IFN (Kotenko et al., 2003), IL-6, IL-10, and CSF1 (Meraz et al., 1996). Several of these cytokines have also been linked to suppression of picornaviruses including EMCV (Ank et al., 2006). Blockade of IFNAR abrogated STAT1 (Y701) phosphorylation from PVSRIPO and EMCV infection (Fig. 19). From this we can conclude endogenous type I

IFNs are the main driver of STAT1 (Y701) phosphorylation during picornavirus infection of these cells.

After blocking downstream type I IFN signaling, we expected to see a substantial increase in EMCV replication. Exogenous IFN treatment inhibited EMCV (Fig. 14B, 16B), and EMCV replication is compromised in innate immune competent DM440, relative to innate immune incompetent DM443 (Fig. 18). Surprisingly, loss of IFN signaling had no detectable effect on EMCV replication or translation (Fig. 19B, C). Likewise, IFNAR blockade had no effect on PVSRIPO replication, but this effect was expected, as throughout this study we found innate immune responses had only minimal effects on PVSRIPO (Fig. 19A, C). It is possible innate antiviral immune responses upstream of the autocrine/paracrine effects of IFNs suppress EMCV, and that PVSRIPO is more resistant to these responses.

3.3 Innate Immune Response to PVSRIPO and EMCV are MDA5-dependent and more inhibitory of EMCV

3.3.1 Introduction

EMCV is inhibited in IFN-pretreated melanoma cells and in untreated IFN-competent melanoma cells, but inhibition in untreated cells is independent of type I IFN signaling. This paradox suggests EMCV activates IFN-independent antiviral immune responses. MDA5 is a PRR for the recognition of cytoplasmic dsRNA and picornaviruses including EMCV and PV (Kato et al., 2006). Activation of MDA5 activates TBK1/IKKε

which phosphorylate and activate IRF3 (Loo & Gale, 2011). IRF3 dimers transcribe type I IFNs, but also transcribe ISGs in an IFN-independent manner (Grandvaux et al., 2002). Thus, it is possible that MDA5 activation by EMCV stimulates IFN-independent upregulation of ISGs by IRF3, activated by TBK1/IKKε, and causes IFN-independent inhibition of EMCV. MDA5 activation by PVSRIPO would likely cause similar IFN-independent upregulation of ISGs but would have minimal effects on PVSRIPO replication. Some evidence also suggests MDA5 binding to viral dsRNA disrupts RNPs required for replication (Yao et al., 2015). Either or both of these functions of MDA5 could have significant consequences for PVSRIPO and EMCV infection.

3.3.2 Results

3.3.2.1 IFN responses to PVSRIPO in melanoma are MDA5-dependent

To determine if innate immune responses in melanoma cells are MDA5-dependent, stable MDA5-depleted DM440 cells were generated by lentiviral shRNA transduction. Immunoblot analysis of MDA5-depleted DM440 cells infected with 0.1 MOI PVSRIPO showed effective depletion of MDA5 and loss of STAT1 phosphorylation (Y701) during infection (Fig. 20A, B). In accordance with earlier observations, only small differences in viral protein 2C or viral cytotoxicity (as measured by eIF4G1 cleavage) between PVSRIPO-infected, MDA5-depleted DM440 cells and the parental cell line were

observed (Fig. 20A). PVSRIPO infection of MDA5-depleted DM440 cells did not trigger release of detectable IFN-β, unlike the WT parental cell line (Fig. 20C).

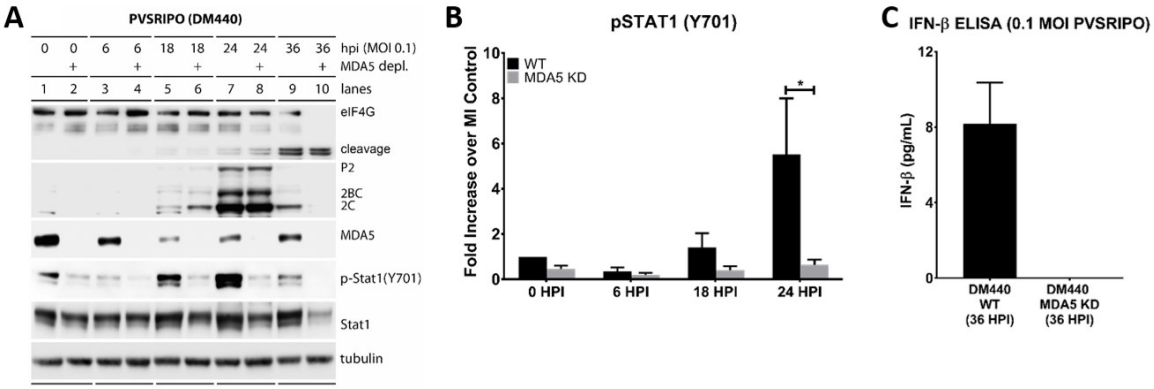

Figure 20: MDA5 is necessary for IFN response to PVSRIPO in DM440 cells
Parental WT and stably MDA5-depleted DM440 cells were MI (0h) or infected with 0.1 MOI PVSRIPO. (A) Immunoblots of cell lysates collected at the indicated hpi analyzed for markers of viral cytotoxicity (eIF4G1), viral translation (P2/2BC/2C), and the innate response [MDA5, (p-STAT1(Y701) and STAT1)]; immunoblots are representative of at least 4 independent experiments. (B) Fold-increase of p-STAT1 (Y701) relative to MI control; mean ± SEM (n=4), p <0.05 (*) WT vs. MDA5-depleted (24 hpi). (C) ELISA of IFN-β in media 36 hpi; mean ± SEM (n=4).

Immunoblots comparing MDA5-depleted and WT parental DM6 melanoma cells infected with 0.1 MOI PVSRIPO showed similar loss of STAT1 (Y701) phosphorylation with only mild effects on 2C translation (Fig. 21A). DM6 cells also showed no detectable differences in viral propagation between MDA5-depleted and parental lines in a multi-step growth curve analysis with 0.01 MOI PVSRIPO (Fig. 21B). Like D440 cells, depletion of MDA5 from DM6 cells completely abrogated secretion of IFN-β from PVSRIPO-infected cells (Fig. 21C).

