Mechanisms of Innate Immune Activation by a Hybrid Alphavirus–Rhabdovirus Vaccine Platform

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Virus-like vesicles (VLV) are infectious, self-propagating alphavirus-vesiculovirus hybrid vaccine vectors that can be engineered to express foreign antigens to elicit a protective immune response. VLV are highly immunogenic and nonpathogenic *in vivo*, and we hypothesize that the unique replication and structural characteristics of VLV efficiently induce an innate antiviral response that enhances immunogenicity and limits replication and spread of the vector. We found that VLV replication is inhibited by interferon (IFN)- α , IFN- γ , and IFN- λ , but not by tumor necrosis factor- α . In cell culture, VLV infection activated IFN production and expression of IFN-stimulated genes (ISGs), such as MXA, ISG15, and IFI27, which were dependent on replication of the evolved VLV-encoded Semliki Forest virus replicon. Knockdown of the pattern recognition receptors, retinoic acid-inducible gene I and melanoma differentiation-associated protein 5 or their intermediary signaling protein mitochondrial antiviral-signaling protein (MAVS) blocked IFN production. Furthermore, ISG expression in VLV-infected cells was dependent on IFN receptor signaling through the Janus kinase (JAK) tyrosine kinases and phosphorylation of the STAT1 protein, and JAK inhibition restored VLV replication in otherwise uninfected cell lines. This work provides new insight into the mechanism of innate antiviral responses to a hybrid virus-based vector and provides the basis for future characterization of the platform's safety and adjuvant-like effects *in vivo*.

Keywords: vesiculovirus, Semliki Forest virus, vesicular stomatitis virus, virus-like vesicle, viral vaccine vector

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Introduction

VACCINATION IS THE most effective preventative treatment option available to combat many infectious diseases and may also be an effective approach for therapeutic intervention to treat chronic infections. Replicating virus-based vaccine platforms have displayed strong immunogenicity and efficacy, but while many live-attenuated and inactivated viral vaccines are successfully utilized in humans today, some have undesirable safety risks (Mort and others 2013). Subunit vaccines are often considered to be a safer option but can produce comparatively weak immune responses without adjuvants (Mort and others 2013).

The virus-like vesicles (VLV) vaccine platform is a capsid-free, self-replicating, antigen expression system that may represent an attractive alternative to other virus-based vectors (Rose and others 2008, 2014; Schell and others 2011; Reynolds and others 2015). VLV encodes a Semliki Forest virus (SFV) replicon and an additional structural protein, the vesicular stomatitis virus glycoprotein (VSV-G) (Rolls and others 1994). Following *in vitro* evolution by 50 passages in BHK-21 cells that led to 10 amino acid changes in SFV nsP1-4, the evolved SFV nonstructural proteins promote high-titer VLV replication in cell culture through increased efficiency of VLV release (Rose and others 2014). VSV-G expression allows for robust and pantropic infectivity, as infectious vesicles composed of SFV replication spherules derived from bulb-shaped plasma membrane invaginations are coated with VSV-G protein and bud from infected cells, spread to uninfected cells, and undergo multiple rounds of infection (Rolls and others 1994; Finkelshtein and others 2013; Rose and others 2014). VLV are non-pathogenic in mice and rhesus macaques, have little risk of genome integration or reversion to pathogenesis, and are immunogenic in the absence of adjuvant (Schell and others 2011; Reynolds and others 2015).

Like VLV, other alphavirus-based vaccine vectors benefit from efficient RNA replication and are utilized as naked RNA replicons, recombinant viral particles, or layered DNA vectors (Lundstrom 2014, 2015). In general, these vectors typically rely on encoded SFV capsid proteins or are cotransfected into cells with helper RNA encoding capsid proteins to facilitate packaging and budding of virus or virus-like particles (Lundstrom 2014, 2015), but some of these approaches are limited to 1 round of infection (Liljestrom and Garoff 1991; Polo and others 2000; Lundstrom 2014, 2019). Whereas all enveloped RNA viruses utilize capsid proteins to package their RNA, VLV replicate and spread without the protection of a capsid, which may promote more direct exposure of viral RNA to pattern recognition receptors (PRRs) and allow for more efficient activation of innate immune mechanisms (Rose and others 2014). Alphavirus non-structural proteins can antagonize interferon (IFN) signaling pathways through multiple mechanisms, including transcription and translation inhibition (Gorchakov and others 2005, 2008; Breakwell and others 2007), but the role of these processes in VLV replication is not known.

VSV-G, the structural component that mediates VLV cellular entry, can confer neurotropism in mice (Chauhan and others 2010; Hastie and others 2013), which is prevented by IFN signaling and antiviral gene activation in the olfactory mucosa and brain (Detje and others 2009; Kalinke

and others 2011; Sorgeloos and others 2013; van den Pol and others 2014). Many neurotropic viruses encode proteins that antagonize the IFN system, such as VSV M, Rabies virus P, and Hendra and Nipah virus P, V, and W, which may contribute to their pathogenesis (Rodriguez and others 2002, 2003; Ahmed and others 2003; Brzozka and others 2005). Interestingly, VLV have no adverse effects when injected directly into the brain of normal mice, but are lethal when injected into the brain of mice lacking the type I IFN receptor, further highlighting the importance of innate immune activation in mediating VLV control and limiting pathogenic effects (van den Pol and others 2017).

