

**Identification and mechanistic  
characterization of a  
noncanonical host-modulating  
function of *Cardiovirus* VPg**

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## Summary

The *Picornaviridae* family is composed of positive-sense, single-stranded RNA (ssRNA) viruses and is well-known for its lytic nature. *Picornaviridae* encode a very small viral protein called 3B, which is also known as viral protein genome-linked (VPg). Until now, VPg has been recognized primarily as a crucial protein essential for viral RNA synthesis, functioning as a primer using the viral ssRNAs as templates.

*Cardiovirus* is a genus within the *Picornaviridae* family that includes the encephalomyocarditis virus (EMCV). Using EMCV as a model, this study identified that EMCV-infected host cells release two critical factors that synergize to induce potent apoptosis. The first factor is host-derived inflammatory cytokine tumor necrosis factor alpha (TNF- $\alpha$ ), which is induced after sensing viral double-stranded RNA. The second factor is EMCV-derived VPg, which is produced during the EMCV life cycle and released extracellularly. This study unexpectedly discovered that VPg from EMCV (VPg<sup>EMCV</sup>), as well as VPg from other *Cardioviruses*, can function as an agonist of the interferon gamma receptor (IFNGR). This “noncanonical” host-responsive function of VPg is specific to the genus *Cardiovirus*, and is not conserved in other genera.

Mechanistically, VPg<sup>EMCV</sup> activates the canonical JAK-STAT1 signaling pathway through IFNGR1/2, mimicking the canonical function of interferon gamma (IFN- $\gamma$ ). This activation leads to STAT1 phosphorylation and induction of interferon stimulated genes (ISGs) such as interferon regulatory factor 1 (IRF-1). IRF-1 induction then promotes inducible nitric oxide synthase (iNOS) expression, leading to nitric oxide (NO) accumulation. NO accumulation sensitizes cells to Caspase 8-dependent cell death, which is ultimately triggered by TNF- $\alpha$  signaling through TNF receptor 1 (TNFR1). Furthermore, VPg<sup>EMCV</sup> binds directly to IFNGR and induces an expression profile very similar to IFN- $\gamma$ , while also downregulating a unique set of genes. Functionally, this NO-dependent cell death attenuates EMCV load both *in vitro* and *in vivo*. In addition, VPg<sup>EMCV</sup> exhibits cross-species activity in both humans and mice, and can act as an antiviral agent through its IFN- $\gamma$ -like properties, exhibiting protective effects against other RNA viruses in both *in vitro* and *in vivo* models.

These findings indicate that VPg<sup>EMCV</sup>, a 20-amino acid viral protein, functions not only in viral RNA synthesis but also as a host-responsive cytokine mimic. The establishment of these findings should open avenues for future research in both fundamental biology and potential clinical applications.

## **Chapter 1**

### **Introduction and Background**

## 1-1 Innate immunity and cell death

Living organisms have acquired several mechanisms to defend themselves against pathogens. Higher mammals, such as humans, possess multiple layers of defense against infection and disease, including both innate and acquired immunity. While both immune systems exist, this research will focus primarily on innate immunity.

Innate immunity functions as the initial defense system against foreign pathogens, prior to activation of acquired immunity, including both physical barriers and host-mediated responses. Although acquired immunity has been studied for a longer period, research into innate immunity expanded rapidly beginning in the 21st century [1]. When a pathogen, such as a virus, enters and infects the body, pattern recognition receptors (PRRs) recognize specific molecular patterns derived from the pathogen, often referred to as pathogen-associated molecular patterns (PAMPs). These signals then propagate rapidly to activate the first line of host defense mechanisms against invading pathogens [1, 2].

One of the most well-known pathways activated by viral infection is the RIG-I-like receptor (RLR) pathway, which serves as a major sensor of RNA viruses and ultimately leads to the production of type I interferons (IFNs) and inflammatory cytokines. This pathway involves receptors such as retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5), and plays a critical role in innate antiviral defense [3]. Another well-known family of receptors is the Toll-like receptor (TLR) family. TLRs are localized either on the cell membrane (e.g., TLR1, TLR2, TLR4, TLR6) or within endosomes (e.g., TLR3, TLR7, TLR9) and are activated by a wide range of foreign pathogens. Each TLR recognizes distinct PAMPs, and activation of these receptors ultimately induces various immune responses, including antiviral defense and inflammatory signaling [4]. Although additional innate immune pathways exist, such as the cyclic GMP-AMP synthase–stimulator of interferon genes (cGAS–STING) pathway, which primarily recognizes DNA viruses and danger signals [5], these pathways will not be discussed further, as they are not directly relevant to this study.

Next, cell death and its well-established mechanisms will be described. Cell death can broadly be classified into regulated and non-regulated (accidental) forms. Regulated cell death is a tightly controlled process that is often initiated as a final defensive measure by host cells. Apoptosis, the most prominent form of programmed

and regulated cell death, is essential for maintaining homeostasis. Programmed cell death generally occurs because it is beneficial to the organism, often functioning as a “last resort” protective mechanism [6]. This process is distinct from necrosis, which is a non-regulated form of cell death that occurs when cells are injured and die prematurely [7]. Other forms of regulated cell death have also been identified, including necroptosis, a programmed form of necrotic cell death [8], ferroptosis, an iron-dependent form of cell death [9], and others. This research focuses primarily on the regulation of apoptosis, which is the most well-characterized form of programmed cell death and was observed in this study.

One defining feature of apoptosis is its dependence on caspases [6]. During apoptosis, cells undergo morphological changes, including cell shrinkage and nuclear fragmentation, which distinguish this process from necrotic cell rupture. Apoptosis is considered one of the most common mechanisms by which cells are eliminated under physiological conditions [6].

Caspases are a family of conserved cysteine proteases. They can be broadly classified into three categories: initiator caspases, executioner caspases, and inflammatory caspases [10]. In mammals, initiator caspases (Caspases 2, 8, 9, and 10) function in the initiation of apoptosis and in the activation of executioner caspases (Caspases 3, 6, and 7), which ultimately execute apoptosis [10]. Inflammatory caspases (Caspases 1, 4, 5, 11) serve roles in inflammatory signaling and can also induce another form of programmed cell death known as pyroptosis [10]. Caspases are activated through proteolytic cleavage of their inactive precursor forms, known as pro-caspases. Upon receiving death stimuli, caspases undergo cleavage and trigger a cascade of downstream events that commonly result in cell death [10].

Two major apoptotic pathways have been described: the intrinsic pathway, which is cell-autonomous, and the extrinsic pathway, which is initiated by extracellular signals and is basically cell-non-autonomous. In the intrinsic apoptotic pathway, mitochondria play a central role. Apoptotic signals converge on mitochondria through multiple mechanisms, and members of the B-cell lymphoma 2 (Bcl-2) family of proteins serve as key regulators of this process [11]. Anti-apoptotic members of this family include Bcl-2 and B-cell lymphoma-extra large (Bcl-xL), whereas pro-apoptotic members, such as Bcl-2 homologous antagonist/killer (BAK) and Bcl-2-associated X protein (BAX), are essential for the induction of apoptosis [11]. Activation of BAK and BAX leads to the release of cytochrome c through the mitochondrial apoptosis-induced

channel (MAC) [12]. Cytochrome c then binds to apoptotic protease activating factor 1 (APAF-1), together with ATP and pro-Caspase 9, forming a complex known as the apoptosome [28]. The apoptosome then mediates cleavage of pro-Caspase 9 into its active form, which then activates Caspase 3 to trigger the apoptotic signaling cascade [11].

In the context of viral infection, cell death can occur either as a consequence of viral-mediated shutdown of host transcription and translation, or as part of the host antiviral response. In many cases, host-induced cell death functions to limit viral replication and reduce viral load. During viral infection, host-induced cell death is often considered to be cell-autonomous and is primarily mediated through intrinsic apoptosis [13].

In contrast, the extrinsic apoptotic pathway enables cells to induce apoptosis in nearby cells through extracellular signaling, functioning like a form of danger signaling. Several ligands can trigger extrinsic apoptosis, including tumor necrosis factor alpha (TNF- $\alpha$ ), Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL) [14]. In the TNF- $\alpha$ -mediated pathway, TNF- $\alpha$  binds to its main receptor, tumor necrosis factor receptor 1 (TNFR1). However, multiple inhibitory mechanisms suppress TNF- $\alpha$ -mediated cell death under most conditions, making TNF- $\alpha$ -induced apoptosis rather uncommon [15]. One hallmark feature of extrinsic apoptosis is the formation of the death-inducing signaling complex (DISC), which includes Fas-associated death domain protein (FADD) and Caspase 8 [15]. Extrinsic apoptosis can also be induced during viral infection, as infected cells produce inflammatory cytokines that promote cell death in surrounding cells [16].

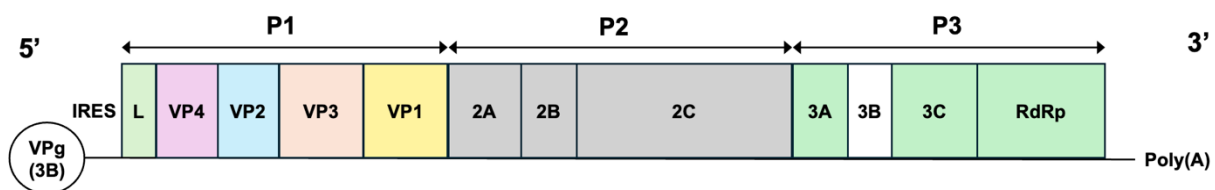
## **1-2 *Picornaviridae* and EMCV-associated host/viral pathways**

### *1-2-1 Overview of Picornaviridae and EMCV*

*Picornaviridae* are a family of non-enveloped, single-stranded, positive-sense RNA viruses. The virion is approximately 30–32 nm in diameter and contains a single ssRNA genome of approximately 6.7 - 10.1 kb in length [17]. The viral capsid consists of 60 protomers, each composed of 4 structural polypeptides: VP1, VP2, VP3, and VP4 [17]. The name *Picornaviridae* is derived from the word “pico,” meaning very small,

combined with “RNA,” reflecting the small size of these RNA viruses [17]. Members of the *Picornaviridae* family primarily infect vertebrates, although the specific host range depends on the virus species [17].

*Picornaviridae* possess a highly conserved genome and share similar protein compositions and life cycles across different genera [17]. The viral genomic RNA is covalently linked at its 5' end to a small viral protein, 3B, also known as viral protein genome-linked (VPg) [17], which will be described in detail later. Downstream of VPg, the long 5' untranslated region (UTR) contains an internal ribosome entry site (IRES) that mediates cap-independent translation [17]. The coding region of the genome is divided into three major regions, P1, P2, and P3, which encode different viral proteins following translation and proteolytic cleavage of a single polyprotein [17]. The P1 region encodes the structural capsid proteins, whereas the P2 and P3 regions encode nonstructural proteins that primarily function in viral replication and host interaction [17], as described later. At the 3' end of the genome, a shorter untranslated region plays an important role in negative-strand RNA synthesis, followed by a poly(A) tail that is heterogeneous in length [17]. Although certain proteins, such as the leader (L) protein, are present in some but not all *Picornaviridae* members, the overall genome structure is largely conserved across the virus family [17]. The basic structure of the *Picornaviridae* genomic RNA is shown below as **Figure 1-1**.



**Figure 1-1. Basic structure of the *Picornaviridae* genomic RNA.**

The *Picornaviridae* family comprises multiple genera, including *Cardiovirus*, *Enterovirus*, *Hepatovirus*, *Rosavirus*, and many others [17]. Two of these genera, *Enterovirus* and *Cardiovirus*, will be explained in detail for their relevance to this study.