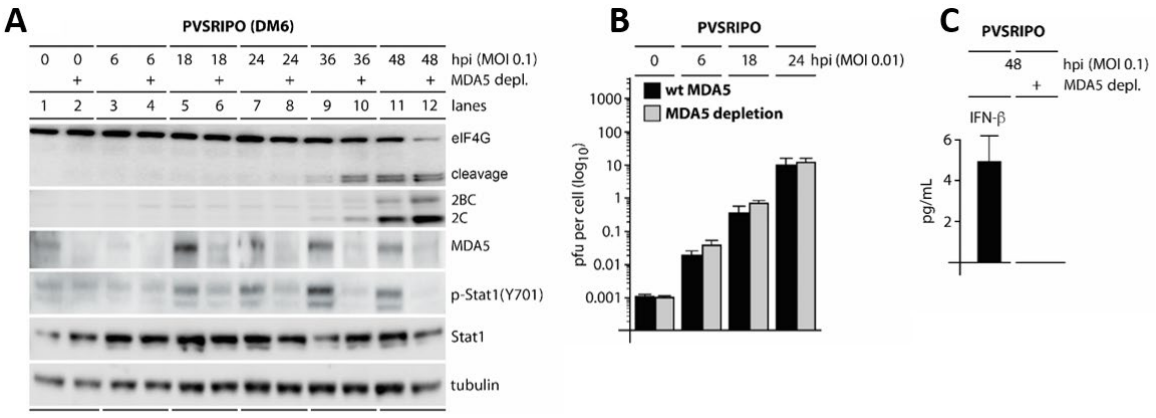

Figure 21: MDA5 is necessary for IFN response to PVSRIPO in DM6 melanoma cells
Parental WT and stably MDA5-depleted DM6 cells were MI (0h) or infected with PVSRIPO. (A) Immunoblots of cell lysates infected at MOI 0.1 or MI collected at the indicated hpi were analyzed as in (Fig. 15A). (B) Multi-step growth curves of 0.01 MOI PVSRIPO; mean ± SEM (n=3). (C) IFN-β ELISA of media from cells infected at MOI of 0.1 48 hpi; mean ± SEM (n=4).

3.3.2.2 MDA5-dependent immune responses inhibit EMCV translation

Earlier data showed intrinsic IFN production was insufficient to significantly inhibit EMCV in melanoma cells, despite EMCV's well-characterized sensitivity to IFNs, but it is possible EMCV propagation is inhibited in DM440 relative to DM443 melanoma cells by other innate antiviral signals. To gauge the intrinsic antiviral capacity of melanoma cells, MDA5-depleted DM440 cells and their WT parental line were infected with 0.1 MOI EMCV. As with PVSRIPO, MDA5-depleted cells showed no detectable STAT1 (Y701) phosphorylation after infection with 0.1 MOI EMCV (Fig. 22A, B). In contrast with PVSRIPO, EMCV infected MDA5-depleted cells showed earlier expression of viral 3Dpol relative to the parental line (18 vs. 24 hpi), as well as accelerated lytic cell

death as measured by loss of cellular proteins and PARP cleavage (Fig. 22A). MDA5-depleted DM6 melanoma cells infected with 0.1 MOI EMCV also lost IFN-β secretion relative to MDA5 WT parental cells (Fig. 21C).

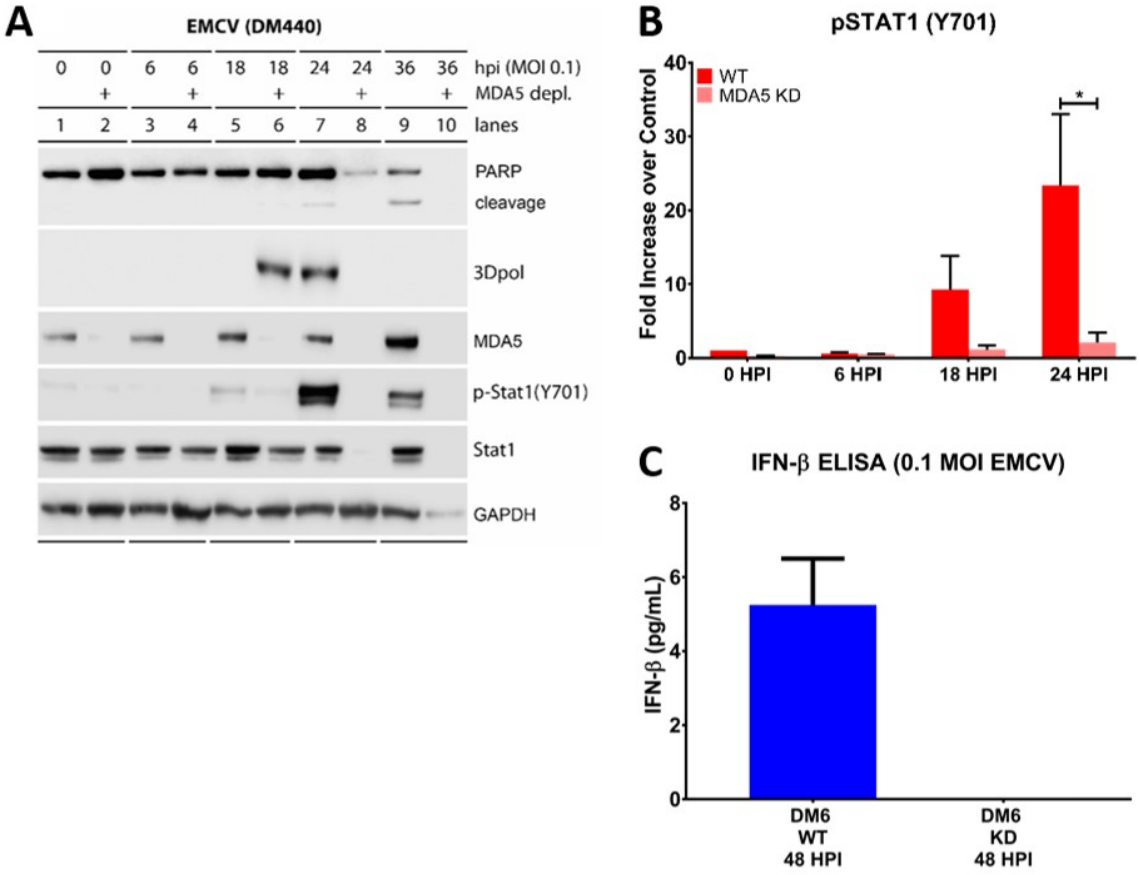

Figure 22: MDA5-depletion in melanoma cells improves EMCV replication and prevents IFN responses

Parental WT and stably MDA5-depleted cells were MI (0h) or infected with 0.1 MOI EMCV for indicated time. (A) Immunoblots of DM440 cell lysates analyzed for markers of viral cytotoxicity (PARP), viral translation (3Dpol), and the innate response [MDA5, (p-STAT1(Y701) and STAT1)]; representative 4 independent experiments. (B) Fold-increase of p-STAT1 (Y701) in DM440 WT and MDA5 KD cells relative to MI control; mean ± SEM (n=4), p <0.05 (*) WT vs. MDA5-depleted (24 hpi). (C) IFN-β ELISA of media from infected MDA5 WT and KD DM6 48 hpi; mean ± SEM (n=4).

3.3.2.3 MDA5-dependent immune responses strongly inhibit EMCV replication

Immunoblots of MDA5-depleted cells suggested loss of MDA5 would increase EMCV viral yield but have little effect on PVSRIPO. We performed multi-step viral growth curves of PVSRIPO and EMCV in WT parental and MDA5-depleted DM440 cells at an MOI of 0.01. PVSRIPO propagation increased in MDA5-depleted cells, with viral titers elevated ~6-fold at 18hpi (Fig. 23A). MDA5 depletion elevated EMCV propagation ~230-fold at 18hpi (Fig. 23B). EMCV yield in WT parental DM440 cells approached the yield in MDA5-depleted DM440 cells over time, suggesting endogenous MDA5 temporarily inhibits EMCV. The small effect MDA5 depletion has on PVSRIPO replication resonates with our finding of unimpeded virus spread/propagation with extremely low MOIs in type I IFN-competent cultures.