The lack of VLV pathogenesis in immunocompetent animals suggests that it is unlikely that the vector effectively evades innate antiviral immunity. Characterizing the induction of innate immune responses and understanding the mechanisms that control VLV replication and spread is essential to provide a definitive understanding of the interaction of the platform with the host immune system. We hypothesized that VLV infection results in robust activation of innate immunity and that VLV replication is highly sensitive to the IFN response.

Materials and Methods

Cells and reagents

All cells were cultured in complete Dulbecco's modified Eagle medium (DMEM) made with the addition of 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mM sodium pyruvate, and 1 × minimal essential media nonessential amino acids. Huh7, BHK-21, A549, BSC-1, and HEK293 cells were seeded into 6-well plates at a density of 3×10^5 cells/well and cultured overnight. Cryopreserved adult primary human hepatocytes (BD Biosciences) were seeded into collagen-coated 6-well plates (Corning Biocoat) at a density of $\sim 5 \times 10^5$ cells/well and cultured overnight before use. The vaccinia virus (VACV) B18R expression plasmid was previously described (Bandi and others 2010). The Human Type I IFN Neutralizing Antibody Mixture (#3900-1) was purchased from PBL Assay Science. Ruxolitinib and Fedratinib were purchased from Advanced Chemblocks and Selleckchem. Recombinant VSV expressing green fluorescent protein (GFP) was provided by Dr. John Rose (Yale University).

VLV production and *in vitro* infection

Sequences encoding GFP or firefly luciferase were cloned into plasmid pCMV-SFVT2AGNJ (provided by Dr. John Rose, Yale University) to create pCMV-SFVGFP2AGNJ and pCMV-SFVLUC2AGNJ, respectively. pCMV-SFVT2AGNJ is a plasmid derived from pCMV-SFVG-P50R (Rose and others 2014) that has been engineered with a *PacI* to *SbfI* cloning site downstream of the SFV subgenomic RNA promoter (Reynolds and others 2015). This plasmid also encodes a ribosomal T2A skipping site downstream of the cloning site but upstream of the VSV glycoprotein. VLV encoding firefly luciferase (VLV-Luc) or enhanced green fluorescence protein (VLV-GFP) (Fig. 1A) were produced using methods described previously (Rose and others 2014). For *in vitro* infection, medium from cells cultured in 6-well

FIG. 1. Type I, II, and III IFNs block VLV replication. **(A)** Diagrams showing genomic organization of VLV-Luc and VLV-GFP. **(B–E)** Huh7 cells were incubated with cytokines at various doses for 18 h in triplicate, infected with VLV, and harvested 18 hpi. Luciferase activity (RLU) following VLV-Luc infection is shown for IFN- α 1, IFN- γ , IFN- λ 1, and TNF- α , respectively. **(F)** Flow cytometry analysis of VLV-GFP-infected Huh7 cells either untreated or pretreated for 18 h with different concentrations of IFN- α 1. For all infections, MOI=0.3. GFP, green fluorescent protein; hpi, hours postinfection; IFN, interferon; MOI, multiplicity of infection; RLU, relative light units; SFV, Semliki Forest virus; TNF, tumor necrosis factor; VLV, virus-like vesicles.

plates was aspirated, and cells were washed with PBS. VLV multiplicity of infection ([MOI]=0.3, 1, or 3) was diluted in serum-free DMEM (200 μ L/well) and added to the cells for 30 min at 37°C. Complete medium was then added and cells were incubated for the time periods indicated in the figure legends.

Luciferase assay

VLV-Luc-infected cells were lysed using 1 $\times$ luciferase lysis buffer (Promega). Luciferase assay reagent was used with an adapted luciferase assay system protocol (Promega). Luciferase assay reagent (100 μ L) and cell lysate (10 μ L) were added simultaneously to a 96-well plate, mixed, and read immediately using a SpectraMax iD3 plate reader (Molecular Devices).

IFN- α , - λ , enzyme-linked immunosorbent assay

The VeriKine Human IFN Alpha (#41100-1) and DIY Human IFN Lambda 1/2/3 (#61840-1) Enzyme-Linked Immunosorbent Assay (ELISA) Kits (PBL Assay Science)

were used as per company assay protocol with media collected from cultured Huh7 cells and human hepatocytes.

RNA purification and measurement of gene expression

Total RNA from cell lines and primary cells was harvested and isolated at the time points postinfection indicated in the figure legends using an RNeasy Mini Kit (Qiagen). The RNA concentration was measured with a Nanodrop ND-1000 Spectrophotometer. Preparation of cDNA was done with equal amounts of total RNA from each sample with a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene expression was measured using reverse transcriptase quantitative polymerase chain reaction (RT-qPCR) normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression and quantified by the $\Delta\Delta C_T$ method using a StepOnePlus real-time PCR system (Applied Biosystems). The primer sequences for RT-qPCR using SYBR Green reagents were as follows: GAPDH, 5'-AAG TAT GAC AAC AGC CTC AAG ATC-3' (sense) and 5'-CTG

TGG TCA TGA GTC CTT C-3' (antisense); interferon lambda 1 (IFN- λ 1), 5'-CTT CCA AGC CCA CCC CAA CT-3' (sense) and 5'-GGC CTC CAG GAC CTT CAG C-3' (antisense); MXA, 5'-ACA GGA CCA TCG GAA TCT TG-3' (sense) and 5'-CCC TTC TTC AGG TGG AAC AC-3' (antisense); IFI27, 5'-CCG TAG TTT TGC CCC TGG-3' (sense) and 5'-CGA GGC GAT TCC CGC CGC-3' (antisense); and ISG15, 5'-AAT GCG ACG AAC CTC TGA GC-3' (sense) and 5'-CAG CCA GAA CAG GTC GTC-3' (antisense). TaqMan[®] Reverse Transcriptase reagents and TaqMan[®] Universal PCR Master Mix (Applied Biosystems) were used with assay-on-demand probes and primers for IFN- α 1 (Hs03044218_g1), IFN- α 2 (Hs00265051_s1), IFN- α 5 (Hs04186137_sH), IFN- β 1 (Hs01077958_s1), C-X-C motif chemokine 10 (CXCL10, Hs00171042_m1), nitric oxide synthase 2 (Hs01075529_m1), guanylate-binding protein 1 (GBP1, Hs00988005_m1), serum amyloid A2 (Hs00761940_s1), serpin family a member 3 (Hs00153674_m1), and haptoglobin (Hs00605928_g1).