*Enterovirus* include many well-known human pathogens associated with a wide range of clinical symptoms. These include *Enterovirus A*, which contains coxsackievirus A (CVA) and enterovirus 71 (EV71); *Enterovirus B*, which includes

viruses such as coxsackievirus B3 (CVB3); and *Enterovirus C*, which includes the poliovirus (PV), among many others [17]. Currently, more than 84 *Enterovirus* types have been identified as causes of human disease, with major clinical syndromes including poliomyelitis, herpangina, and hand-foot-and-mouth disease (HFMD) [17, 18].

In contrast, *Cardiovirus* are less known in terms of human disease. This genus contains relatively few viruses, and most are not considered as major human pathogens compared with *Enterovirus*, although some possess zoonotic potential [19, 20]. Until now, 6 *Cardiovirus* species (*Cardiovirus A*, *B*, *C*, *D*, *E*, and *F*) have been identified [17]. *Cardiovirus A*, also known as the mengovirus, includes the encephalomyocarditis virus (EMCV) [19], which is the primary virus utilized in this study and will be discussed in detail later. *Cardiovirus B* includes viruses such as Theiler's murine encephalomyelitis virus (TMEV), which primarily causes disease in mice [21], and Saffold virus (SAFV), which was the first *Cardiovirus* isolated from human stool samples [22]. SAFV is therefore considered as a rare *Cardiovirus* with the potential to cause severe disease in humans [22]. The crystal structures of EMCV, TMEV, and SAFV have been elucidated in previous studies [23, 24].

Among these viruses, EMCV is the primary focus of this study and was first isolated in 1945 [19]. EMCV primarily infects domestic animals such as pigs and is thought to spread efficiently via rodents or bats [25]. Experimental infection of mice with EMCV induces severe clinical symptoms, including paralysis, with prominent infection of the heart and brain [19, 26]. Although zoonotic transmission of EMCV has been reported, such cases are considered relatively rare [19]. Therefore, research on EMCV has historically been more prevalent in agricultural and veterinary contexts, rather than in studies of human pathology [19].

### *1-2-2 Life cycle of EMCV*

Members of the *Picornaviridae* family follow a conserved life cycle [17], and in this study, the life cycle is described using the encephalomyocarditis virus (EMCV) as a representative model. Infection begins when the EMCV virion attaches to an entry receptor on the host cell surface [19]. Although the specific receptor varies among *Picornaviridae*, several membrane proteins have been reported to serve as functional

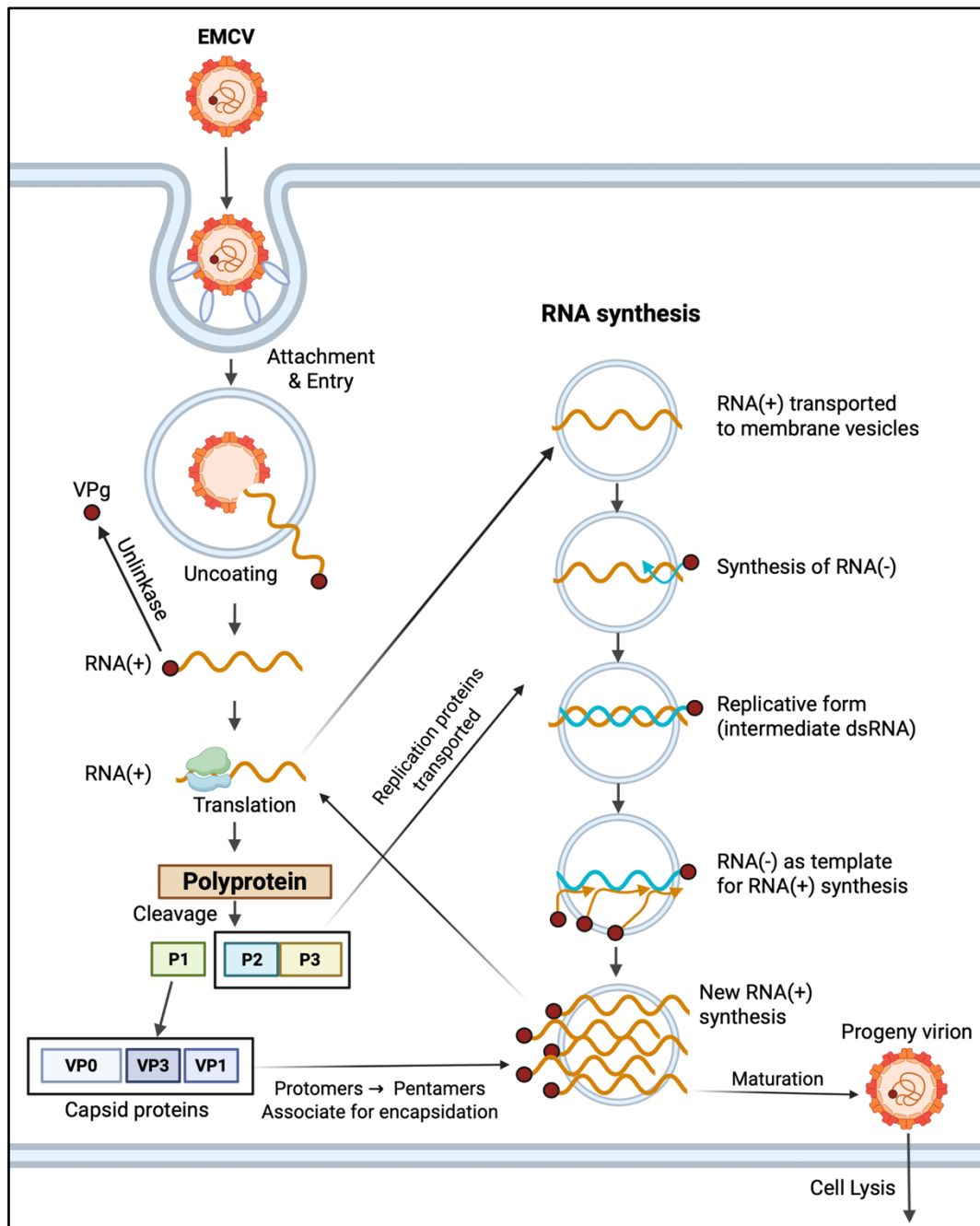
receptors for EMCV [27]. Among these proteins, ADAM metallopeptidase domain 9 (ADAM9) has been identified as an important receptor for EMCV entry [27, 28].

Following receptor binding and viral entry, uncoating occurs and the viral genomic RNA is released into the cytoplasm [19]. At this stage, VPg is cleaved from the 5' end of the viral RNA by a host enzyme known as VPg unlinkase, which has been recently identified as a protein named tyrosyl-DNA phosphodiesterase 2 (TDP2) [29, 30]. The positive-sense viral RNA (RNA(+)) then functions directly as mRNA and is translated into a single polyprotein [19]. This polyprotein is then cleaved by virus-encoded proteases, and the resulting precursor proteins are further processed into mature functional viral proteins [19]. These include many functional proteins, which the known functions of some of these proteins derived from polyprotein processing will be described in detail later.

At this stage of replication, a new VPg functions as a primer for viral RNA synthesis [19]. VPg is modified by the attachment of pUpU at the third tyrosine (Y) residue, enabling initiation of RNA synthesis [31]. Using this mechanism, RNA(+) is replicated to produce negative-sense RNA (RNA(-)) [19]. The synthesis of RNA(-) results in the formation of a double-stranded RNA (dsRNA) intermediate known as the replicative form (RF) [19, 32]. The RNA(-) then serves as a template for the synthesis of additional RNA(+) genomes [19].

The newly synthesized RNA(+) can follow multiple routes/fates: they can (1) be translated again following VPg cleavage, (2) serve as templates for further rounds of viral RNA replication, or (3) be packaged into progeny virions [19]. In encapsidated genomes, VPg is attached to the 5' end of viral positive-sense ssRNA, allowing the virus to initiate the same life cycle following infection of a new host cell after cell lysis [19].

Capsid proteins VP0, VP1, and VP3 can assemble to form a protomer [17, 19]. Five protomers then assemble into a pentamer, and twelve pentamers will further assemble to form what is called an icosahedral capsid [17, 19]. During virion maturation, the precursor protein VP0 is cleaved into VP2 and VP4, resulting in the formation of mature progeny virions, which are ultimately released from infected cells through cell lysis [19]. Host cell lysis is the dominant mechanism for virion release and is a hallmark of *Picornaviridae* infection [17, 19]. The life cycle of EMCV is shown as **Figure 1-2** below.



**Figure 1-2. Schematic illustration of the EMCV life cycle.**

(This figure was created using BioRender.)

### 1-2-3 Host response during EMCV infection, and EMCV proteins

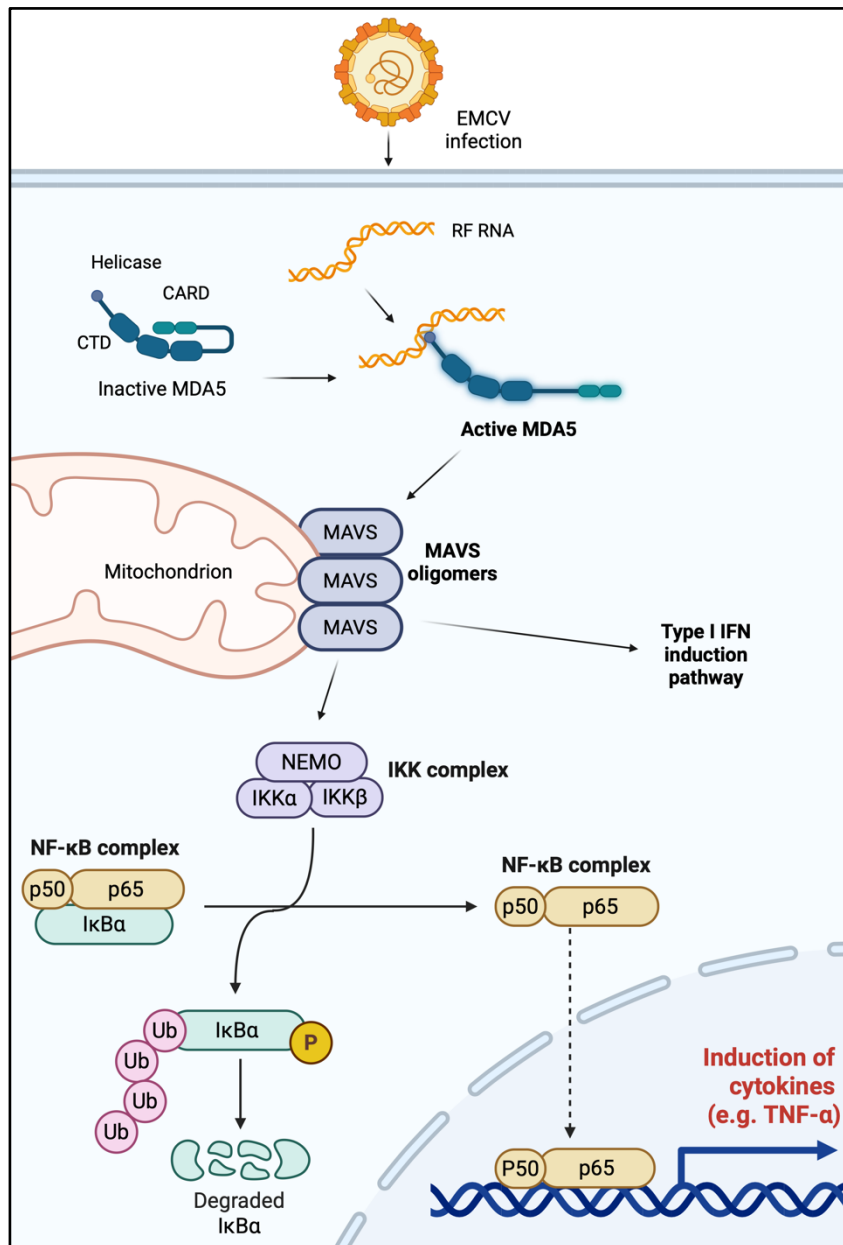
EMCV is frequently used as a model RNA virus in immunology research, as its viral dsRNA is well known to be sensed primarily by MDA5 [33]. This is relatively uncommon, as more RNA viruses are sensed by RIG-I rather than MDA5 [33]. MDA5

mainly recognizes long dsRNA and shares similar domains with RIG-I, including the caspase activation and recruitment domain (CARD) at the N-terminus, a central RNA helicase domain, and a C-terminal domain (CTD) [33]. Previous studies have demonstrated that the RF dsRNA generated during the EMCV life cycle is recognized as a PAMP by MDA5 [32, 33].