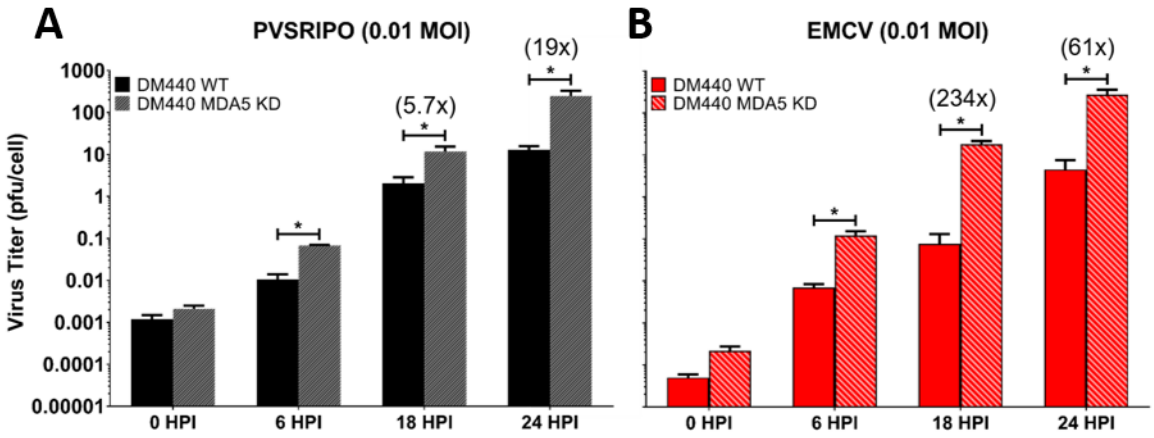

Figure 23: MDA5-depletion enhances replication of EMCV more than PVSRIPO
(A, B) Multi-step growth curves of WT parental and MDA5-depleted DM440 cells infected with 0.01 MOI PVSRIPO (A) or EMCV (B), fold change in viral yield is indicated for 18 and 24 hpi with both viruses; mean ± SEM (n=3), $p < 0.05$ (*) WT vs. MDA5-depleted (6, 18, 24 hpi).

3.3.2.4 EMCV is susceptible to endogenous innate antiviral responses upstream of IFNs and downstream of MDA5 activation

Our work showed MDA5 inhibits EMCV in melanoma cells, but endogenous type I IFNs do not. It also showed PVSRIPO subverts both MDA5- and IFN-activated innate antiviral immune responses. Two mechanisms could explain these effects of MDA5 on PVSRIPO and EMCV: 1) MDA5 may have direct antiviral effects, i.e. associate with viral dsRNA and disrupt viral protein:dsRNA RNP complexes (Yao et al., 2015); or 2) MDA5 activation of the transcription factors IRF3/7 via TBK1/IKKε could upregulate ISGs to intercept the EMCV viral life cycle (Anderson et al., 1999; Chebath et al., 1987). In either case, PVSRIPO likely subverts the response through an unknown mechanism,

but most likely through host cell translation shutdown by viral $2A^{pro}$, not through proteolytic degradation of innate immune modifiers.

To determine if MDA5 binding alone inhibits EMCV or if downstream activation was required, we treated DM440 cells with the small molecule kinase inhibitor BX795, a relatively specific inhibitor of TBK1/IKKε, and infected with PVSRIPO or EMCV (Clark et al., 2009). Pretreatment of DM440 cells with BX795 abolished STAT1 (Y701) phosphorylation after infection with 0.1 MOI PVSRIPO or EMCV (Fig. 24A, B). BX795 pretreatment significantly increased EMCV translation but only had modest effects on PVSRIPO translation (Fig. 24C). At peak viral translation, PVSRIPO 2C expression was enhanced ~1.8-fold (Fig. 24A, C). In contrast, EMCV viral protein expression was elevated ~40-fold with BX795 pretreatment (Fig. 24B, C). As with MDA5 depletion, EMCV-infected cells pretreated with BX795 underwent lytic cell death by 24 hpi, but control cells were still alive at this time point, based on loss of cellular and viral protein signal (Fig. 24B). Pre-treatment of DM440 cells with BX795 roughly mimics the effects of MDA5 depletion on PVSRIPO translation, EMCV translation, and innate immune activation, indicating activation of TBK1/IKKε downstream of MDA5 is a key component of this antiviral innate immune response.

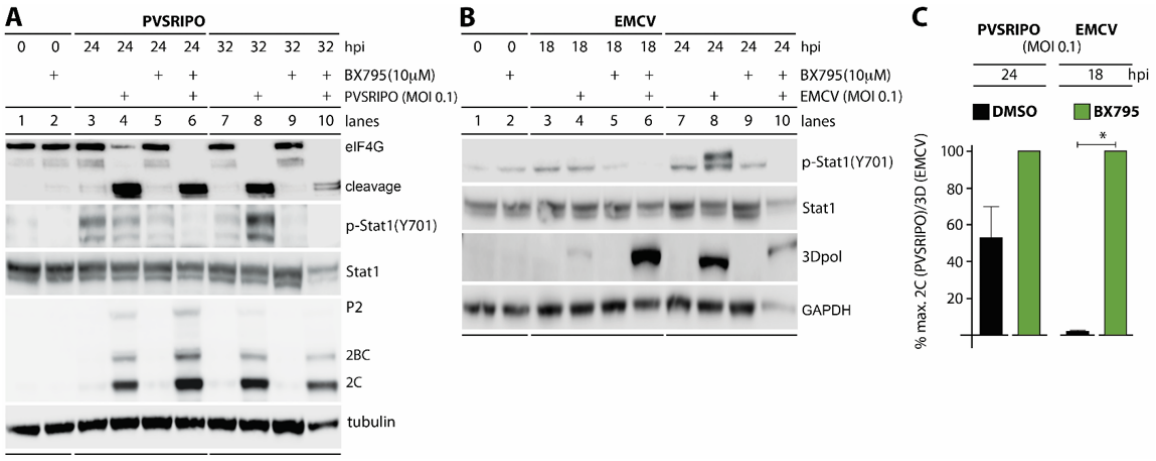

Figure 24: TBK1/IKKε inhibition promotes EMCV translation with little effect on PVSRIPO translation

DM440 cells were pretreated with 10μM BX795 (1h) or equal amount DMSO. (A, B) Cell lysates were collected at the indicated hpi from control cells and cells infected with 0.1 MOI PVSRIPO (A) or EMCV (B) and analyzed by immunoblot for markers of viral translation (P2/2BC/2C or $3D^{pol}$), cytotoxicity (eIF4GI cleavage) and innate immune response [p-STAT1(Y701), STAT1]; immunoblots are representative of 2 independent experiments. (C) Viral protein expression as percent of maximum detected viral protein; mean ± SEM (n=2), p <0.05 (*), BX795 vs. mock-treated control.