Viral RNA transfection

Huh7 cells were infected with VSV (MOI=10, 6 h) or VLV (MOI=10, 12 h), and RNA was harvested and quantified as previously described. Total RNA was transfected into freshly seeded Huh7 cells using the *TransIT*-mRNA Transfection Kit (Mirus Bio).

siRNA transfection

Silencer select predesigned siRNAs specific for retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated factor 5 (MDA5), mitochondrial antiviral-signaling protein (MAVS), stimulator of IFN genes (STING), and negative control #1 siRNA (Ambion) were transfected into Huh7 cells at 200 pmol/well using Lipofectamine 2000 (2 μ L/well) for 6 h at 37°C. Following aspiration, cells received complete DMEM (2 mL/well) and were incubated for 48 h before infection with VLV-Luc for 18 h.

SDS-PAGE and Western blot analysis

Protein samples from Huh7 cells infected with VLV-Luc were collected in 2 $\times$ Laemmli sample buffer (Bio-Rad) +5% BME and separated by 10% SDS-PAGE. Separated proteins were transferred to nitrocellulose membranes, which were blocked with PBS, 0.05% Tween 20, and 5% nonfat dry milk for 1 h at room temperature. Blocked membranes were incubated overnight with the specified primary antibody at 4°C. Primary antibodies included a 1:500 dilution of rabbit anti-phospho-STAT1 (Tyr701) [#9171], a 1:1,000 dilution of rabbit anti-STAT1 [#9172] from Cell Signaling Technology, and a 1:1,000 dilution of goat anti-actin (I-19) [sc-1616] from Santa Cruz Biotechnology. Secondary antibody incubation was carried out with anti-rabbit [phosphorylated STAT1 (pSTAT1), STAT1, phosphorylated STAT3 (pSTAT3), STAT3] and anti-goat (actin) horseradish peroxidase-labeled antibodies. Clarity western ECL reagent (Bio-Rad) was used for detection with a ChemiDoc MP Imaging System (Bio-Rad). Images were acquired using Image Lab software.

Statistical analysis

All data were analyzed using GraphPad Prism software (Version 7). All data are expressed as mean $\pm$ standard error of

the mean. Student's *t*-test was used to determine statistical significance, shown as *P* value below 0.05. **P*<0.05; ***P*<0.01; and ****P*<0.001; not significant (n.s.). Log transformations were performed for some non-normally distributed data sets.

Results

VLV are sensitive to type I, II, and III IFN

IFN- α/β and IFN- λ act on unique receptors and have a variety of antiviral effects that inhibit viral replication (Kotenko and others 2003; Malmgaard 2004; Odendall and Kagan 2015). However, the potential for *in vitro* study of IFN activity against VLV is limited, as the BHK-21 cell line that best supports VLV propagation has defective IFN responses (Otsuki and others 1979). The Huh7 human hepatocyte-derived carcinoma cell line is also somewhat susceptible to VLV infection and produces and responds to IFNs (Melen and others 2000; Pagliaccetti and others 2008), although the IFN pathway is partially impaired in these cells (Keskinen and others 1999; Melen and others 2000). Huh7 cells therefore served as an ideal model for this work as they uniquely support VLV replication (although less efficiently than BHK-21 cells) and express IFN and IFN-stimulated genes (ISGs) following viral infection. Huh7 cells were pretreated with IFN- α 1, IFN- γ , IFN- λ 1, or tumor necrosis factor (TNF)- α for 18 h and subsequently infected with VLV-Luc, a vector that expresses a luciferase reporter (Fig. 1A). Luciferase activity was reduced in a dose-dependent manner following IFN- α 1, IFN- γ , or IFN- λ 1 treatment relative to untreated cells (Fig. 1B–D), with at least a 50% decline being achieved at IFN concentrations as low as 1 U/mL (IFN- α 1, IFN- γ) or 1 ng/mL (IFN- λ 1). This inhibition is similar to the antiviral activity of these cytokines against other viruses, such as HCV and VSV in Huh7 cells (Pagliaccetti and others 2008). In contrast, treatment with TNF- α did not affect luciferase activity (Fig. 1E). Similar to VLV-Luc, we also observed a dose-dependent reduction in GFP⁺ cells pretreated with various concentrations of IFN- α 1 followed by infection with the VLV-GFP vector (Fig. 1A, F). Consistent with our hypothesis, the reduction in luciferase activity and proportion of GFP⁺ cells show that IFNs inhibit VLV replication.