Upon recognition of viral dsRNA, MDA5 is known to undergo a conformational change that exposes its CARD domain [34], enabling downstream signaling through a protein called mitochondrial antiviral signaling protein (MAVS), which is localized to the mitochondrial membrane [35]. Once activated, MAVS is known to form oligomers/aggregates [36].

Downstream signaling from MAVS diverges into two major pathways. One pathway leads to the induction of type I IFNs and inflammatory cytokines through activation of TANK-binding kinase 1 (TBK1) and inducible I $\kappa$ B kinase (IKKi), mediated by adaptor molecules such as tumor necrosis factor receptor-associated factors (TRAFs) [37]. TBK1 and IKKi play critical roles in the phosphorylation of interferon regulatory factors 3 and 7 (IRF-3 and IRF-7), which then dimerize and translocate into the nucleus [38]. This step is essential for the transcriptional induction of type I IFNs following viral infection [38].

Another major signaling pathway activated downstream of MAVS is the nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $\kappa$ B) pathway. This pathway is highly dependent on the inhibitor of nuclear factor kappa-B kinase (IKK) complex, which consists of the catalytic subunits IKK $\alpha$  and IKK $\beta$ , as well as the regulatory subunit NF- $\kappa$ B essential modulator (NEMO), also known as IKK $\gamma$  [39]. The IKK complex phosphorylates I $\kappa$ B $\alpha$ , leading to its degradation and release of the NF- $\kappa$ B subunits p50 and p65 (also known as RelA) [39]. The activated NF- $\kappa$ B complex then translocates into the nucleus, where it binds to NF- $\kappa$ B response elements and induces inflammatory cytokines such as interleukin 6 (IL-6) and TNF- $\alpha$  [39]. Detailed information regarding TNF- $\alpha$  signaling will be provided in the next section, as it is highly relevant to this research. The innate immune signaling pathways activated by EMCV infection and MDA5-mediated recognition of RF dsRNA, with a focus on NF- $\kappa$ B activation and TNF- $\alpha$  induction, is summarized in **Figure 1-3**.



**Figure 1-3.**  
Innate immune  
signaling pathways  
induced by  
EMCV infection,  
with a focus on the  
NF-κB pathway.

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BioRender.)

Functional proteins derived from EMCV will also be described here. As explained earlier, during the EMCV life cycle, a large polyprotein is synthesized and cleaved into functional proteins. The protein composition of EMCV is similar to that of other members of the *Picornaviridae* family (see **Figure 1-1** for the general genomic RNA structure of *Picornaviridae*). In total, 13 functional proteins have been identified for EMCV [19]. These include the L protein (7.9 kDa), followed by the P1 region, which encodes VP4 (7.3 kDa), VP2 (28.6 kDa), VP3 (25.1 kDa), and VP1 (30.5 kDa) [19, 40]. The P2 region encodes the proteins 2A (16.9 kDa), 2B (17 kDa), and 2C (36.5 kDa) [19, 40]. In addition, a variant form of 2B, known as the 2B\* protein and composed of

129 amino acids, is produced as a result of programmed ribosomal frameshifting, although its function remains unknown [19, 40]. Finally, the P3 region encodes proteins 3A (9.9 kDa), 3B/VPg (2.3 kDa), 3C (21.6 kDa), and 3D (51.9 kDa) [19, 40].

Although it is beyond the scope of this section to describe the functions of all EMCV proteins in detail, several key examples will be highlighted. The VP1-VP4 proteins primarily function as structural components of the viral capsid, as described earlier [19, 40]. Protein 2C possesses ATPase activity and plays an important role in viral replication [41, 42]. Protein 3B/VPg serves as a primer for viral RNA synthesis and is essential for viral genome replication [19, 40]. The 3C protein, often referred to as 3C<sup>pro</sup>, functions as a protease that is critical for processing the polyprotein into mature viral proteins [19, 43]. The 3D protein, often referred to as 3D<sup>pol</sup>, functions as the viral RNA polymerase and is essential for viral RNA replication [19, 44].

Several EMCV proteins have also been reported to modulate host functions. EMCV, like many members of the *Picornaviridae* family, is known to strongly suppress innate immune responses, particularly type I IFN signaling [45, 46]. In the context of EMCV infection, for example, protein 2C [45] and the structural protein VP2 [46] have been reported to suppress type I IFN responses and function as IFN- $\beta$  antagonists. Other EMCV proteins have been reported for modulation of other host defense mechanisms, such as cell death [47-51]. The L and 2A proteins are well-known to inhibit apoptosis in infected host cells [47-49]. The 2B protein has been reported to activate the NLR family pyrin domain-containing 3 (NLRP3) inflammasome [50], which is a critical mediator of Caspase 1 activation and is also associated with pyroptotic cell death. Furthermore, the 2C and 3D proteins have been reported to induce autophagy [51]. Altogether, these observations indicate that EMCV encodes multiple proteins that manipulate host defense systems, resulting in complex host-pathogen interactions.

### **1-3 TNF- $\alpha$ and its associated signaling pathways**

TNF- $\alpha$ , as explained previously, is an inflammatory cytokine belonging to the TNF family and is mainly induced upon activation of the NF- $\kappa$ B signaling cascade. TNF- $\alpha$  is produced mainly by macrophages, but it is also expressed by many other cell types, including lymphocytes, NK cells, neutrophils, mast cells, and even neurons [52, 53]. As briefly mentioned earlier, the primary physiological role of TNF- $\alpha$  is not the

induction of apoptosis, but rather the regulation of immune responses. TNF- $\alpha$  is strongly associated with inflammatory responses such as fever, and dysregulation of TNF- $\alpha$  signaling has been implicated in a wide range of pathological conditions, including Alzheimer's disease [54], cancer [52], depression [55], and inflammatory bowel disease [56].

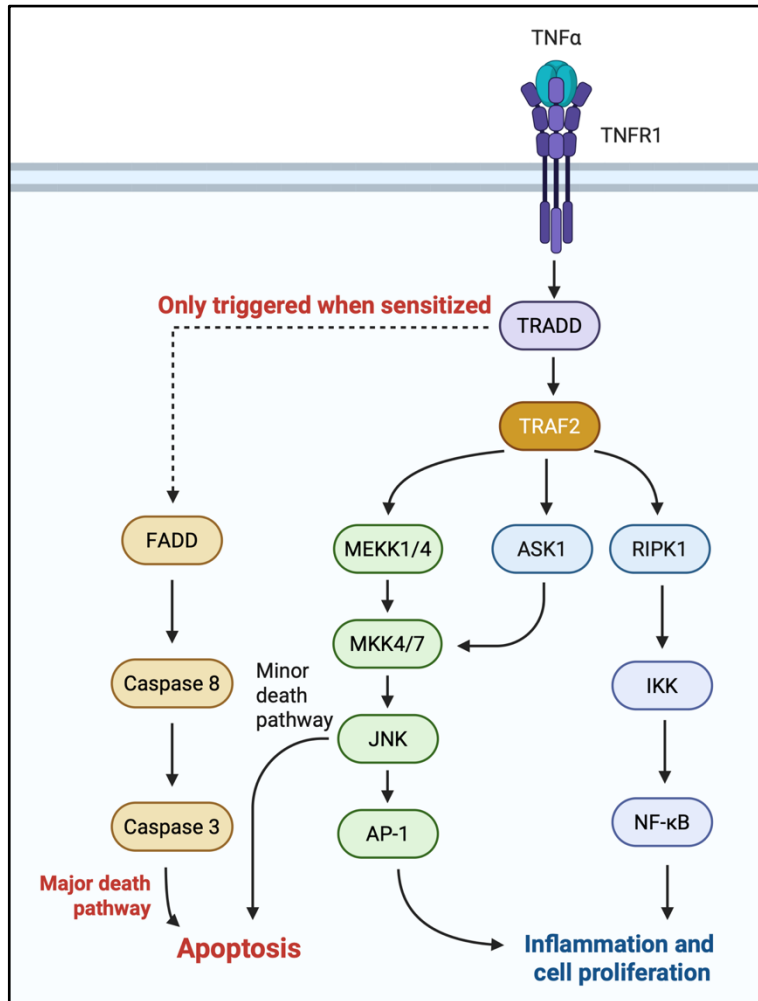
TNF- $\alpha$  exists in two major forms: a soluble form (sTNF- $\alpha$ ) with a molecular weight of approximately 17 kDa and a membrane-bound form (mTNF- $\alpha$ ) with a molecular weight of approximately 26 kDa [57, 59]. sTNF- $\alpha$  is generated by cleavage of mTNF- $\alpha$  [57, 59]. Both forms bind to 2 different receptors, TNFR1 and TNFR2 [52, 58], and TNF- $\alpha$  is known to function in a biologically active homotrimeric state upon receptor binding [59, 129]. However, among these receptors, TNFR1 is mainly associated with the induction of cell death via the extrinsic apoptotic pathway [52, 58, 62]. Therefore, in the context of this study, only downstream signaling pathways of TNFR1 will be discussed in detail. TNF- $\alpha$  activity can be neutralized therapeutically using monoclonal antibodies such as infliximab or a dimeric fusion protein, etanercept (also known as ENBREL), which consists of the soluble TNFR2 extracellular domain fused to the Fc region of an IgG1 antibody, and is known to bind TNF- $\alpha$  with high affinity [60].

Following binding of TNF- $\alpha$  to TNFR1, multiple downstream signaling pathways are activated through recruitment of adaptor proteins to the receptor. A central adaptor molecule in this process is TNFR1-associated death domain protein (TRADD). 3 major signaling pathways downstream of TRADD are commonly described [61, 62]. The first pathway involves activation of the NF- $\kappa$ B signaling cascade, forming a positive feedback loop that enhances inflammatory cytokine induction. In this pathway, TRADD recruits TNF receptor-associated factor 2 (TRAF2) and receptor-interacting protein kinase 1 (RIPK1), a serine-threonine kinase, leading to activation of the IKK complex and NF- $\kappa$ B signaling [61, 62]. This pathway primarily promotes inflammatory responses, cell survival, and cell proliferation, and is generally considered anti-apoptotic [61, 62].

The second pathway involves activation of the mitogen-activated protein (MAP) kinase signaling cascade. This pathway includes activation of MAP kinase kinases (MAP3Ks), such as apoptosis signal-regulating kinase 1 (ASK1) or MEKK1/4, which then activate downstream MAP kinase kinases, including MKK4/7, leading to activation of the c-Jun N-terminal kinase (JNK) pathway [61-63]. Activation of JNK

results in phosphorylation of transcription factors such as c-Jun and activating transcription factor 2 (ATF2), which regulate genes involved in cell differentiation and proliferation [61-63]. In the MAP kinase pathway, downstream activator protein 1 (AP-1) can also induce inflammatory cytokines such as TNF- $\alpha$  [64]. Under certain contexts, JNK activation has also been associated with the induction of apoptosis, although this pathway is generally not considered the dominant mechanism for TNF- $\alpha$ -induced cell death [63, 65].