MDA5 depletion and TBK1/IKKε inhibition both accelerated EMCV replication at similar rates. Thus, MDA5-dsRNA binding is likely not responsible for antiviral effects on EMCV. Rather, downstream upregulation of ISGs by MDA5 activation, through TBK1/IKKε-IRF3/7 signaling, likely inhibits EMCV. To further test this hypothesis, MDA5-depleted DM440 cells were pretreated with poly(I:C) and infected with 0.1 MOI PVSRIPO or EMCV. Extracellular poly(I:C) upregulates IFNs and ISGs through TLR3 and TBK1/IKKε activation (Alexopoulou et al., 2001; Brownell et al.,

2014). Cells were also co-treated with IFNAR2-blocking antibody, as in Section 3.2.2.5, to exclude the effect of secreted type 1 IFNs after poly(I:C) treatment.

Treatment of MDA5-depleted DM440 cells with extracellular poly(I:C) for 6 hours induced STAT1 (Y701) phosphorylation and upregulation of IFIT1 (Fig. 25A). Co-treatment of poly(I:C) and IFNAR2 monoclonal antibody (mAb) induced expression of IFIT1 without STAT1 (Y701) phosphorylation, indicating poly(I:C) induces ISG upregulation independently of type I IFNs, presumably through TLR3 activation (Fig. 25A).

Poly(I:C) pretreated MDA5-depleted DM440 cells infected with PVSRIPO had upregulated IFIT1 expression and STAT1 (Y701) phosphorylation, but the combination of poly(I:C) treatment and PVSRIPO infection also upregulated STAT1 and IFIT1 in the absence of IFNAR2 mAb (Fig. 25B). This indicates poly(I:C), PVSRIPO, and autocrine/paracrine type I IFNs have cumulative effects on expression of ISGs. It also belies claims PV directly targets the innate immune system for degradation by viral proteins. As expected, any changes in PVSRIPO translation were not statistically significant under any test conditions (Fig. 25B, D).

Pretreated cells infected with EMCV showed similar IFIT1 and STAT1 expression patterns to PVSRIPO-infected cells (Fig. 25B, C). Unlike PVSRIPO, however, poly(I:C) pretreatment significantly inhibited EMCV viral protein expression (Fig. 25C, D).

Inhibition was preserved, albeit to a lesser degree, in poly(I:C) pretreated cells co-treated with IFNAR2 blocking antibody (Fig. 25C, D). Thus, PRR activation and autocrine/paracrine IFNs, have distinct but cumulative antiviral effects on EMCV. They also have distinct but cumulative upregulation of ISGs. PVSRIPO, not EMCV, subverts these effects in melanoma.

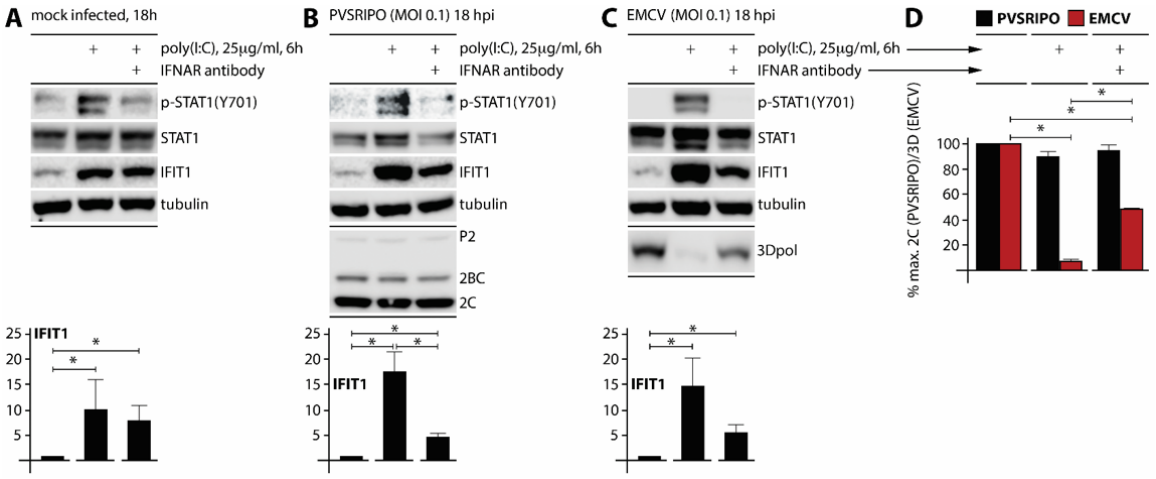

Figure 25: TLR3 activation in MDA5-depleted DM440 cells inhibits EMCV, not PVSRIPO, upstream of Type 1 IFNs

MDA5-depleted DM440 cells were treated overnight with 1µg/mL isotype-matched control or IFNAR2 blocking antibody, co-treated with 25µg/mL poly(I:C) or vehicle for 6h. (A, B, C top panels) Lysates were collected from mock- (A), 0.1 MOI PVSRIPO- (B), and 0.1 MOI EMCV- (C) infected cells 18 hpi and analyzed by immunoblot for markers of viral translation [P2, 2BC, 2C (PVSRIPO); $3D^{pol}$ (EMCV)], and innate immune response [p-STAT1(Y701), STAT1, and IFIT1)]; immunoblots are representative of 2 independent experiments. (A, B, C bottom panels) Fold-increase in IFIT1 relative to untreated controls; mean ± SEM (n=2), p <0.05 (*). (D) Viral protein expression after PVSRIPO (2C) or EMCV ($3D^{pol}$) infection as percent of maximum detected viral protein; mean ± SEM (n=2), p <0.05 (*) for all $3D^{pol}$ differences, no statistically significant differences in 2C.

3.3.3 Discussion

When IFNs bind to their receptor it triggers phosphorylation of STAT1 by JAK1/Tyk2. Phosphorylated STAT1 (Y701) is an integral part of transcription factor complexes which upregulate many ISGs and other antiviral genes in response to IFNs (Darnell et al., 1994). Depletion of MDA5 from IFN competent melanoma cells eliminates STAT1 (Y701) phosphorylation during infection with PVSRIPO (Fig. 20A, B). MDA5-depleted melanoma cells also did not secrete IFN-β during infection with PVSRIPO, while WT parental lines did (Fig. 20C). This is unsurprising, as MDA5 is the major PRR for detection of picornavirus infection and activation of the antiviral innate immune response (Gitlin et al., 2006; Kato et al., 2006). MDA5-depleted melanoma cells conveyed only a minor translational advantage to PVSRIPO over parental cells, and both parental and MDA5-depleted cells showed widespread lytic cell death with similar kinetics (Fig. 20A). These results are in keeping with earlier observations that neither exogenous nor endogenous IFNs have major impact on PVSRIPO cytopathogenicity. In addition, the requirement for MDA5 provides insight into the mechanism of PVSRIPO's immune stimulation.