VLV primarily induce IFN- λ in Huh7 cells and primary human hepatocytes

While nearly all cells respond to IFN- α/β , only epithelial cells of the intestinal tract, respiratory tract, and reproductive barriers, hepatocytes, and blood/brain barrier, respond to IFN- λ (Lazear and others 2015; Kotenko and Durbin 2017). To investigate the ability of VLV to activate an antiviral response, Huh7 cells were infected with VLV or VSV for 6 and 18 h, and the induction of type I and III IFN was measured by an RT-qPCR-based assay and ELISA. VSV is an enveloped virus that, like VLV, encodes the structural protein VSV-G and is used in this study for comparison. Infection over an 18-h period with VLV, but not VSV, stimulated IFN- λ 1 mRNA expression (Fig. 2A, B). At 6 and 18 hpi, the relative mRNA levels of multiple type I IFN subtypes, including IFN- α 2, 5, and IFN- β 1 in cells infected with VLV and VSV (Fig. 2C), showed a similar pattern as IFN- α 1 (Fig. 2A, B), with a limited increase in expression

FIG. 2. VLV primarily induce IFN- λ in Huh7 cells and primary hepatocytes. Relative expression of IFN- λ 1 and IFN- α 1 mRNA was measured by RT-qPCR at the indicated time points in (A) VLV-Luc- and (B) VSV-GFP-infected Huh7 cells. (C) Relative expression of type I IFN subtypes (α 2, α 5, β 1) was measured at 6 and 18 hpi in uninfected, VSV-, or VLV-infected cells. (D) IFN- λ and IFN- α protein in the media of Huh7 cells infected with VLV or VSV was quantified by ELISA. (E) Primary human hepatocytes were infected with VLV-Luc or left uninfected (-) and harvested 18 hpi. Relative fold change in mRNA expression of IFN- α 1 and IFN- λ 1 was measured using RT-qPCR (left y-axis), and IFN- α and IFN- λ protein expression was measured by ELISA (right y-axis). For all infections, MOI=1 for VLV and 0.1 for VSV. * p < 0.05; ** p < 0.01; *** p < 0.001. ELISA, enzyme-linked immunosorbent assay; LOD, limit of detection; ND, not detected; PHH, primary human hepatocytes; RT-qPCR, reverse-transcriptase quantitative polymerase chain reaction; VSV, vesicular stomatitis virus.

FIG. 3. Unlike VSV, VLV infection does not antagonize IFN- λ production. IFN- λ 1 mRNA expression in Huh7 cells 10 h after (A) VSV-GFP infection (MOI=0.2) or transfection of total RNA from VSV-infected (MOI=10, 6 h) Huh7 cells, or (B) VLV infection (MOI=1) or transfection of total RNA from VLV-infected (MOI=10, 12 h) Huh7 cells. (C) GFP mRNA expression in Huh7 cells 10 h after VSV infection (MOI=0.2) or transfection of total RNA from VSV-infected (MOI=10, 6 h) Huh7 cells. Relative fold change in mRNA expression was measured using RT-qPCR. *** p < 0.001.

at 6 hpi for VLV and 18 hpi for VSV. At 18 hpi, IFN- λ protein was also substantially increased following infection with VLV, but not VSV (Fig. 2D). In contrast, IFN- α protein was modestly elevated by infection with VSV, but was below the assay limit of detection following VLV infection (Fig. 2D), consistent with IFN gene expression (Fig. 2A, B).

To confirm the results observed in the Huh7 cell line, we also determined if VLV activate IFN- λ production in non-transformed primary human hepatocytes. Following VLV infection, IFN- λ 1 mRNA expression and protein production were both increased (Fig. 2E). In parallel to the findings in Huh7 cells, primary human hepatocytes displayed no detectable evidence of IFN- α/β mRNA or protein expression following VLV infection (Fig. 2E). Collectively, these results display the capability of VLV to activate an IFN response, at least in hepatocyte-derived cells.

Unlike VSV, VLV infection does not antagonize IFN- λ production

VLV are composed of VSV-G and an evolved SFV replicon (Fig. 1A) and unlike VSV, induce IFN- λ expression following infection of Huh7 cells (Fig. 2). VSV likely evades innate immune activation by actively antagonizing IFN production, as evasion is lost in the absence of viral proteins (Du and others 2016). To investigate if VLV employ a similar evasion mechanism, we used a previously established strategy to identify the IFN induction activity of VLV (Rehwinkel and others 2010; Feng and others 2012; Du and others 2016). Huh7 cells were infected with either VSV or VLV, and total RNA from infected cells was collected, purified, and transfected into uninfected Huh7 cells. As expected (Du and others 2016), transfection of VSV RNA elicited a robust IFN- λ response, whereas VSV

FIG. 4. VLV infection promotes the expression of ISGs and IFN-induced inflammatory mediators. Huh7 cells were infected with VLV-Luc, treated with IFN- α 1 (100 U/mL) or IFN- λ 1 (10 ng/mL), or not infected (-). Relative fold change in mRNA expression of the ISGs (A) MXA, (B) IFI27, and (C) ISG15 was measured 18 h later. (D) Relative fold change in IFN- λ 1, MXA, ISG15, and IFI27 mRNA expression 48 and 72 h after VLV-Luc infection. (E) Primary human hepatocytes were infected with VLV-Luc or left uninfected (-) and harvested at 18 hpi. (F) Huh7 cells were infected with VLV-Luc or not infected (-), and expression of IFN-induced inflammatory mediators CXCL10, iNOS, and GBP1 and acute phase proteins α 1-antichymotrypsin, serum amyloid A, and haptoglobin was measured. In all experiments, relative fold change in mRNA expression was measured using RT-qPCR. For all infections, MOI = 1. * p < 0.05; *** p < 0.001. CXCL10, C-X-C motif chemokine 10; GBP1, guanylate-binding protein 1; iNOS, inducible nitric oxide synthase; ISGs, IFN-stimulated genes; n.s., not significant.

infection did not (Fig. 3A). Expression of GFP in infected and transfected cells confirmed the infection and transfection of the cells by VSV-GFP (Fig. 3C). In contrast to VSV, infection and transfection of VLV RNA induced comparable IFN- λ expression (Fig. 3B), implying that unlike VSV, VLV do not efficiently interfere with the IFN- λ activation machinery.