The third pathway is the death signaling pathway downstream of TNFR1. In this pathway, TRADD associates with Fas-associated death domain protein (FADD), leading to recruitment and activation of Caspase 8 [61, 62]. Activated Caspase 8 can initiate downstream activation of executioner caspases such as Caspase 3/Caspase 7, resulting in apoptotic cell death [66]. However, under most physiological conditions, this pathway is suppressed due to the strong anti-apoptotic signaling mainly mediated by NF- $\kappa$ B and some other survival pathways, and therefore TNF- $\alpha$ -induced apoptosis is generally considered minor unless cells are sensitized by alterations in cell death regulatory mechanisms [61, 62]. The major TNF- $\alpha$  signaling pathways downstream of TNFR1 are summarized in **Figure 1-4**.



**Figure 1-4.**  
**Downstream signaling**  
**pathways activated by**  
**TNFR1.**

(This figure was created using  
BioRender.)

## 1-4 IFN- $\gamma$ and its receptor complex

### 1-4-1 Comparison of type I and type II IFNs, and biological functions of IFN- $\gamma$

Another important component of this study is interferon gamma (IFN- $\gamma$ ), which is introduced briefly in this section. IFN signaling can be broadly classified into 3 types. Type I IFNs include several subtypes, such as IFN- $\alpha$ , IFN- $\beta$ , IFN- $\epsilon$ , IFN- $\omega$ , and some others [67, 68]. In contrast, type II IFNs consist solely of IFN- $\gamma$  [67, 68]. There is also a third category, type III IFNs, which include interferon-lambda (IFN- $\lambda$ ) [69]; however, this group will not be discussed further, as it is not directly relevant to this study.

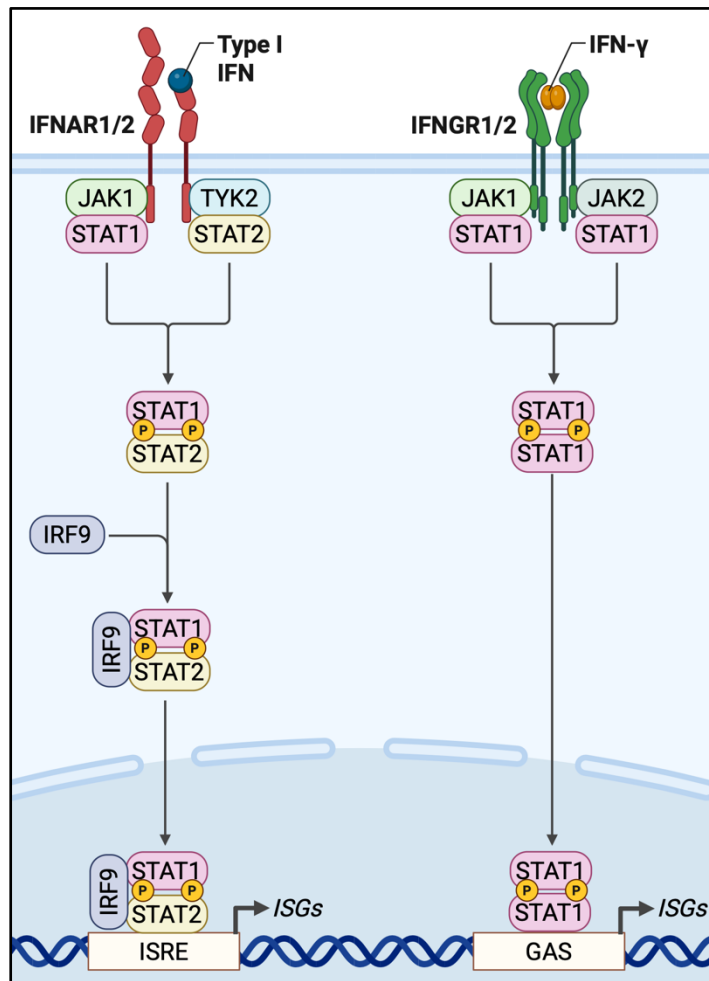
Type I IFNs, particularly IFN- $\alpha$  and IFN- $\beta$ , are well known for their role in inducing antiviral responses through the induction of interferon-stimulated genes

(ISGs) early during viral infection [5, 67, 68]. In contrast, although type II interferons (IFN- $\gamma$ ) share the ability to induce antiviral responses, their receptor usage, downstream signaling mechanisms, and the cell types responsible for their production are quite distinct. IFN- $\gamma$  is predominantly produced by natural killer (NK) cells and natural killer T cells upon activation of these cells [70, 71], while type I IFNs also function to activate NK cell responses, indicating a close relationship between type I IFNs and type II IFNs [68, 70, 71].

The receptor for type I IFNs is called the type I interferon receptor (IFNAR), which consists of two subunits, IFNAR1 and IFNAR2 [67, 72]. This receptor complex is distinct from the type II IFN receptor, known as the interferon gamma receptor (IFNGR), which is composed of the receptor subunits IFNGR1 and IFNGR2 [68, 73]. Despite the differences in receptor composition, both IFNAR and IFNGR activate a common downstream signaling pathway known as the Janus kinase–signal transducer and activator of transcription (JAK-STAT) signaling pathway [67, 68].

Although the overall signaling pathways are similar, there are clear mechanistic differences between type I and type II IFN signaling. IFNAR signaling utilizes the kinases JAK1 and tyrosine kinase 2 (TYK2), and involves both STAT1 and STAT2 [67, 68]. Upon binding of type I IFNs to the IFNAR1/2 complex, JAK1 and TYK2 are activated, leading to phosphorylation of STAT1, primarily at tyrosine 701 (Y701), and STAT2, primarily at tyrosine 690 (Y690) [67, 68]. Phosphorylated STAT1 and STAT2 then associate with interferon regulatory factor 9 (IRF9) to form a complex known as interferon-stimulated gene factor 3 (ISGF3) [67, 68]. This complex translocates into the nucleus, where it binds to interferon-stimulated response elements (ISREs) in the genome to induce the expression of ISGs [67, 68].

In contrast, IFNGR signaling does not involve TYK2, but instead utilizes JAK1 and JAK2 [67, 68, 73]. Activation of IFNGR leads to phosphorylation of STAT1, but not STAT2, leading to the formation of STAT1 homodimers [67, 68, 73]. Recruitment of IRF9 is also not required in IFNGR signaling [67, 68]. The phosphorylated STAT1 homodimer directly translocates into the nucleus, where it binds to gamma-activated sequence (GAS) elements in the genome and induces ISGs [67, 68]. A schematic comparison of type I and type II interferon signaling pathways is shown in **Figure 1-5**.



**Figure 1-5. Comparison of downstream signaling components between type I and type II IFN signaling.**

(This figure was created using BioRender.)

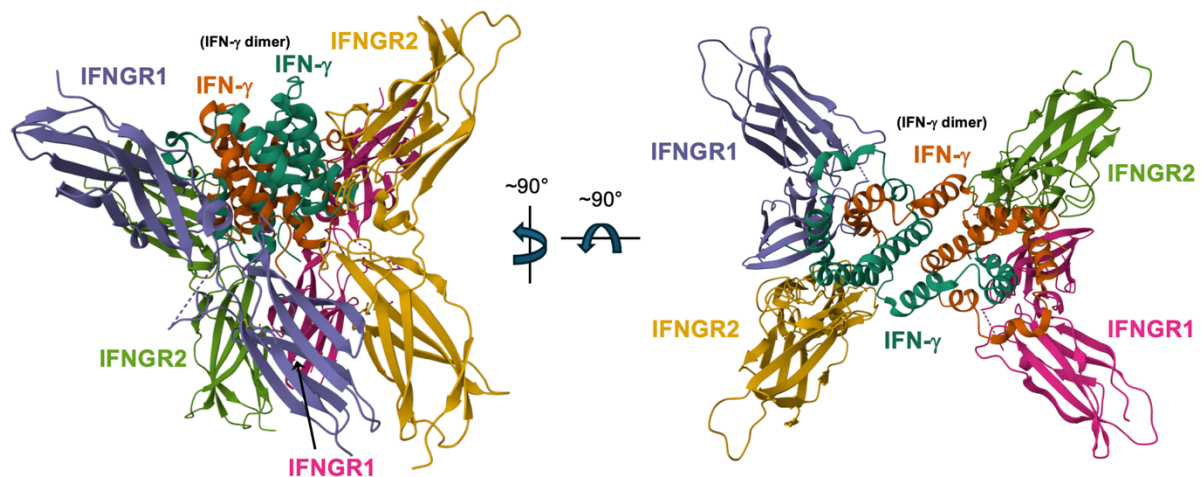
Although both type I and type II interferons induce ISGs, the expression profiles differ substantially, resulting in distinct biological outcomes [67, 68]. As for the context of this study, only the biological functions of type II IFN signaling will be discussed. One feature of IFN- $\gamma$  signaling is the strong induction of interferon regulatory factor 1 (IRF-1), a transcription factor that plays key roles in immune regulation and apoptosis [74, 75]. IRF-1 can further bind to ISREs and promote the expression of additional ISGs, which includes inducible nitric oxide synthase (iNOS) [74, 76]. Induction of iNOS then leads to increased production of nitric oxide (NO), which exerts diverse effects on host cells [74, 76]. NO has been reported to either inhibit or promote cell death depending on its concentration and context [74, 76, 77]. High levels of NO beyond physiological ranges are associated with cytotoxic effects [77], and recent studies have reported synergistic interactions between NO and inflammatory cytokines such as TNF- $\alpha$  [78, 79].

Beyond this example, IFN- $\gamma$  signaling regulates a wide range of additional biological functions. These include promotion of dendritic cell maturation [80],

upregulation of major histocompatibility complex (MHC) class I and class II expression on antigen-presenting cells (APCs) to enhance T cell activation [80, 81], increased phagocytic activity in macrophages [82], and induction of programmed death-ligand 1 (PD-L1) expression in neutrophils [83] and myeloid-derived suppressor cells [84], which serves as a ligand for the immune checkpoint receptor PD-1 [83, 84]. Although NK cells and natural killer T cells are major producers of IFN- $\gamma$ , paracrine signaling from CD4<sup>+</sup> and CD8<sup>+</sup> T cells further amplify IFN- $\gamma$  production during immune responses [74, 85]. IFN- $\gamma$  is also recognized as a critical mediator of anti-tumor immunity, contributing to cancer cell elimination through mechanisms that include apoptosis induction via the JAK-STAT1–IRF-1 signaling axis [74, 86]. Altogether, IFN- $\gamma$  represents a key cytokine with multifarious roles in host defense and immune homeostasis.