Overexpression of MDA5 in melanoma causes terminal differentiation, growth arrest, and apoptosis (Kang et al., 2002). MDA5 activation by enteroviruses has also been linked to, and may cause, autoimmune diseases in humans, including type 1 diabetes

(Horwitz et al., 1998; Sadler, 2018). Exploiting this existing mechanism to generate immune responses against TAAs, rather than normal host antigens, may hold significant cancer immunotherapeutic potential (Zitvogel et al., 2015). MDA5 activation by synthetic dsRNA or overexpression of MDA5 is a proposed treatment for glioblastoma, melanoma, and pancreatic cancer (Besch et al., 2009; Glas et al., 2013; Schnurr & Duewell, 2014). However, oncogenic constitutive activation of Ras-Raf-ERK1/2 suppresses MDA5 pro-apoptotic signaling (Augustine et al., 2010; Lin et al., 2006). All three of the melanoma cell lines used in this paper share this constitutive activation phenotype, and yet are still killed by PVSRIPO, and two of them showed robust activation of innate immune responses dependent on MDA5 (Fig. 20A, 21A) (Augustine et al., 2010). Thus, PVSRIPO activates type I IFN responses in melanoma cells despite oncogenic Ras-Raf-ERK1/2 mutations in an MDA5-dependent manner, which may be an important part of the virus's clinical application. PVSRIPO also has IFN- and MDA5-independent cytopathogenic effects in cancer cells.

As with PVSRIPO, STAT1 (Y701) phosphorylation and IFN-β production were compromised in MDA5-depleted melanoma cells during EMCV (Fig. 22). Unlike PVSRIPO, loss of MDA5 also stimulated EMCV replication (Fig. 23B). However, endogenous IFN signaling had no detectable effect on EMCV replication (Fig. 19). Thus, MDA5 has antiviral effects independent of IFNs and subsequent autocrine/paracrine

activation. These effects inhibit EMCV significantly more than PVSRIPO. Thus, PVSRIPO likely evades multiple components of the innate antiviral immune response, including MDA5 activation and IFN stimulated responses.

We considered two explanations for the effects of MDA5 on EMCV in infected host cells. First, MDA5 may exert antiviral effects directly, e.g. by binding to viral dsRNA and disrupting RNP assemblies (Yao et al., 2015). Second, antiviral transcriptional programs, upregulated by IRF3/7 and NF-κB, could inhibit EMCV independent of type I IFNs (Schmid et al., 2010).

Inhibition of TBK1/IKKε with the kinase inhibitor BX795 in DM440 melanoma cells recapitulated the effects of MDA5 depletion on STAT1 (Y701) phosphorylation (Fig. 22A, 24B) (Clark et al., 2009). Most likely this was a result of a loss of type I IFN production by inhibition of signal transduction to IRF3/7 by the MDA5/MAVS signaling cascade which relies on TBK1/IKKε (Fitzgerald et al., 2003). BX795 also induced robust increases in EMCV 3Dpol expression, relative to control, 18 hpi (Fig. 24C). This was the same pattern of EMCV translation in MDA5-depleted melanoma cells (Fig. 22A). Thus, signal transduction downstream of MDA5 activation is required for antiviral immune responses against EMCV, not simply MDA5 binding to viral dsRNA.

Extracellular poly(I:C) activates antiviral immune responses through TLR3 (Alexopoulou et al., 2001). The TLR3 signal transduction pathway shares TBK1/IKKε,

IRF3/7, and NF-κB with the MDA5 signal pathway, but uses a distinct adapter complex (Kawai & Akira, 2006). By using poly(I:C) to stimulate TLR3 in MDA5-depleted melanoma cells we were able to further dissect the innate immune components necessary to inhibit EMCV. To eliminate influence of type I IFNs, IFNAR-blocking antibody was added in combination with poly(I:C) treatment (Tsugawa et al., 2014).

Poly(I:C) stimulated upregulation of IFIT1 and p-STAT1(Y701) in MDA5-depleted melanoma cells during treatment with either IFNAR2 antibody or isotype control antibody (Fig. 25A). This implies TLR3, and likely MDA5, stimulation upregulates antiviral genes independently of IFNs. Under the same conditions, PVSRIPO-infected cells showed similar patterns of IFIT1 upregulation and STAT1 (Y701) phosphorylation, but IFIT1 was substantially upregulated in cells treated with a combination of control antibody, poly(I:C), and PVSRIPO (Fig. 25B). This may be a combinatorial effect of two potent innate immune stimuli, namely poly(I:C) and PVSRIPO infection. Combinatorial effects were lost when cells were treated with IFNAR2 blocking antibody, which implies the effect was downstream of IFN signaling (Fig. 25B). No immune stimulatory treatments had any significant effect on PVSRIPO translation, as has been the norm throughout this study (Fig. 25D).

MDA5-depleted cells treated with poly(I:C) mounted effective antiviral immune responses against EMCV infection (Fig. 25C). As these experiments were performed in

MDA5-depleted cells, we exclude the possibility MDA5 had direct antiviral effects on EMCV. More likely, EMCV is inhibited by upregulation of multiple antiviral immune responses downstream of PRR activation. Antiviral effects against EMCV were detectable, but reduced, when cells were co-treated with poly(I:C) and IFNAR2 blocking antibody (Fig. 25D). While upregulation of ISGs independently of IFNs is a component of the antiviral immune response to EMCV, IFNs still play a significant role, especially in cells already under potent innate immune stimulation. However, it is notable both PVSRIPO and EMCV showed significant cumulative upregulation of IFIT1 with poly(I:C) treatment. This combination of MDA5 and TLR3 agonists may be an effective paradigm for future cancer therapy. Regardless, PVSRIPO appears to evade many different antiviral innate immune responses in cancer cells.

4. Conclusion

OVs are slowly becoming accepted cancer in cancer immunotherapy regimens. However, there is still disagreement on the role of the relationship between OVs and the innate immune responses of treated cancer cells in the therapy response. Type I IFN responses are clearly important for effective stimulation of anti-tumor immunity by OVs, but they also inhibit virus replication (Prestwich et al., 2009). There is also significant variability in the interplay of OVs and infected cancer cell based on cell type, concentration of virus, and type of virus. Our work focuses on the engineered oncolytic poliovirus PVSRIPO and its interaction with type I IFN responses in infected melanoma, breast cancer, and glioma cells. By using EMCV as an IFN-sensitive comparison picornavirus and low MOIs we found surprising interactions between cancer cell innate immune responses and virus infection. We determined 1) PVSRIPO stimulates type I IFN immune responses in multiple treated cancer types while simultaneously evading, but not inhibiting, the innate immune response; and 2) Innate immune responses in melanoma from EMCV and PVSRIPO are MDA5-dependent, but MDA5 has IFN-independent antiviral effects on EMCV.