VLV promote the expression of ISGs and IFN-induced inflammatory mediators

The autocrine/paracrine loop of IFN signaling increases the expression of antiviral ISGs (Schneider and others 2014; Schoggins 2014). To identify VLV-mediated activation of the antiviral state, we measured the mRNA fold change of the representative ISGs MXA, ISG15, and IFI27 following VLV infection of Huh7 cells. Compared with exogenous IFN treatment, infection with VLV similarly induced MXA, IFI27, and ISG15 mRNA expression (Fig. 4A–C). Because IFN- λ can lead to prolonged ISG activation (Bolen and others 2014), we also examined gene expression at longer time points after VLV infection. MXA, ISG15, and IFI27 all remained substantially increased at 48 and 72 hpi, along with IFN- λ 1 (Fig. 4D). To confirm ISG induction in primary cells, the experiments were repeated using primary human hepatocytes, and infecting these cells with VLV also increased MXA, ISG15, and IFI27 mRNA expression (Fig. 4E). Therefore, VLV infection triggers antiviral gene expression in both transformed and primary cells.

To further explore the innate immune responses elicited by VLV infection, we analyzed the mRNA expression of proinflammatory mediators that are activated by IFN signaling (Saha and others 2010), as well as other inflammatory factors (Gabay and Kushner 1999). The results showed that IFN-induced mediators, such as CXCL10 (4,830 mean fold induction), inducible nitric oxide synthase (526-fold induction), and IFN-induced GBP1 (1,385-fold induction), were potently expressed following VLV infection (Fig. 4F). In contrast to IFN-induced mediators, the expression of acute-phase proteins (APPs) α 1-antichymotrypsin (32-fold induction), serum amyloid A (22-fold induction), and haptoglobin (no induction), which are primarily activated by IL-6 in the liver in response to injury or inflammation (Gabay and Kushner 1999), were only weakly activated following VLV infection (Fig. 4F). Taken together, these results suggest a putative role of the IFN signaling pathway in controlling VLV replication, as opposed to other IFN-independent innate pathways such as APPs.

VLV-mediated ISG expression is primarily driven by IFN- λ

Because IFN- α is a more potent inducer of ISG expression and antiviral activity in Huh7 cells compared with IFN- λ (Marcello and others 2006; Pagliaccetti and others 2008; Bolen and others 2014), it is conceivable that a small amount of IFN- α produced following VLV infection is responsible for stimulating a relatively large proportion of ISG expression. We therefore determined the extent to which

FIG. 5. VLV-mediated ISG expression is primarily driven by IFN- λ . (A–C) HEK293 cells were transfected with plasmid expressing the secreted VACV B18R IFN- α / β -binding protein or empty vector control. (A) Huh7 cells were treated with conditioned media from the transfected cells and IFN- α 1 (100 U/mL), IFN- γ (100 U/mL), or IFN- λ 1 (10 ng/mL), and MXA mRNA expression was measured at 24 h post-treatment. (B, C) Huh7 cells were treated with conditioned media from the transfected cells, and infected with VLV (MOI=0.3) and (B) MXA or ISG15 mRNA and (C) luciferase activity (RLU) was measured 24 h after infection. (D) Cells were uninfected (–) or infected with VLV (MOI=1) and concurrently treated with a cocktail of IFN- α / β - and IFN- α / β R-blocking antibodies at the indicated dilutions, and MXA and ISG15 mRNA expression was measured 18 h after infection. In all experiments, relative mRNA fold change was measured by RT-qPCR. ** p < 0.01; *** p < 0.001. VACV, vaccinia virus.

FIG. 6. UV inactivation of VLV abrogates IFN and ISG induction. VLV were inactivated using 200 mJ/cm² UV light, and Huh7 cells were infected with VLV-Luc, UV-inactivated VLV, or UV-treated serum-free DMEM. (A) UV abrogation of VLV replication verified by luciferase assay. (B–E) Relative fold change in mRNA expression of (B) IFN-λ1, (C) MXA, (D) ISG15, and (E) IFI27 was measured 18 hpi using RT-qPCR. For all infections, MOI=1. **p*<0.05; ***p*<0.01. DMEM, Dulbecco's modified Eagle medium; UV, ultraviolet.

ISG expression is dependent upon either IFN-α or IFN-λ produced after VLV infection. VACV B18R is a secreted IFN-α/β-binding protein (Alcami and others 2000) that does not inhibit IFN-λ (Huang and others 2007; Bandi and others 2010). HEK293 cells were transfected with a B18R expression vector or empty vector, and media were transferred to Huh7 cells with IFN treatment (Fig. 5A) or VLV infection (Fig. 5B, C). As expected, B18R blocked IFN-α- but not IFN-λ- or IFN-γ-mediated ISG expression in Huh7 cells (Fig. 5A). In comparison, the overall ISG fold induction following VLV infection, although reduced, remained substantial even in the presence of B18R (Fig. 5B), and was associated with only a small increase in VLV replication (Fig. 5C). To further confirm these findings, VLV infection of Huh7 cells was also performed in the presence of a cocktail of IFN-α/β-neutralizing antibodies, which showed a similar significant induction in ISG expression even in the presence of the antibodies (Fig. 5D). Because IFN-λ and IFN-α have additive cooperative activities on ISG expression in Huh7 cells (eg, an ~50-fold increase in ISG15 expression from IFN-λ plus ~3-fold increase from IFN-α would equal an ~150-fold total increase) (Pagliaccetti and others 2008), these results imply that the majority of ISG induction following VLV infection is mediated by IFN-λ rather than IFN-α/β, consistent with the predominance of IFN-λ induction (Fig. 2).