#### *1-4-2 The IFN- $\gamma$ -IFNGR receptor complex*

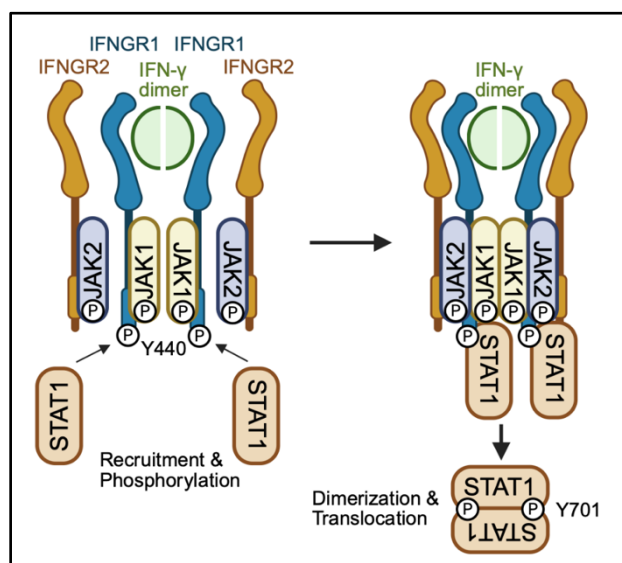
The mechanism of how IFN- $\gamma$  binds to IFNGR, and the receptor complex composed of IFN- $\gamma$  and IFNGR1/2 will be briefly discussed. One hallmark of IFN- $\gamma$  is that it exists as a homodimer, while type I IFNs such as IFN- $\alpha$  and IFN- $\beta$  are monomeric [87, 88]. A major difference between the active receptor complexes of IFNAR and IFNGR is their stoichiometry. Type I interferons form a ternary signaling complex with IFNAR, commonly described as a 1:1:1 complex of type I IFN:IFNAR1:IFNAR2 [87, 88], whereas the IFNGR signaling complex forms a 2:2:2 hexameric assembly with IFN- $\gamma$ :IFNGR1:IFNGR2 subunits [87]. The complete structure of the IFN- $\gamma$ –IFNGR 2:2:2 hexameric complex was determined in 2019 [87]. Although the complex contains multiple interaction sites, one example is that an IFNGR2 mutation (T168N) impairs assembly of the signaling complex [87]. The overall structure of the IFN- $\gamma$ -IFNGR 2:2:2 hexameric complex is shown as **Figure 1-6**.



**Figure 1-6. Crystal structure of the 2:2:2 hexameric IFN- $\gamma$ -IFNGR1/2 complex.**

(Structural data were retrieved from the RCSB Protein Data Bank, PDB ID: 6E3L, deposited by Jude K. M., Mendoza J. L., and Garcia K. C., released February 2019.)

Regarding activation of the JAK-STAT1 pathway downstream of IFNGR, IFN- $\gamma$  binding promotes the assembly of the receptor complex, enabling activation of JAKs. In general, JAK1 is associated with IFNGR1 and JAK2 is associated with IFNGR2 [89]. Upon receptor activation, the JAK kinases are activated and phosphorylate tyrosine residues on the cytoplasmic region of IFNGR1, creating docking sites for STAT1 [89]. A well-known STAT1 recruitment site in human IFNGR1 is phosphorylated tyrosine 440 [90]. STAT1 is then phosphorylated at tyrosine 701, which is critical for STAT1 dimerization, nuclear translocation, and DNA binding [91]. Phosphorylated STAT1 forms a homodimer, translocates into the nucleus, and induces ISG expression by binding to GAS elements [91, 92]. This receptor activation schematic is shown below as Figure 1-7.



**Figure 1-7. Schematic of IFNGR1/2 activation by IFN- $\gamma$ , including STAT1 activation mechanisms.**

(This figure was created using BioRender.)

## **1-5 VPg: its canonical role and a newly-identified function in this study**

Finally, the most relevant protein in this study, VPg (3B) of *Cardiovirus*, is briefly introduced and its significance in the context of this study is outlined. As described in previous sections, VPg is a very small viral protein, approximately 2.3 kDa in *Cardiovirus* and composed of only 20 amino acids [19, 40]. The precise amino acid length of VPg varies among members of the *Picornaviridae* family; for example, *Enterovirus* VPg consists of 22 amino acids rather than 20 [93]. The detailed sequences of VPg and their variations will be described and discussed later in this study.

Canonically, VPg is a critical protein during viral RNA synthesis, where it functions as a primer for viral genome replication [19, 40]. Although the essential role of VPg in the *Picornaviridae* life cycle has been well-established through numerous previous studies, additional functions of VPg have not been reported. In this study, a previously unrecognized function of VPg was identified that can be regarded as a “noncanonical” role. Specifically, EMCV VPg was found to function as an IFNGR agonist, triggering downstream signaling events that closely resemble those induced by IFN- $\gamma$ . To the best of my knowledge, this host-modulating activity of VPg has not been previously described in any study.

This study explains in detail of the process through which this function of VPg was discovered, including elucidation of its mechanism and characterization of its biological effects. Furthermore, the study explores the impact of this cytokine-mimic-like activity of VPg within the broader context of host-pathogen interactions during EMCV infection.

## **Chapter 2**

### **Materials and Methods**

## 2-1 List of cell lines

Cell lines were originally obtained from authenticated sources such as ATCC or JCRB. Knockout cell lines were all derived from authenticated WT HeLa cells (ATCC) or THP-1 cells (JCRB). All cell lines were confirmed negative for mycoplasma contamination using MycoAlert (Lonza Bioscience).

Cell lines listed below were cultured in DMEM medium (Nacalai Tesque Inc.) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and 1% Antibiotic-Antimycotic Mix (A/A) (Nacalai Tesque Inc.).

- HeLa (ATCC)
- HeLa BAK/BAX DKO (this study)
- HeLa TNFR1 KO (this study)
- HeLa IFNGR1 KO (this study)
- HeLa IFNGR2 KO (this study)
- HeLa IFNGR1/2 DKO (this study)
- HeLa CASP8 KO (this study)
- HeLa RIG-I KO (referenced in [94])
- HeLa MAVS KO (referenced in [94])
- HeLa IRF-3 KO (referenced in [94])
- HeLa PKR KO (referenced in [94])
- HeLa TRIF KO (referenced in [94])
- HEK293T (ATCC)
- A549 (ATCC)
- L929 (ATCC)
- RAW 264.7 (ATCC)
- B16-F10 (ATCC)
- Vero (ATCC)

THP-1 (JCRB) and THP-1 BAK/BAX DKO (this study) cells were cultured in RPMI-1640 medium (Nacalai Tesque Inc.) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and 1% Antibiotic-Antimycotic Mix (Nacalai Tesque Inc.).

Primary human dermal fibroblasts, adult (HDFa, PromoCell) were cultured in Fibroblast Basal Medium 2 (PromoCell) supplemented with components from the Fibroblast Growth Medium 2 Kit (PromoCell), prepared according to the manufacturer's instructions.

## **2-2 Mice and ethical approval**

Mice (strain: C57BL/6) used in this study were purchased from CLEA Japan, Inc. To maintain experimental consistency, all mice were of the same sex (female) and 12 weeks old. Mice were handled in accordance with the regulations on animal experimentation at Kyoto University and the fundamental guidelines for the proper conduct of animal experiments and related activities in academic research institutions under the jurisdiction of the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

All studies involving C57BL/6 mice were reviewed and approved by the Animal Experimentation Committee of Kyoto University (approval number: 253001). Mice were maintained under specific pathogen-free (SPF) conditions, and virus infection experiments (EMCV, IAV) were conducted in a biosafety level 3 (BSL-3) facility.

## **2-3 Viruses and infection procedures**

Encephalomyocarditis virus (EMCV, strain isolated from chimpanzee) was purchased from ATCC and amplified in Vero cells. EMCV titers were determined by plaque assay using Vero cells (see Section 2-18 for protocol). Sendai virus (SeV, strain Cantell) was purchased from ATCC and propagated in embryonated chicken eggs. Newcastle disease virus (NDV, strain Miyadera/51) was propagated in embryonated chicken eggs and was of origin as previously described [95]. Vesicular stomatitis virus (VSV, strain Indiana) was amplified in BHK-21 cells and was of origin as previously described [96]. Influenza A virus (IAV, H1N1 strain PR8) was propagated in embryonated chicken eggs. Poliovirus (PV, strain type I Mahoney) originated from a cDNA clone [97]. Coxsackievirus B3 (CVB3, strain Nancy) originated from a cDNA clone [98]. Enterovirus 71 (EV71, strain Isehara VP1-145Q variant) was generated from the original WT virus VP1-145E [99] and selected for this study due to its high infectivity

toward HeLa cells. All Enterovirus strains (PV, CVB3, EV71) were kindly provided by Dr. Satoshi Koike (Tokyo Metropolitan Institute of Medical Science).

For all virus infection experiments, cells (HeLa, unless otherwise indicated) were seeded at the indicated densities and incubated overnight at 37 °C. The next day, cells were infected with viruses at the indicated MOIs in DMEM without serum or antibiotics. DMEM without the live virus was applied to cells for all Mock-infection conditions. 1 hour after viral adsorption at 37 °C, the virus-containing medium was removed and replaced with DMEM (10% FBS/1% A/A). All virus-related experiments were conducted in biosafety level 2 or level 3 facilities.

## **2-4 Reagents and treatment conditions**

Caspase inhibitors Z-VAD-FMK, Z-IETD-FMK, Z-LEHD-FMK, and Z-DEVD-FMK were purchased from R&D Systems. Tofacitinib was purchased from AdipoGen Inc. NG-Nitro-L-arginine methyl ester (L-NAME) was purchased from Cayman Chemical Company. N-(3-(Aminomethyl)benzyl)acetamidine (1400W) was purchased from Selleck Chemicals. These reagents were pre-treated to cells 2 hours before the following experiments unless otherwise indicated. The exact treatment concentrations are provided in the figure legends.

Recombinant human TNF- $\alpha$ , murine TNF- $\alpha$ , human IFN- $\gamma$ , and human IFN- $\beta$  were purchased from PeproTech. 3-Morpholinosydnonimine hydrochloride (SIN-1) was purchased from Cayman Chemical Company. Treatment times and concentrations for recombinant cytokines and SIN-1 are described in the figure legends.

## **2-5 Synthetic peptides used in this study**

All synthetic peptides used in this study were synthesized and purchased from Eurofins Genomics. Peptides were diluted in RNase-free water before treatment. Peptide concentrations and treatment durations are specified in the figure legends. All VPg peptide sequences are described throughout the study and summarized in **Figure 4-2**.

## **2-6 Transfection procedures**

### Transfection of PX458 (spCas9(BB)-2A-GFP) CRISPR-Cas9 backbone plasmids:

HeLa or THP-1 cells were seeded overnight ( $1 \times 10^5$  cells/mL). Lipofectamine 2000 (2  $\mu$ L; Thermo Fisher Scientific) diluted in 250  $\mu$ L Opti-MEM (Thermo Fisher Scientific), and 1  $\mu$ g of PX458 backbone plasmid diluted in 250  $\mu$ L Opti-MEM were mixed and incubated at room temperature for 20 minutes, then added to cells in 2 mL of culture medium (DMEM for HeLa, RPMI-1640 for THP-1). Cells were incubated at 37 °C for 24 hours before sorting (see Section 2-7).

### Transfection of overexpression plasmids:

HEK293T cells were seeded overnight ( $1 \times 10^5$  cells/mL). Lipofectamine 2000 (7.5  $\mu$ L) diluted in 500  $\mu$ L Opti-MEM, and 6  $\mu$ g of Myc-IFNGR1 or Myc-IFNGR2 plasmids (OriGene) diluted in 500  $\mu$ L Opti-MEM were mixed, incubated at room temperature for 20 minutes, and added to cells in 1.5 mL DMEM. Cells were incubated at 37 °C for 48 hours before use.

### Transfection of siRNA (gene knockdown):

HeLa cells were seeded overnight ( $1 \times 10^5$  cells/mL). Lipofectamine RNAiMAX (2  $\mu$ L; Thermo Fisher Scientific) diluted in 250  $\mu$ L Opti-MEM, and 10 nM siRNA (Silencer Select siRNA, Thermo Fisher Scientific) diluted in 250  $\mu$ L Opti-MEM were mixed, incubated at room temperature for 20 minutes, and added to cells in 2 mL DMEM. Cells were incubated at 37 °C for 48 hours prior to following experiments.

siRNA IDs (Silencer Select siRNAs): siNOS2 #1: s9618, siNOS2 #2: s9619, siIRF-1: s7501

## **2-7 Knockout generation by CRISPR-Cas9 and guide RNAs**

The spCas9(BB)-2A-GFP (PX458) backbone plasmid used for knockout generation was a gift from Dr. Feng Zhang (Addgene #48138; referenced in [100]). The plasmid was digested with BbsI (New England Biolabs) and purified using the Monarch DNA Gel Extraction Kit (New England Biolabs). Forward and reverse 20-mer oligonucleotides corresponding to the sgRNA spacer sequences were annealed and

phosphorylated using T4 polynucleotide kinase (PNK) (TOYOBO). All guide sequences were designed using CRISPick (Broad Institute; [101, 102]).