4.1 PVSRIPO Initiates and Subverts Innate Antiviral Immune Responses in Cancer Cells

4.1.1 Conclusion

Of the three tested BRAF-mutant melanoma cells, two secreted IFN-β in response to 0.1 MOI PVSRIPO infections. These two cell lines also phosphorylated STAT1 (Y701) during infection, indicating intact autocrine and/or paracrine responses to IFNs. The single tested breast cancer cell line shared this phenotype. Thus, intact innate immune responses may be more common in cancer than initially realized, and engineered IFN-susceptibility therefore may not be an effective strategy for limiting OV tropism to neoplastic cells (Stojdl et al., 2000; Westcott et al., 2015). Unlike most OVs, PVSRIPO tropism is limited by expression of the poliovirus receptor CD155, expressed in a wide variety of cancer types, and by dysregulated translation initiation mechanisms that facilitate viral cap-independent translation of the HRV2 IRES (Bevelacqua et al., 2012; Brown et al., 2014b; Ochiai et al., 2006)

PVSRIPO translation, replication, and cytotoxicity is not compromised in IFN-competent cancer cells even at exceptionally low MOI, unlike the related picornavirus EMCV. PVSRIPO also replicates more efficiently and is more cytotoxic than EMCV in IFN-pretreated melanoma and glioma cells, regardless of intrinsic IFN responses. Likewise, pathogenic PV replication and cytotoxicity is less inhibited by IFN pretreatment than EMCV (Morrison & Racaniello, 2009). The mechanism for this

difference is not completely understood. However, decreased EMCV cytopathogenicity by type I IFNs correlates with preserved cellular translation in IFN treated cells, normally compromised during EMCV infection (Munoz & Carrasco, 1981). IFNs do not prevent translation inhibition by proteolytic cleavage of eIF4G1 by $2A^{pro}$ during PV infection (Krausslich et al., 1987; Munoz & Carrasco, 1983). Cellular translation inhibition is extremely cytotoxic. This difference in host cell translation shutdown may explain why expression of PV $2A^{pro}$ in mutant EMCV partially rescues virus replication in IFN treated HeLa cells (Morrison & Racaniello, 2009). PVSRIPO expresses PV $2A^{pro}$ and cleaves eIF4G1 in IFN-treated and -untreated cells. Thus, it is possible PVSRIPO's relative IFN insensitivity is due to global host cell translation shutdown by $2A^{pro}$ but not direct inhibition of IFNs or ISGs.

Experiments with pathogenic PV and other enteroviruses showed evidence they cleave MDA5 and its signal transducer MAVS during infection (Barral et al., 2007; Feng et al., 2014a; Mukherjee et al., 2011). These cleavages were linked to both caspase-dependent and -independent, viral protease-dependent mechanisms. PVSRIPO does not cleave MDA5 or MAVS in infected melanoma cells at physiologically relevant MOIs. Nor does it prevent the release of IFN-β or upregulation of ISGs after TLR3, MDA5, or type I IFN stimulation. Thus, we found no evidence PVSRIPO significantly or directly inhibits innate immune responses. It is possible pathogenic PV has innate immune

inhibitory strategies not present in PVSRIPO. This is unlikely as their ORFs are largely identical (Gromeier et al., 1996). MDA5/MAVS cleavage was typically observed in cells infected with enteroviruses at MOIs ≥10. All infections in this work were MOIs ≤0.1. It is possible this difference in infectious titer is the source of the disagreements between this work and the published record. Further research is necessary to elucidate the mechanisms involved in innate immune evasion by PVs and, by extension, PVSRIPO.

4.1.2 Future Directions

One of the most striking observations in this study was PVSRIPO's productive replication and cytotoxicity in cancer cells pretreated with type I IFNs. The mechanism for this phenotype remains somewhat mysterious. Both PVSRIPO and WT PV inhibit host cell translation with extremely cytotoxic consequences regardless of IFN treatment or IFN competency of the infected cell (Munoz & Carrasco, 1983). However, determining if host cell translation shutdown is required for PVSRIPO cytotoxicity and replication in IFN pretreated cancer cells is fraught with technical difficulties.

Low expression of innate immune proteins and PVSRIPO cytotoxicity in cancer cells makes detection of altered immune signaling difficult. However, it may be possible to study PVSRIPO resistance and relationship with host cell innate immune signaling in an immune cell model. Immune cells like DCs and macrophages express higher levels of relevant proteins, making detection more straight-forward than in cancer cell lines.

PVSRIPO is also less cytotoxic in these cells, and they are natural locations of PV infection (Brown et al., 2017; Shen et al., 2017).

Our lab detects host cell translation shutdown using a puromycin-incorporation assay. Puromycin inhibits protein elongation by incorporating into nascent polypeptide strands prematurely terminating protein synthesis. The abundance of puromycin-containing proteins can be quantified by immunoblot with puromycin-specific antibodies and measure global host translation (Bryant et al., 2018). Co-expression of enterovirus $2A^{pro}$ and infection of EMCV should rescue EMCV replication in IFN pretreated cells. We can then use the puromycin-incorporation assay to correlate global host translation shutdown by $2A^{pro}$ and rescue of EMCV replication. Mutant $2A^{pro}$, as well as varying time and quantity of $2A^{pro}$ expression, may allow finer resolution of the mechanisms involved.

PVSRIPO appears to be resistant to endogenous IFNs in infected monocyte-derived cells (Brown et al., 2017). THP-1 cells differentiated by TPA-stimulation, treated with IFNAR blocking antibody, and then infected with PVSRIPO showed no increase in PVSRIPO translation but did lose responses to type I IFNs, e.g. p-STAT1(Y701) (Fig. 26). Monitoring PVSRIPO replication, translation, and cytotoxicity in IFN-pretreated THP-1 cells could give insight into manipulation or evasion of the innate immune system by PVSRIPO in a relevant cellular context. We could also examine global host cell

translation with the puromycin-incorporation assay to determine if translation shutdown correlates with PVSRIPO replication in immune cells with potent antiviral signaling. Repeating these experiments in differentiated monocytes isolated from human peripheral blood mononuclear cells (PBMCs) would provide connections between clinical results of PVSRIPO treatment and *in vitro* experiments.

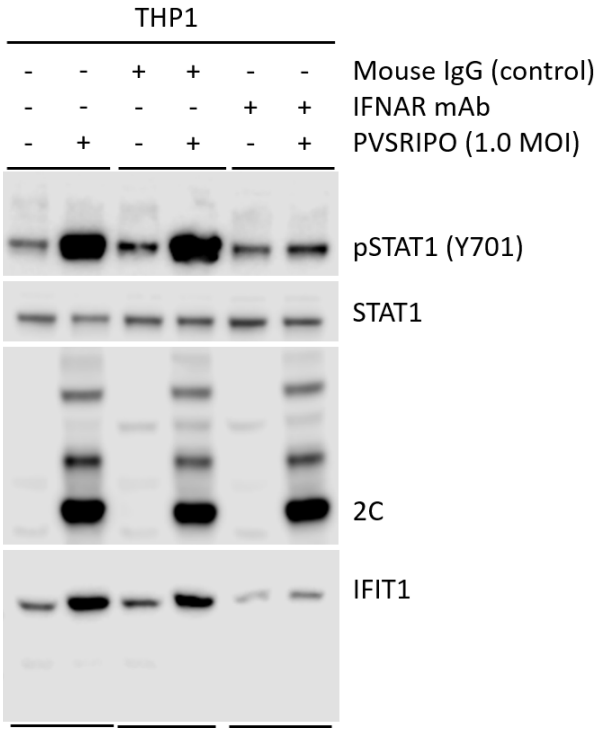

Figure 26: IFNAR blockade does not affect PVSRIPO translation in THP1 cells
Immunoblots of lysates from THP-1 cells pretreated with no Ab, IFNAR mAb, or isotype-matched mouse IgG (control) and MI or infected with 1.0 MOI PVSRPO for 24 h probed for viral translation (2C), and type I IFN response [pSTAT1(Y701), STAT1, IFIT1]. Done in collaboration with Zachary McKay.