VLV replication is required for IFN and ISG induction

The process of VLV RNA replication, which occurs in the absence of capsid proteins in the cytoplasm, may be re-

sponsible for robust innate immune activation. Hence, we hypothesized that nonreplicating VLV would fail to activate the IFN and ISG response. To determine if antiviral gene activation requires VLV replication, we used an ultraviolet (UV) crosslinking treatment to inactivate VLV. Media ± VLV-Luc were exposed to 200 mJ/cm² UV light, and the abolishment of replication following UV treatment was shown by a loss of luciferase activity in VLV-Luc-infected Huh7 cells (Fig. 6A). In contrast to untreated VLV, UV-treated, nonreplicating VLV failed to induce expression of IFN-λ1, MXA, IFI27, and ISG15 mRNA in Huh7 cells (Fig. 6B–E). Therefore, VLV replication is required to activate the IFN response.

Cytosolic PRRs and MAVS are required for VLV-mediated induction of IFN and ISG

PRRs respond to nucleic acids and stimulate the production of antiviral IFNs. RIG-I and MDA5 specifically respond to viral RNA and induce IFN production following VSV and SFV infection (Melchjorsen 2013; Chan and Gack 2016). To provide insight into the mechanism of cellular recognition of VLV and subsequent activation of IFN and ISG, we determined the role of RIG-I and MDA5 in this process by utilizing siRNA to ablate the expression of these proteins. Knockdown of either RIG-I or MDA5 was sufficient to significantly block VLV-mediated induction of IFN-λ1 and MXA, and cotransfection of both siRIG-I and siMDA5 exhibited a cooperative effect (Fig. 7A). VLV infection following siRNA transfection was verified by measuring luciferase mRNA expression (Fig. 7B), which as expected was increased following PRR knockdown, indicative of enhanced VLV replication (Fig. 7B).

FIG. 7. RIG-I, MDA5, and MAVS knockdown abrogates VLV-mediated induction of IFN and ISG. Huh7 cells were transfected with 200 pmol siRNA to knockdown RIG-I, MDA5, RIG-I and MDA5 (100 pmol each siRNA), MAVS, STING, or negative control (siCTRL). Knockdown of (A) RIG-I, MDA5, and RIG-I + MDA5 was followed by VLV-Luc infection and % relative expression of (A) IFN-λ1 and MXA and (B) Luciferase was measured. Knockdown of (C) MAVS or (D) STING was followed by VLV-Luc infection and % relative expression of IFN-λ1 and MXA were measured. (E) Expression of each gene following siRNA knockdown was measured to verify efficiency. *Dashed lines* indicate that fold mRNA change is measured relative to siCTRL, which represents 100% gene expression. All infections were done 48 h after siRNA transfection, MOI=1. * $p < 0.05$; *** $p < 0.001$. MAVS, mitochondrial antiviral-signaling protein; MDA5, melanoma differentiation associated factor 5; RIG-I, retinoic acid-inducible gene I; siCTRL, negative control siRNA; STING, stimulator of IFN genes.

In the IFN pathway, the downstream intermediary signaling molecule, MAVS, can be activated by both RIG-I or MDA5 to stimulate IFN and ISG induction (Vazquez and Horner 2015). To determine its role in VLV-mediated IFN and ISG expression, we used siRNA specific for MAVS. Knockdown of MAVS blocked IFN-λ1 and MXA mRNA expression in VLV-infected cells (Fig. 7C). IFN induction by cytosolic DNA is activated by the cyclic-GMP-AMP synthase/stimulator of IFN genes (cGAS-STING) pathway (Ma and Damania 2016). STING knockdown failed to block IFN-λ1 or MXA mRNA expression in VLV-infected cells (Fig. 7D). The efficiency of siRNA knockdown of each gene was verified (Fig. 7E). We conclude that VLV is recognized by cytosolic PRRs RIG-I and MDA5 in Huh7 cells, which signal downstream through MAVS to activate the IFN response.

Janus kinase signaling is required for VLV-mediated STAT1 phosphorylation and ISG induction

Both the IFN-α receptor (IFNAR) and IFN-λ receptor (IFNLR) signal through intracellular Janus kinase (JAK) tyrosine kinases, which phosphorylate STAT proteins to

activate ISG expression (Odendall and Kagan 2015). To determine if ISG induction by VLV is signaled downstream of IFNAR and IFNLR through pSTAT1, we examined pSTAT1 in VLV-infected Huh7 cells by Western blot. No pSTAT1 was detectable at 6 hpi, regardless of MOI. However, at 18 hpi, a weak pSTAT1 signal is detectable at MOI=1 and a stronger signal is detected after infection at MOI=3 (Fig. 8A). In comparison, VLV infection failed to activate pSTAT3 (Fig. 8B).