#### Guide RNA sequences:

Human BAK Forward: 5' – (CACCG)GTTGATGTCGTCCCCGATGA – 3'

Human BAK Reverse: 5' – (AAAC)TATCGGGGACGACATCAAC(C) – 3'

Human BAX Forward: 5' – (CACCG)TCGGAAAAAGACCTCTCGG – 3'

Human BAX Reverse: 5' – (AAAC)CCGAGAGGTCTTTTTCCGA(C) – 3'

Human CASP8 Forward: 5' – (CACCG)CTACCTAAACACTAGAAAGG – 3'

Human CASP8 Reverse: 5' – (AAAC)CCTTTCTAGTGTTTAGGTAG(C) – 3'

Human IFNGR1 Forward: 5' – (CACCG)GGTACTCCCAATATACGTA – 3'

Human IFNGR1 Reverse: 5' – (AAAC)TATCGTATATTGGGAGTACC(C) – 3'

Human IFNGR2 Forward: 5' – (CACCG)GACTTGTAACAGTACACTC – 3'

Human IFNGR2 Reverse: 5' – (AAAC)GAGTGTACTGTTTACAAGTC(C) – 3'

Other HeLa knockouts used in this study were generated in a previous study [94], and guide RNA sequences are referenced within the paper.

Annealing and phosphorylation were performed using the following thermal cyclor program: 37 °C for 30 min, 95 °C for 5 min, followed by a ramp-down at 5 °C/min to 25 °C. Annealed oligonucleotides were ligated into the PX458 backbone using Ligation High (TOYOBO) for 10 minutes at room temperature. Transformation was performed using DH5α competent cells (TOYOBO). Plasmids were sequenced using a human U6 promoter primer (sequence: GACTATCATATGCTTACCGT) to confirm correct insertion. Plasmid DNA was amplified and purified using NucleoBond Xtra Midi (Macherey-Nagel).

24 hours after transfection (see Section 2-6), GFP-positive cells were sorted using an SH800 cell sorter (SONY Biotechnology Inc.). Single-cell colonies were isolated and expanded. Knockout validation was performed by confirming both direct loss of protein expression (Western blotting; see Section 2-15) and loss of known functional activity: loss of STAT1 phosphorylation by IFN-γ treatment for IFNGR1 KO, IFNGR2 KO, and

IFNGR1/2 DKO, abrogation of cell death by Actinomycin D for BAK/BAX DKO, abrogation of cell death by TNF- $\alpha$  + cycloheximide for CASP8 KO.

## **2-8 Amido Black survival assay and quantitative analysis**

HeLa and other adherent cells were seeded at  $4 \times 10^5$  cells/mL in DMEM (10% FBS, 1% A/A). After treatment or infection (typically 24 hours unless otherwise indicated), cells were washed twice with PBS (Nacalai Tesque Inc.) and fixed with 4% paraformaldehyde (PFA) (Nacalai Tesque Inc.) for 10 minutes. After removal of PFA, cells were stained with Amido Black 10B solution (0.1% Amido Black 10B, 10% acetic acid, 50% methanol; all from Nacalai Tesque Inc.) for 30 minutes. Excess stains were removed by gentle washing with running water, plates were dried completely, and scanned.

For quantitative survival analysis, stains were eluted using 50 mM NaOH, and absorbance was measured at 630 nm - 405 nm using a microplate reader (Bio-Rad). Relative cell survival was calculated using a standard curve normalized to 100% (control well) and 0% (empty well). Absorbance values were used as a proxy for relative cell survival.

## **2-9 Trypan Blue survival quantification**

THP-1 cells were diluted appropriately and stained with Trypan Blue solution (Nacalai Tesque Inc.) at a 1:1 ratio with the cell culture supernatant. Samples were loaded onto a hemocytometer and counted manually (average of 4 areas, in duplicate). Cell survival percentages were calculated as follows:

$$\text{Cell survival (\%)} = [1 - (\text{number of dead cells} / \text{total number of cells})] \times 100$$

## **2-10 Annexin V/PI apoptosis assay**

THP-1 cells were washed twice with PBS (each wash followed by centrifugation at 1500 rpm for 5 minutes) and resuspended in 1 mL of 1X Binding Buffer (0.01 M HEPES,

pH 7.4; 0.14 M NaCl; 2.5 mM CaCl<sub>2</sub>). Cells were stained with Annexin V-FITC (BioLegend; 1:100 dilution) and Propidium Iodide (PI; BioLegend; 1:100 dilution) for 15 minutes in the dark at room temperature. Samples were kept on ice and analyzed within 1 hour using flow cytometry (BD FACSVerse). Flow cytometry data were analyzed using FlowJo software (BD Biosciences).

## **2-11 ATP-based survival quantification**

HeLa cells were seeded at  $4 \times 10^5$  cells/mL in DMEM (10% FBS/1% A/A). After treatment, infection, etc. (typically 24 hours unless indicated otherwise), cells were washed carefully with PBS. CellTiter-Glo reagent (Promega) was added to cells at a 1:1 ratio with DMEM, and cells were lysed for 2 minutes on an automatic shaker. After waiting for 10 minutes (stabilization of signal), luminescence values were measured using a TriStar<sup>2</sup> multimode microplate reader (BERTHOLD). Relative cell survival was calculated from luminescence measurements after constructing a standard curve, in which luminescence values were normalized to 100% (control well) and 0% (empty well).

## **2-12 Quantification of late-stage apoptosis by ELISA**

HeLa cells were seeded at  $1 \times 10^5$  cells/mL. At 9 hours post-treatment, cells were washed twice carefully with PBS, then lysed using 200  $\mu$ L of lysis buffer from the Cell Death Detection ELISA<sup>PLUS</sup> kit (Roche). Cells were incubated at room temperature for 30 minutes, centrifuged (5000 rpm, 5 min), and 20  $\mu$ L of the supernatants (corresponding to the cytoplasmic fraction) were used for ELISA using the same kit. Absorbance at 450 nm was measured using a TriStar<sup>2</sup> multimode microplate reader (BERTHOLD). Apoptosis levels were estimated from the values, setting the mean values of (control - blank) as 1.

## **2-13 Quantification of TNF- $\alpha$ by ELISA**

Supernatants or fractions were collected for measurement of total TNF- $\alpha$  levels by ELISA. A human TNF- $\alpha$  ELISA kit (R&D Systems) was used for quantification,

following the manufacturer's protocol. Absorbance at 450 nm was measured using a TriStar<sup>2</sup> multimode microplate reader (BERTHOLD), and values were calculated from the standard curve generated using known concentrations of TNF- $\alpha$  provided in the kit.

## **2-14 Bradford assay**

Total protein concentrations were quantified in fractions using the Bradford Protein Assay Kit II (Bio-Rad), using BSA as the standard. Absorbance at 595 nm was measured for protein quantification using a microplate reader (Bio-Rad).

## **2-15 SDS-PAGE, Western blotting, and list of antibodies**

Sample preparation for cells: Samples were lysed in 1% NP-40 lysis buffer (50 mM Tris-HCl pH 8.0 (Nacalai Tesque Inc.), 150 mM NaCl (Nacalai Tesque Inc.), 10% NP-40 Substitute (Nacalai Tesque Inc.)) including a protease/phosphatase inhibitor cocktail (0.1 mM Na<sub>3</sub>VO<sub>4</sub> (Nacalai Tesque Inc.) and 1/100 Protease Inhibitor Cocktail (Nacalai Tesque Inc.)). Cell lysates were centrifuged (16000 g, 10 minutes), and the supernatant was collected. Sample protein concentrations were quantified using Bradford assay (see Section 2-14) and adjusted to 30  $\mu$ g/lane unless specified otherwise.

For all samples from here, samples were diluted 2-fold using 2X SDS sample buffer (250 mM Tris-HCl pH 6.8 (Tris-base: Nacalai Tesque Inc.), 2% SDS (Nacalai Tesque Inc.), 10% glycerol (Nacalai Tesque Inc.), 5% 2-mercaptoethanol (Nacalai Tesque Inc.), 0.005% bromophenol blue (Nacalai Tesque Inc.)), and heated for 5 minutes at 100 °C for protein denaturation.

SDS-PAGE: Samples were loaded into either 14-well or 18-well commercial 5–20% polyacrylamide gels (ATTO), and separated in 1X SDS running buffer (Nacalai Tesque Inc.) at 200 V for 40-70 minutes at room temperature.

Western blotting: After SDS-PAGE, proteins were transferred to a PVDF membrane (Merck Millipore) using a semi-dry transfer device (Trans-Blot SD Transfer Cell, Bio-

Rad) at 100 mA/membrane for 100 min. The PVDF membrane was pre-soaked in methanol (Nacalai Tesque Inc.) for 2 minutes, equilibrated for 5 minutes, and proteins were transferred using transfer buffer (25 mM Tris-base, 192 mM glycine (Nacalai Tesque Inc.), 20% methanol). The PVDF membrane was blocked using 5% BSA (Nacalai Tesque Inc.) in 1X TBS-T (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.1% Tween 20 (Nacalai Tesque Inc.)) for 60 minutes after transfer, and then incubated with primary antibodies (diluted in 5% BSA in 1X TBS-T) overnight at 4 °C with rotation.

List of primary antibodies and its dilution:

Anti-GAPDH (Santa Cruz Biotechnology, sc-32233), dilution: 1:1000

Anti-Bak (CST, #12105), dilution: 1:1000

Anti-Bax (abcam, ab53154), dilution: 1:1000

Anti-Caspase 8 (CST, #9746), dilution: 1:500

Anti-Cleaved PARP (CST, #9541), dilution: 1:1000

Anti-Mengo 3DPol (Santa Cruz Biotechnology, sc-65633), dilution: 1:500

Anti-iNOS (Santa Cruz Biotechnology, sc-7271), dilution: 1:500

Anti-STAT1 (CST, #9172), dilution: 1:1000

Anti-pSTAT1 (Y701) (CST, #9167), dilution: 1:1000

Anti-pSTAT2 (Y690) (CST, #4441), dilution: 1:1000

Anti-Myc-Tag (CST, #2276), dilution: 1:1000

Anti-IkB $\alpha$  (Santa Cruz Biotechnology, sc-371), dilution: 1:500

Anti-HSP70 (Santa Cruz Biotechnology, sc-32239), dilution: 1:1000

Anti-IFNGR1 (Proteintech, 10808-1-AP), dilution: 1:1000

Anti-IFNGR2 (Proteintech, 10266-1-AP), dilution: 1:500

Anti-VPg<sup>EMCV</sup> (IBL, this study), dilution: 1:5000. This polyclonal anti-VPg<sup>EMCV</sup> (anti-3B) antibody was created by Immuno-Biological Laboratories (IBL), at a final concentration of 1 mg/mL confirmed with low endotoxin levels (<0.3 EU/mg/mL). For generation of the antibody, a peptide with an addition of cysteine in the N-terminal region (C+GPYNETARVKPKTLQLLDIQ) was first synthesized, and bovine thyroglobulin was used as the carrier protein. Antibody was pooled from the serum of 3 rabbits and column-purified. All steps from peptide synthesis to column purification were conducted by IBL.