Antiviral immunity is partially driven by co-evolution of pathogenic viruses and the immune system of their host organism (Cagliani et al., 2014). PV is adapted for

infection of primates, including humans, so PVSRIPO's immune evasion and activation may be highly dependent on host species. Bone marrow-derived dendritic cells (BMDCs) from human CD155-tg C57Bl/6 mice showed attenuated PVSRIPO replication relative to BMDCs from human CD155-tg IFNAR knock-out C57Bl/6 mice (Fig. 27A). IFNAR knock-out also abrogated STAT1 (Y701) phosphorylation in mouse BMDCs during PVSRIPO infection, consistent with earlier results (Fig. 27B). However, blocking IFNAR on DCs derived from human PBMCs did not increase expression of PVSRIPO proteins, despite this treatment preventing downstream innate immune signaling (Fig. 27C). This species difference may explain results showing PV pathogenicity and tissue tropism as being dependent on type I IFN signaling in mice (Ida-Hosonuma et al., 2005). Significant *in vitro* and *in vivo* work would be necessary to test this hypothesis.

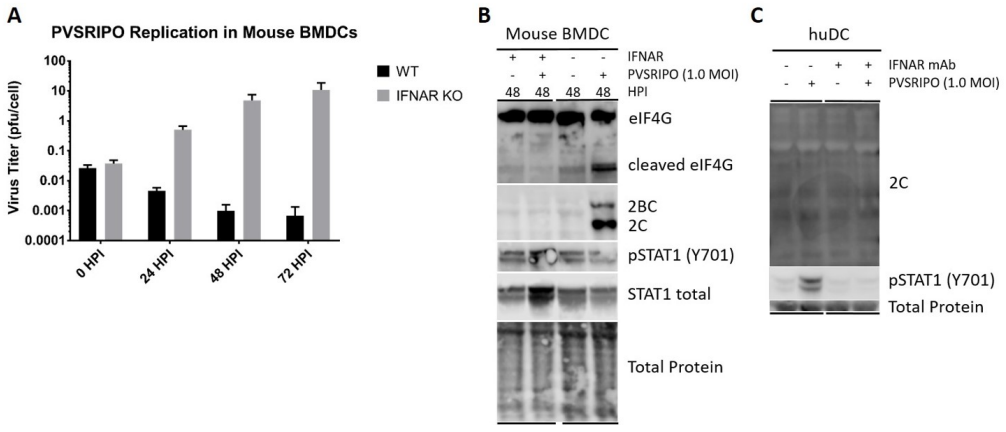

Figure 27: Lack of IFNAR signaling increases PVSRIPO replication in mouse BMDCs but does not affect PVSRIPO replication in human DCs

(A, B) BMDCs from IFNAR -/- PVR-tg mice and IFNAR +/+ PVR-tg were infected with 1.0 MOI PVSRIPO for indicated time. (A) Multi-step growth curve; mean ± SEM (n=3). (B) Immunoblots of cell lysates probed for viral translation (2BC/2C), cytotoxicity (eIF4G1 cleavage) and type I IFN response [pSTAT1(Y701) and STAT1]; representative blot of two independent experiments. (C) Immunoblots from lysates of human DCs pretreated with IFNAR mAb (+) or isotype control (-) for 24 h and infected at 1.0 MOI PVSRIPO for 24h probed for viral translation (2C), cytotoxicity, and type I IFN response [pSTAT1(Y701)]; representative blot of three independent experiments.

4.2 MDA5 Activation has IFN-Independent Antiviral Effects

4.2.1 Conclusion

MDA5 detects picornaviruses in normal cells, but little was known about its effects in cancer cells. Early experiments on MDA5 activation by synthetic agonists indicated it may be an effective cancer therapy via apoptosis and decreased proliferation, but effects were inconsistent and inhibited by oncogenic signaling (Kang et al., 2002; Lin et al., 2006). We found PVSRIPO activates innate immune responses, particularly type I IFN responses, dependent on MDA5. However, PVSRIPO replication

and cytotoxicity are neither inhibited nor strongly promoted by MDA5 activation. PVSRIPO treatment also activates DCs and promotes anti-cancer CTL responses more strongly than common synthetic agonists (Brown et al., 2017). Thus, PVSRIPO may activate DCs in an MDA5-dependent manner as well. PVSRIPO's MDA5-independent cytotoxicity and replication, combined with potent natural MDA5 activation may make it an ideal adjuvant for cancer immunotherapy.

We found EMCV replication and cytotoxicity is reduced in IFN-competent melanoma cells and by IFN pretreatment of melanoma cells. Surprisingly, EMCV is not promoted by loss of endogenous IFNAR signaling. However, MDA5 depletion abrogated IFN-β secretion by EMCV-infected melanoma cells and promoted EMCV replication. EMCV translation and replication were most inhibited in IFN-competent melanoma cells before IFN-β or STAT1 (Y701) phosphorylation were detectable. This implies MDA5 has IFN-independent antiviral mechanisms which inhibit EMCV.

Inhibition of TBK1/IKKε, kinases downstream of MDA5/MAVS which phosphorylate IRF3/7, with BX795 prevented autocrine/paracrine activation of STAT1 by PVSRIPO and EMCV, and promoted EMCV translation. Thus, MDA5 activation is a key step in achieving an antiviral state after picornavirus infection of cancer cells. IRF3 transcription factors upregulate ISGs like IFIT1 and OAS1 independently of IFNs (Grandvaux et al., 2002; Ourthiague et al., 2015; Schmid et al., 2010). Overexpression of

OAS1 inhibits EMCV replication, opening the possibility other ISGs inhibit EMCV (Chebath et al., 1987). Treatment of MDA5-depleted melanoma cells with the TLR3 agonist poly(I:C) showed IFN independent upregulation of IFIT1 and reduced translation of EMCV. These effects were more robust when IFNAR signaling was intact but were detectable when IFNAR blocking antibodies were present. Thus, EMCV is inhibited by early expression of antiviral proteins upstream of type I IFNs and downstream of PRR activation. These results suggest redundancy within the antiviral innate immune system is a vital cellular strategy to survive and prevent infections, and that these mechanisms are intact even in cancer cells.

4.2.2 Future Directions

Our *in vitro* studies in this work suggest MDA5-dependent innate immune responses triggered by PVSRIPO treatment of tumors may activate anti-tumor immunity. However, PVSRIPO infused into syngeneic tumors in human CD155-tg mice may infect stromal cells, including DCs and macrophages, leading to their activation. This would confound *in vivo* experiments to test the importance of tumor-cell intrinsic MDA5 signaling. PVSRIPO treatment of SUM149 breast cancer xenografts in athymic mice triggers intratumoral neutrophil infiltration and upregulation of pro-inflammatory cytokines (Holl et al., 2016). This model serves as an ideal platform to determine the importance of tumor-initiated innate immune responses to PVSRIPO treatment without

confounding factors from infection of other cell types. We are generating MDA5-depleted SUM149 cells using stable lentiviral transduction of shRNA and plan on implanting these cells in athymic mice. By comparing intratumoral neutrophil infiltration, upregulation of ISGs, serum levels of pro-inflammatory cytokines, tumor size, and survival post-treatment of mice with MDA5-depleted and MDA5-WT SUM149 tumors we can determine the influence of MDA5-dependent innate immune activation specifically in tumors.