Both IFNAR and IFNLR utilize JAK1 and TYK2 kinases to activate ISG expression, whereas in some cell types, IFN-λ can additionally activate JAK2 (Lee and others 2012; Odendall and others 2014). To determine if VLV-mediated activation of ISG relies on JAK signaling, Huh7 cells were treated with the small molecule JAK inhibitors, Ruxolitinib and Fedratinib (JAK1/2- and JAK2-specific, respectively) before VLV infection (Curran and others 2017; Febvre-James and others 2018). Treatment with either Ruxolitinib or Fedratinib was sufficient to block STAT1 phosphorylation 18 hpi at MOI=3 (Fig. 8C). VLV-mediated induction of MXA, but not IFN-λ1, mRNA was significantly inhibited following Ruxolitinib treatment (Fig. 8D), and VLV

FIG. 8. JAK inhibition abrogates VLV-mediated STAT1 phosphorylation and ISG induction. Western blot analysis of Huh7 cell protein expression of (A) pSTAT1 and STAT1, and (B) pSTAT3 and STAT3 following infection with VLV (MOI and infection time are shown), treatment with IFN- α (100 U/mL) or no infection (-, serum-free DMEM). (C) pSTAT1 and STAT1 protein expression following VLV infection (MOI=3) for 18 h in the presence of JAK inhibitors Rux (1 μ M) or Fed (1 μ M). Relative fold change in mRNA expression of (D) IFN- λ 1 and MXA and (E) Luciferase were measured using RT-qPCR in Huh7 cells infected with VLV-Luc (MOI=3) in the presence of Rux (1 μ M), vehicle control (10 μ L serum-free DMEM), or not infected (-). ** $p < 0.01$; *** $p < 0.001$. Fed, Fedratinib; JAK, Janus kinase; pSTAT1, phosphorylated STAT1; pSTAT3, phosphorylated STAT3; Rux, Ruxolitinib.

FIG. 9. Inhibition of JAK signaling enhances VLV replication in otherwise resistant cells. Cells were treated with +Rux (1 μ M), +Fed (1 μ M), or serum-free DMEM, infected with VLV-Luc, and harvested 18 hpi. Luciferase activity (RLU) following VLV-Luc infection is shown for cell types (A) BHK, (B) A549, (C) BSC-1, and (D) HEK293. For all infections, MOI=3. All differences compared to the no drug control were statistically significant, $P < 0.001$, except (A) BHK-labeled n.s. BHK, baby hamster kidney.

infection was verified by measuring luciferase mRNA expression (Fig. 8E). This result was expected as JAK signaling occurs downstream of IFN expression but upstream of ISG activation. It is unclear if IFNAR, IFNLR, or both receptors signal through JAK1/2 to stimulate ISG in this system; however, the predominance of IFN- λ induction (Fig. 2) and significant ISG induction in the presence of type I IFN inhibitors (Fig. 5) suggests that IFNLR may play the primary role in these cells. Altogether, these results show that VLV-induced JAK signaling leads to STAT1 phosphorylation and ISG activation.

Inhibition of JAK signaling enhances VLV replication in otherwise resistant cells

VLV robustly infect and replicate in baby hamster kidney (BHK) cells (Fig. 9A), a cell line known to lack a functional IFN response (Otsuki and others 1979). Cell lines known to express IFNs in response to viral infection, such as human lung (A549), African green monkey kidney (BSC-1), and human kidney (HEK293) fail to support VLV replication (Fig. 9B–D). To determine if IFN signaling inhibition would permit VLV replication in uninfected cell lines, we infected A549, BSC-1, and HEK293 cells in the presence of JAK inhibitors, Ruxolitinib or Fedratinib. Inhibition of JAK signaling resulted in increased VLV replication in each of these cells (Fig. 9B–D). As expected, due to the IFN insensitivity of BHK cells, treatment with Ruxolitinib or Fedratinib had no effect on VLV replication in these cells (Fig. 9A). This finding shows that VLV replication is blocked in these cell lines by a mechanism utilizing JAK signaling, likely the IFN response.

Discussion

The advantages of VLV as a vaccine platform include strong immunogenicity and a lack of pathogenesis *in vivo* (Rose and others 2008, 2014; Schell and others 2011; Reynolds and others 2015). Previous research on VLV has primarily focused on the ability of this platform to induce adaptive immunity and has not addressed the cellular and molecular mechanisms of immunogenicity and pathogenesis. As an infectious, yet capsid-free, hybrid viral vector, it has previously been speculated that VLV may resemble the primitive viruses that existed before the evolution of protective capsid and nonstructural proteins, such as the VSV nucleocapsid and matrix proteins (Rose and others 2014). The relatively simple, capsid-free design of VLV may leave the vector particularly susceptible to innate recognition by cytosolic PRRs.

In Huh7 cells, we initially determined that type I, II, and III IFN reduce VLV replication in a dose-dependent manner. VLV appeared to be more sensitive to IFN- α/β compared with IFN- λ . This is not surprising, as IFN- α is thought to act rapidly and to more potently stimulate ISG production at its peak, whereas the antiviral effects of IFN- λ are longer lasting but relatively weaker (Zhou and others 2007; Bolen and others 2014; Jilg and others 2014). Collectively, these results highlight the ability of IFN to control VLV replication and suggest why IFN-responsive cell lines may be resistant to VLV replication *in vitro*.