The next day, the PVDF membrane was washed 3 times for 10 min with 1X TBS-T, then incubated for 1 hour at room temperature with AP-conjugated secondary antibodies. Either anti-mouse (CST, #7056) or anti-rabbit (CST, #7054) AP-conjugated secondary antibodies were diluted in 5% BSA in 1X TBS-T at 1:4000. The PVDF membrane was washed 3 times for 10 min with 1X TBS-T, rinsed briefly with 1X TBS, then bands were detected using BCIP-NBT solution (Nacalai Tesque Inc.). The reaction was stopped when bands were clearly visible by rinsing with RNase-free water. After the membrane was completely dried, blots were scanned.

## **2-16 Neutralization of TNF- $\alpha$ using etanercept**

Etanercept (also called ENBREL) was purchased from Pfizer. Etanercept (10  $\mu$ g/mL) was mixed with the acid-EMCV-inactivated supernatant (see Section 2-17 for preparation), and neutralization was performed for 2 hours. The etanercept–TNF- $\alpha$  complex was removed using Dynabeads Protein G (Invitrogen), following the manufacturer's protocol for removal. The depleted mixture was diluted 2-fold using DMEM (10% FBS, 1% A/A) and treated to cells.

## **2-17 Virus inactivation procedures**

Following viral infection (see Section 2-3), the supernatant was collected and centrifuged (12000 g, 5 min) to remove debris.

Inactivation using acid: 1 N HCl was used to acidify infected culture supernatant to pH 2.0, then stored at 4 °C for 7 days to inactivate the virus. After 7 days, the supernatant was neutralized to pH 7.0 by slowly adding 500 mM NaOH.

Inactivation using heat: The supernatant was heated to 100 °C for 10 minutes using a heat block, then rapidly cooled on ice. This inactivation procedure was also used to inactivate the activity of most cytokines in the supernatant, such as TNF- $\alpha$ .

Inactivation (removal) using centrifugal filter units: The supernatant was filtered using a 10 kDa cut-off filter (Amicon Ultra 10K, Merck Millipore), following the manufacturer's instructions. The flowthrough was collected as the sample with removal of active virions and a large portion of cytokines such as TNF- $\alpha$ .

Following all inactivation methods, complete loss of viral activity was confirmed by plaque assay (see Section 2-18) and by observing loss of cytopathic effects (CPEs) in HeLa cells.

## **2-18 Quantification of virus titers by plaque assay**

Vero or L929 cells were seeded at  $2 \times 10^5$  cells/mL overnight. 10-fold (1 log) serial dilutions of virus-containing samples (supernatants) were prepared using DMEM without serum or antibiotics (see Section 2-3 for infection protocol). Vero or L929 cells were infected with diluted virus-containing samples (Mock conditions were also prepared). After 1 hour of adsorption, virus-containing supernatants were removed and replaced with 1.5% Avicel PH-101 (Fluka/Sigma-Aldrich) in DMEM (10% FBS, 1% A/A). For VSV and EMCV infections, cells were incubated for 24 hours at 37 °C. Supernatant containing Avicel PH-101 was removed completely, wells were washed carefully with PBS, and fixed with 4% PFA for 10 minutes. After fixation, wells were washed gently with water. After drying, 0.5% Crystal Violet solution (2.5 g crystal violet (Nacalai Tesque Inc.) in 20% methanol (Nacalai Tesque Inc.)) was added to wells for 30 minutes. Wells were then washed gently with water and dried. After complete drying, plaques were counted in triplicate, and PFU/mL was calculated from the dilution factor.

## **2-19 RNA extraction**

RNA was extracted from cells or mouse serum (isolated and centrifuged from blood) using TRIzol reagent (Invitrogen), followed by standard chloroform/phenol extraction and ethanol precipitation for purification of total RNA. DNase I (Roche) was used according to the manufacturer's protocol for removal of DNA. Extracted RNA was quantified using NanoDrop 2000 (Thermo Fisher Scientific), then used for RT-qPCR (see Section 2-20) or RNA-seq analysis (see Section 2-29).

## **2-20 RT-qPCR and list of primers**

RNA concentrations were adjusted after quantification (see Section 2-19), and RNA was reverse-transcribed using ReverTra Ace qPCR Master Mix with gDNA remover (TOYOBO). The manufacturer's protocol was followed for gDNA removal and reverse

transcription. cDNA was used for real-time PCR (quantitative PCR, qPCR) using instrument StepOnePlus (Applied Biosystems). THUNDERBIRD SYBR qPCR Mix (TOYOBO) was used for qPCR analysis, mixing cDNA and 3  $\mu$ M each of forward and reverse primers to quantify relative expression levels ( $\Delta\Delta$ CT, relative to GAPDH) of specific genes.

All primers used for qPCR analysis in this study are as follows:

mGapdh Forward: 5' – CCCCAGCAAGGACACTGAGCAAG – 3' (ref: [103])  
mGapdh Reverse: 5' – GGGGTCTGGGATGGAAATTGTGAGG – 3' (ref: [103])  
EMCV Forward: 5' – TCATTAGCCATTTCAACCCA – 3' (ref: [104])  
EMCV Reverse: 5' – GAGATACAAACCCGCCCTAA – 3' (ref: [104])  
mNos2 Forward: 5' – CAGCTGGGCTGTACAAACCTT – 3' (ref: [105])  
mNos2 Reverse: 5' – CATTGGAAGTGAAGCGGTTCG – 3' (ref: [105])  
mTnf Forward: 5' – GGCATGGATCTCAAAGACAACC – 3' (ref: [103])  
mTnf Reverse: 5' – CAGGTATATGGGCTCATACCAG – 3' (ref: [103])  
hGAPDH Forward: 5' – TTGGCTACAGCAACAGGGTG – 3' (ref: [106])  
hGAPDH Reverse: 5' – GGGGAGATTCAGTGTGGTGG – 3' (ref: [106])  
hUBE2L6 Forward: 5' – ACGAGAACGGACAGATTTGC – 3'  
hUBE2L6 Reverse: 5' – TTGGCAAGTCTTGGTGCAAG – 3'  
hIDO1 Forward: 5' – ATTTGTCTGGCTGGAAAGGC – 3'  
hIDO1 Reverse: 5' – ACTGAAAGACGCTGCTTTGG – 3'  
hTAP1 Forward: 5' – GTGGGAAAATGGTACCAGTTGC – 3'  
hTAP1 Reverse: 5' – TCGTTGGCAAAGCTTCGAAC – 3'  
hC1R Forward: 5' – TGCCAACGGGAAGAACATTG – 3'  
hC1R Reverse: 5' – ACAGCAGATCCACAGCATTG – 3'  
hWARS1 Forward: 5' – ACGGCCAAGCAGATCAAAAC – 3'  
hWARS1 Reverse: 5' – ACACGTCCACATCACAGTTG – 3'  
hH4C3 Forward: 5' – CAAGCGCATTTCCGGTCTTATC – 3'  
hH4C3 Reverse: 5' – TGTGACAGTTTTGCGCTTGG – 3'  
hDUSP1 Forward: 5' – AACGTCTCAGCCAATTGTCC – 3'  
hDUSP1 Reverse: 5' – TGAAGTCAATGGCCTCGTTG – 3'  
hC19orf48P Forward: 5' – AGAAGGTGGATAAGTGGGCTAC – 3'  
hC19orf48P Reverse: 5' – ATAGGCCTGGTCACAATAGGG – 3'  
hTGIF1 Forward: 5' – TTTTGGCTCGTCCATCAGTG – 3'

hTGIF1 Reverse: 5' – TTTTGGCCGCTATCTGCTG – 3'  
hDDIT4 Forward: 5' – AAGACACGGCTTACCTGGATG – 3'  
hDDIT4 Reverse: 5' – AAGTGTTTCATCCTCAGGGTCAC – 3'

## **2-21 Size-exclusion chromatography**

Sephadex G-25 (Sigma-Aldrich) was pre-treated in 1X Tris-Acetate-EDTA buffer (Nacalai Tesque Inc.) for 3 hours at room temperature. This slurry was autoclaved, cooled overnight at 4 °C, and packed into a 20 mL column for separation. Purified water (RNase-free water) was used as the pre-wash buffer (total of 30 mL) and as the elution buffer. A total of 250 µL of acid-inactivated EMCV supernatant (see Section 2-17 for preparation) was size-fractionated into 20 fractions (1 mL each) under sterile conditions. F0 indicates the first flowthrough when the sample was applied. Post-fractionation, samples were evaporated once using centrifugal concentration and then reconstituted in 250 µL of DMEM (10% FBS/1% A/A). These fractions were treated to cells at a 1:1 concentration (fractions: fresh DMEM (10% FBS/1% A/A)) for 24 hours.

## **2-22 Size-dependent separation using centrifugal filter units**

HeLa cells were infected with EMCV (MOI 0.1) for 24 hours (see Section 2-3 for virus infection procedures). The virus-infected supernatant was filtered using a 50 kDa cut-off filter (Amicon Ultra 50K, Merck Millipore) following the manufacturer's instructions. The concentrated supernatant (>50 kDa) containing active virions was discarded, and the flowthrough (<50 kDa) was collected. This <50 kDa flowthrough was further filtered using a 10 kDa cut-off filter (Amicon Ultra 10K, Merck Millipore) following the manufacturer's instructions. The concentrated supernatant (<50 kDa >10 kDa) and the flowthrough (<10 kDa) were both collected, and the volume of the concentrated supernatant (<50 kDa >10 kDa) was adjusted back to its original volume using DMEM (10% FBS, 1% A/A). This flowthrough (<10 kDa) was further filtered using a 3 kDa cut-off filter (Amicon Ultra 3K, Merck Millipore) following the manufacturer's instructions. The concentrated supernatant (<10 kDa >3 kDa) and the flowthrough (<3 kDa) were both collected, and the volume of the concentrated supernatant (<10 kDa >3 kDa) was adjusted back to its original volume using DMEM (10% FBS, 1% A/A).

All collected fractions were treated to HeLa cells either at their original concentration (direct treatment) or after 10-fold dilution in DMEM, with or without recombinant human TNF- $\alpha$  (10 ng/mL). Cell survival or apoptosis was quantified by ATP levels (see Section 2-11) or DNA fragmentation by ELISA (see Section 2-12).

## **2-23 Total nitrite measurement by Griess quantification**

Culture supernatants or mouse serum were quantified for total nitrite levels using Griess quantification with the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical Company), following the manufacturer's protocol. Total nitrite was measured after reducing all nitrate using nitrate reductase contained in the kit. Absorbance at 540 nm was measured using a microplate reader (Bio-Rad). Total nitrite levels were quantified as a readout of nitric oxide (NO) production, using the nitrite standard provided in the kit.

## **2-24 Neutralization attempt and pulldown of VPg<sup>EMCV</sup>**

HeLa cells were Mock-infected or infected with EMCV for 24 h at MOI 0.1 (see Section 2-3 for infection protocol). Supernatants were heat-inactivated for inactivation of viruses and cytokines (see Section 2-17).

For neutralization attempt: Mock and EMCV-inactivated supernatants, or synthetic VPg<sup>EMCV</sup> solution (2 ng/mL) in DMEM (10% FBS/1% A/A), were mixed with anti-VPg<sup>EMCV</sup> antibody (10  $\mu$ g/mL) overnight (4 °C) on a rotator. Conditions without addition of anti-VPg<sup>EMCV</sup> were also prepared. Supernatants and synthetic VPg<sup>EMCV</sup> solutions, with or without antibody, were diluted 2-fold using DMEM (10% FBS/1% A/A) and treated to HeLa cells for 24 hours (see Section 2-8 for survival quantification).