Oncolysate from cancer cells killed by PVSRIPO potently activates DCs but activation requires direct infection of DCs with PVSRIPO (Brown et al., 2017). We compared the effects of oncolysate from PVSRIPO-treated MDA5-depleted and parental DM6 melanoma cells on DCs by detection of secreted IFN-β and IL-12, both indicators of DC activation (Brown et al., 2017). These experiments showed cytokine upregulation 24 hours post-oncolysate regardless of MDA5-depletion from melanoma cells (Fig. 28). Thus, at this time point, DC activation by PVSRIPO infection likely overshadows other activation signals. It is possible DCs treated with oncolysate from MDA5-positive cells will show earlier or more prolonged activation than oncolysate from MDA5-depleted cells, but further experiments are necessary. It is also possible other cytokines necessary for intratumoral immune cell infiltration are upregulated in an MDA5-dependent, IFN-

independent manner. CXCL10 is a pro-inflammatory cytokine regulated in this way and is a strong chemoattractant signal for a variety of immune cells (Brownell et al., 2014).

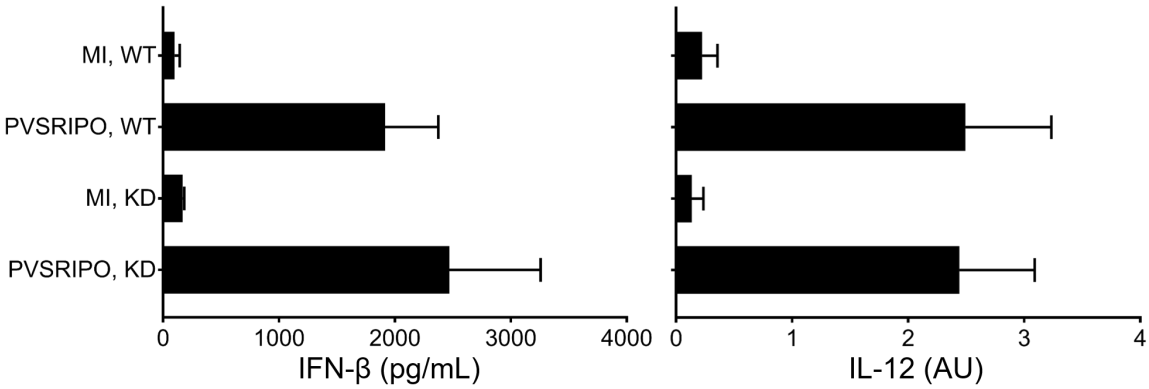

Figure 28: Oncolysate from MDA5 WT and MDA5-depleted DM6 cells activate DCs Oncolysate from MDA5 WT and MDA5-depleted DM6 melanoma cells infected with 0.1 MOI PVSRIPO or MI for 48 hours was collected and incubated with immature human DCs for 24 hours; media from DCs was collected and analyzed by ELISA for IFN-β and IL-12; mean +/- SEM, (n = 3). IL-12 expressed in absorbance units (AU).

PRRs have IFN-dependent and -independent effects on EMCV, and best evidence suggests upregulation of ISGs is a key part of this inhibition. Proteomics and RNA-Seq comparing MDA5-depleted and MDA5 WT cells infected with EMCV or PVSRIPO may provide insights into antiviral mechanisms targeting picornaviruses and identify new antiviral proteins and pathways. MDA5-depleted melanoma cells may not be the ideal model for these experiments, due to low expression levels of the proteins. However, THP-1 cells are susceptible to EMCV and PVSRIPO infection, and can be transduced by lentivirus (Wies et al., 2013). Proteomics and RNA-Seq of EMCV and PVSRIPO infected MDA5-depleted THP-1 cells would therefore serve as an ideal model

to study the range of ISGs differentially regulated under these conditions and would be a starting point to determine differential effects of ISGs on PVSRIPO and EMCV.

This work focuses on MDA5 activation in cancer cells by PVSRIPO infection and the relative impact of this activation on PVSRIPO and EMCV. MDA5 was required for innate immune activation by both viruses, but a combination of the TLR3 agonist poly(I:C) with viral infection was a more potent innate immune agonist. TLR3 has also been linked to innate immune activation by pathogenic PV in mice, despite broad agreement within the field that MDA5 is the primary intracellular sensor of picornavirus infection (Oshiumi et al., 2011). Lysis of PV-infected cells may release TLR3 agonist dsRNA which is then phagocytosed by DCs and serves as a PAMP along with intracellular viral dsRNA (Tatematsu et al., 2013). To determine the importance of TLR3 and MDA5 with PVSRIPO we could use a DC activation system and knock-down or inhibit TLR3 and MDA5 signaling independently. It may also be worth investigating the possibility of combining PVSRIPO with approved TLR3 agonists for improved cancer treatments.

Appendix A

Figure 1 was adapted by permission from John Wiley and Sons: Cancer "Oncolytic polio virotherapy of cancer," Michael C. Brown, Elena Y. Dobrikova, Mikhail I. Dobrikov, Ross W. Walton, Sarah L. Gemberling, Smita K. Nair, Annick Desjardins, John H. Sampson, Henry S. Friedman, Allan H. Friedman, Douglas S. Tyler, Darrell D. Bigner, Matthias Gromeier, © 2014.

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Biography

Ross William Walton was born in Anaheim, CA, USA on November 14, 1986. He spent most of his young years in Lancaster, CA where he was an avid competitive swimmer and Boy Scout. In 2005 he was accepted to the University of California, Berkeley and graduated May 2008 with a Bachelor of Arts degree in Molecular and Cell Biology. After graduating he conducted research in Robert Glaeser's lab at the Lawrence Berkeley National Laboratory on the 3-dimensional structure of single-particle proteins using electron microscopy.

In 2012 Ross was accepted to Duke University in the Molecular Genetics and Microbiology Ph.D. program at Duke University where he joined the lab of Matthias Gromeier. His research revolved around exploring the relationship between the recombinant oncolytic poliovirus PVSRIPO and the innate immune system. His publications are mentioned below. While at Duke Ross received the Duke Center for Virology Travel Award (2017) and the Duke Molecular Genetics and Microbiology Distinguished Fellows Travel Award (2018). Ross was also a Duke Viral Oncology Training grantee (2013-2014). Ross presented his research at the annual meeting of the Society for the Immunotherapy of Cancer (2017) and the inaugural Duke GradX Talks (2014). Ross will graduate in December 2018 with a Ph.D. in Molecular Genetics and Microbiology.

Publications:

Walton RW, Brown MC, Sacco MT, & Gromeier M. (2018). Engineered Oncolytic Poliovirus PVSRIPO Subverts MDA5-Dependent Innate Immune Responses in Cancer Cells. *Journal of Virology*, JVI.00879-18. https://doi.org/10.1128/JVI.00879-18 *in press*

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