Alphavirus nonstructural proteins inhibit the IFN system (Gorchakov and others 2005, 2008; Breakwell and others

2007), but the potent induction of IFN and ISGs after VLV infection compared with VSV infection implies a lack of significant anti-IFN activity. Interestingly, during initial development, VLV were evolved by passage through IFN-defective BHK cells and accumulated various mutations in the SFV ns proteins, including nsP1 and 2 (Rose and others 2014). These mutations may have resulted in the loss of anti-IFN activity displayed by WT SFV, and may contribute to VLV safety and immunogenicity. Notably, research studying the anti-IFN activity of SFV ns proteins has focused on IFN- α/β responses and has not considered the impact on IFN- λ production. The substantial overlap between the 2 antiviral IFN pathways may suggest that SFV ns proteins have some capacity to blunt both type I and III IFN responses, but this has not been studied.

Cytosolic RNA sensors RIG-I and MDA5 were both found to be necessary for the production of an optimal IFN and ISG response to VLV infection, while DNA sensors, cGAS-STING, were dispensable. In general, RIG-I binds short dsRNA (<300 bp), and MDA5 binds long dsRNA (>1,000 bp) (Reikine and others 2014). However, in some cases, both play a role in nucleic acid recognition. For example, both RIG-I and MDA5 activate IFN signaling in Rotavirus-infected intestinal epithelial cells (Broquet and others 2011). It remains to be determined if both PRRs directly recognize VLV RNA or have alternative, indirect roles in the activation of IFN production. In addition, the contribution of other PRRs such as Toll-like receptors to VLV sensing may differ in other cell types.

VLV induce more IFN- λ than IFN- α/β in Huh7 cells and primary human hepatocytes. This bias toward IFN- λ may be explained by the preferential expression of IFN- λ by human epithelial cells and hepatocytes in response to viral infection (Lazear and others 2015; Kotenko and Durbin 2017). However, VSV infection elicited an IFN- α -specific response in Huh7 cells and failed to activate any detectable IFN- λ expression. Notably, VSV produces proteins known to antagonize IFN production, such as matrix (M) protein that can blunt the IFN response by blocking nuclear export of cellular mRNA (Ahmed and others 2003). In contrast to VSV infection, the transfection of VSV RNA induced the production of IFN- λ , showing that VSV proteins likely block IFN- λ production during infection.

Type I and type III IFN are activated by common cytosolic receptors, including RIG-I and MDA5, but different downstream mechanisms might explain their differential activation by VSV and VLV. Through its C-terminal transmembrane domain, MAVS can anchor into the mitochondrial, peroxisome, or endoplasmic reticulum membranes and signal through IRF3/NF- κ B/p38 to activate IFN- α/β and IFN- λ (Seth and others 2005; Dixit and others 2010; Odendall and others 2014). To promote signaling MAVS must be membrane bound, suggesting that its localization differentially regulates IFN induction (Vazquez and Horner 2015). Independent of IRF3/NF- κ B/p38, human intestinal epithelial cells can signal through peroxisomal MAVS (MAVS-pex) and IRF1 to induce IFN- λ , whereas mitochondrial MAVS (MAVS-mito) and Erk activate IFN- α/β (Odendall and others 2014). VLV infection may promote MAVS-pex, rather than MAVS-mito accumulation, resulting in a predominance of type III IFN expression.

An adjuvant-like effect of VLV *in vivo* may be attributed to the potential of viral RNA to be readily recognized by

innate receptors, stimulate antiviral genes, and create an immunogenic environment upon infection. IFN signaling can contribute to the humoral and cellular adaptive responses, such as the differentiation, maturation, and function of dendritic cells, and the activation, expansion, and memory formation of CD8⁺ T cells (Kadowaki and Liu 2002; Egli and others 2014). Intentional stimulation of the RIG-I pathway by synthetic RIG-I agonists intended to act as adjuvants has been studied to activate inflammatory immune responses and increase the immunogenicity of vaccines and cancer immunotherapies (Nielsen and others 2017). We found that VLV intrinsically activate ISG and proinflammatory mediator expression. One such mediator, CXCL10, recruits T cells and dendritic cells and promotes T cell adhesion and is critical for dendritic cell-mediated development of CD8⁺ T cell responses (Dufour and others 2002; Padovan and others 2002; Majumder and others 2012). It is possible that CXCL10 or other VLV-induced mediators are responsible for driving immunogenicity and antigen-specific protective responses *in vivo*, perhaps by providing a link between the innate and adaptive immune systems (Tough 2004; Kolumam and others 2005; Pantel and others 2014).

In IFN-responsive cell lines, blocking ISG activation through inhibition of JAK signaling allowed for enhanced VLV replication in cells that are otherwise resistant to infection. Manipulating uninfected cell lines to support VLV replication could allow for more expansive *in vitro* study and may have implications for large-scale VLV production. Importantly, this work validates the idea that VLV are IFN stimulators in different cell types, and these cytokines in turn negatively regulate VLV replication. Although unstudied, VLV spread *in vivo* is also likely dictated, at least in part, by IFN expression. A further understanding of the impact of infection route on vector spread and control will be necessary to determine vaccination targets and strategies using this platform.

Future investigation remains an important step to the application of VLV as a vaccine platform in humans. In this study, we have outlined a mechanism of innate immune stimulation and control mediated by this capsid-free self-replicating virus-based vector. These new insights improve our understanding of VLV/host interactions and can be used to support the design and optimization of future treatments utilizing this system. The scope of this work is limited to cell culture study and now warrants a transition to *in vivo* study to better understand the tropism, spread, and control of VLV infection, and to characterize the link between innate stimulation and the development of adaptive immune responses.

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Author Disclosure Statement

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