For pulldown of VPg<sup>EMCV</sup>: Dynabeads Protein G (Invitrogen) was resuspended into a tube (50  $\mu$ L for 1 mL of supernatant or solution), and the supernatant was discarded on a magnetic rack. Anti-VPg<sup>EMCV</sup> (10  $\mu$ g) was used for each condition, and binding of anti-VPg<sup>EMCV</sup> was performed following the manufacturer's instructions. Conditions with Protein G beads but without anti-VPg<sup>EMCV</sup> were also prepared. After removal of binding buffer (with or without the antibody), Mock and EMCV-infected supernatants, or

synthetic VPg<sup>EMCV</sup> solution (2 ng/mL), were mixed with the bead–antibody complex and incubated overnight on a rotator (4 °C). The next day, these supernatants or synthetic VPg<sup>EMCV</sup> solutions were collected on a magnetic rack (excluding the beads), diluted 2-fold using DMEM (10% FBS/1% A/A), and treated to cells for 24 hours (see Section 2-8 for survival quantification).

## **2-25 Confirmation of VPg<sup>EMCV</sup> by Western blotting**

HeLa cells were seeded at  $2 \times 10^5$  cells/mL and infected with EMCV at MOI 0.1 for 9 hours (see Section 2-3 for infection protocol) in 6-well plates. The entire culture supernatant (2 mL) was collected, centrifuged (5000 rpm, 5 min) to remove debris, concentrated to 100X using centrifugal concentration (dried up), and resuspended in 1% NP-40 lysis buffer (see Section 2-15 for composition). Cell lysates were collected using 200  $\mu$ L 1% NP-40 lysis buffer (total of  $4 \times 10^5$  cells in 200  $\mu$ L). The 100X concentrated supernatants (1-, 2-, and 4-fold dilutions for EMCV, and 1-fold for Mock), cell lysates (1-, 2-, and 4-fold dilutions for EMCV, and 1-fold for Mock), and synthetic VPg<sup>EMCV</sup> of known concentrations (2-fold serial dilutions starting from 10 ng) were subjected to Western blotting (see Section 2-15). For this experiment, 20  $\mu$ L of samples were applied to each lane during SDS-PAGE.

## **2-26 Digestion of VPg<sup>EMCV</sup> by PDEI and Western blotting**

HeLa cells were seeded at  $2 \times 10^5$  cells/mL and infected with EMCV at MOI 0.1 for 24 hours (see Section 2-3 for infection protocol) in 6-well plates. The entire culture supernatant (2 mL) was collected, centrifuged (5000 rpm, 5 min) to remove debris, and filtered using a 50 kDa cut-off filter (Amicon Ultra 50K, Merck Millipore). Portions of the flowthrough were digested with  $>0.1$  U/mL (1/1000 dilution of original stock) snake venom-derived phosphodiesterase I (PDEI, Sigma-Aldrich) at 37 °C for 2–12 hours, as indicated. Conditions without adding PDEI were also prepared. Digested supernatants were heated at 100 °C for 10 min for complete inactivation of PDEI, concentrated to 100X using centrifugal concentration (dried up), and resuspended in 1% NP-40 lysis buffer (see Section 2-15 for composition). The 100X concentrated supernatants, as well as synthetic VPg<sup>EMCV</sup> of known concentrations (2-fold serial

dilutions starting from 10 ng), were subjected to Western blotting (see Section 2-15). For this experiment, 20  $\mu$ L of samples were applied to each lane during SDS-PAGE.

## **2-27 Dot blotting, quantification of VPg<sup>EMCV</sup> using ImageJ, and comparison of activities of VPg<sup>EMCV</sup>**

For dot blotting: A PVDF membrane was pre-soaked in methanol for 1 minute, soaked in RNase-free water for 2 minutes, and equilibrated in 1X TBS-T for 10 minutes. Two layers of filter paper were soaked in 1X TBS-T, and the wet PVDF membrane was placed on the filter paper. Synthetic VPg<sup>EMCV</sup> was adjusted to 1  $\mu$ g/mL (1 ng/ $\mu$ L) and serially diluted (2-fold), then 1  $\mu$ L of known concentrations of VPg<sup>EMCV</sup> was blotted in triplicates (n = 3). The <3 kDa flowthrough (EMCV-infected supernatant; see Section 2-3 for infection protocol) collected from size-based filtration (see Section 2-22) was concentrated 10X using a centrifugal concentrator, and 1  $\mu$ L of the supernatant was blotted in quadruplicates (n = 4). The PVDF membrane was dried for 2 hours, blocked with 5% BSA in TBS-T for 1 hour, incubated with anti-VPg<sup>EMCV</sup>, and blots were detected using BCIP-NBT (see Section 2-15 for entire protocol).

Quantification using ImageJ: After detection of dot blots, images were opened in ImageJ (original reference: [107]). Blots were converted to grayscale and color-inverted before measurement. Integrated Density (IntDen) values were used to quantify blot intensities. A reference curve was created using known concentrations of synthetic VPg<sup>EMCV</sup> (n = 3), and the concentration (ng/mL) of VPg<sup>EMCV</sup> in the <3 kDa flowthrough post-EMCV infection was quantified using this reference curve (n = 4). The average VPg<sup>EMCV</sup> concentration in the supernatant was calculated from the 4 values obtained.

Concentration adjustment and comparison of activities: After obtaining the average VPg<sup>EMCV</sup> concentration in the supernatant, the concentration of the <3 kDa flowthrough was adjusted to approximately 1 ng/mL using DMEM (10% FBS, 1% A/A) and serially diluted 2-fold from 1 ng/mL. Similarly, synthetic VPg<sup>EMCV</sup> solution (in DMEM with 10% FBS and 1% A/A) was serially diluted 2-fold starting from 1 ng/mL. Both natural (<3 kDa flowthrough) and synthetic VPg<sup>EMCV</sup> were treated to HeLa cells for 24 hours together with recombinant human TNF- $\alpha$  (10 ng/mL). At 24 h post-treatment, relative

survival for each condition was quantified by Amido Black 10B staining and elution (see Section 2-8).

## **2-28 Confirmation of IFNGR-VPg<sup>EMCV</sup> binding by co-immunoprecipitation**

HEK293T cells were seeded overnight at  $2 \times 10^5$  cells/mL, then transfected with Myc-IFNGR1, Myc-IFNGR2, or their combination (see Section 2-6).

For pulldown attempts of supernatant-derived (natural) VPg<sup>EMCV</sup> and synthetic VPg<sup>EMCV</sup> (**Figure 3-34**), cells were treated with either 10X concentrated <50 kDa-filtered EMCV supernatant (see Section 2-22 for protocol) or 100 ng/mL synthetic VPg<sup>EMCV</sup> for 1 hour (non-treated conditions were also prepared).

For pulldown attempts comparing WT and mutant VPgs (**Figure 3-35**), cells were treated with 100 ng/mL VPg<sup>EMCV</sup>, VPg<sup>EMCV-Q15A</sup>, VPg<sup>EMCV-L16A</sup>, or VPg<sup>EMCV-Q15A/L16A</sup>.

Cells were washed carefully twice with PBS, then lysed in cold 200  $\mu$ L of 1% NP-40 lysis buffer (see Section 2-15 for composition). Lysates were centrifuged at 16000 g for 10 minutes to remove cell debris.

Dynabeads Protein G from the kit (Invitrogen) were resuspended and transferred to tubes (50  $\mu$ L beads were used for 100  $\mu$ L lysis buffer). Anti-Myc-Tag antibody (CST, #2276; 2.5  $\mu$ L) was used for each condition, and antibody binding was performed following the manufacturer's protocol. 100  $\mu$ L of lysis buffer were mixed with the bead-antibody complex and incubated on a rotator at 4 °C overnight.

Beads were washed according to the manufacturer's protocol and eluted using 40  $\mu$ L of a 1:1 mixture of elution buffer (from the kit) and 2X SDS sample buffer (see Section 2-15 for composition). The bead-elution buffer mixture was heated for 5 minutes at 100 °C for denaturation, and beads were removed using a magnetic rack.

20  $\mu$ L of the immunoprecipitated eluate, 10  $\mu$ L of lysate input (1/5 concentration), and 1/5 of the total VPg input were subjected to SDS-PAGE and Western blotting (see Section 2-15).

## **2-29 RNA-seq analysis and using OlvTools**

Preparation of samples: HeLa cells were seeded at  $1 \times 10^5$  cells/mL overnight and treated with either recombinant human IFN- $\beta$  (1000 U/mL), recombinant human IFN- $\gamma$  (10 ng/mL), or VPg<sup>EMCV</sup> (1 ng/mL) for 24 hours. Non-treated (N.T.) controls were

also prepared. Samples were collected as biological duplicates ( $n = 2$ ). RNA was extracted after 24 hours (see Section 2-19 for protocol).

RNA quality check and RNA-seq analysis (conducted by Takara Bio): RNA samples were sent to Takara Bio for RNA-seq analysis. RNA quality was assessed using the TapeStation system (Agilent). Libraries were constructed using the SMART-seq v4 Ultra Low Input RNA Kit (Takara Bio), following the manufacturer's protocol. Sequencing was performed using the NovaSeq system (Illumina), and raw data were converted into FASTQ files by Takara Bio. Gene expression data were generated using the DRAGEN Bio-IT Platform (Illumina). These data were used for heatmaps (quantitative data) in **Figure 3-37c** and **Figure 3-39a**, as well as for quantitative analyses in **Figure 3-37d**, **Figure 3-37e**, and **Figure 3-39b**.

Further analyses using OlvTools: FASTQ files provided by Takara Bio were further analyzed using OlvTools (BxINFO, Tokyo, Japan). Transcript abundances were estimated using Salmon against the human transcriptome GRCh39. Gene-level abundances were calculated using the tximport package. Differential expression analysis was performed using the edgeR package after applying the filterByExpr function to remove lowly expressed genes.

Genes with  $|FC| > 1$  and  $FDR < 0.05$  were considered differentially expressed. Volcano plots in **Figure 3-36** were generated using OlvTools. Data from this analysis were used for ranking the top 50 upregulated genes by FDR (**Figure 3-37a**, **Figure 3-37b**), as well as for generating heatmaps (top gene list) of upregulated genes (**Figure 3-37c**) and downregulated genes (**Figure 3-39a**).

## **2-30 Washout experiment of VPg<sup>EMCV</sup>**

Initial experiment: HeLa cells were seeded at  $4 \times 10^5$  cells/mL in DMEM (10% FBS, 1% A/A) overnight. Cells were treated with 1 ng/mL or 100 ng/mL VPg<sup>EMCV</sup>, with or without recombinant human TNF- $\alpha$  (10 ng/mL), for 10 minutes. Non-treated (N.T.) conditions were also prepared. After 10 minutes, cells were carefully washed twice with PBS to remove residual VPg<sup>EMCV</sup> or TNF- $\alpha$ , and fresh DMEM (10% FBS, 1% A/A) was added. 24 hours later, supernatants were collected for further assays, and

remaining cells were quantified for survival using Amido Black 10B staining and elution (see Section 2-8).

Following experiment: Fresh HeLa cells were seeded at  $4 \times 10^5$  cells/mL in DMEM (10% FBS, 1% A/A) overnight. Supernatants collected from the initial experiment were used. N.T. supernatants and supernatants from treatments with 1 ng/mL or 100 ng/mL VPg<sup>EMCV</sup> without recombinant TNF- $\alpha$  were centrifuged (5000 rpm, 10 minutes) to remove debris. These supernatants were applied to HeLa cells at a 1:1 ratio (fresh DMEM:supernatant), with or without recombinant human TNF- $\alpha$  (10 ng/mL), for 24 hours, followed by Amido Black 10B survival assay (see Section 2-8).

### **2-31 Assessment of antiviral activity**

Cell survival quantification: HeLa cells were seeded at  $2 \times 10^5$  cells/mL overnight. The following day, 2-fold serial dilutions of IFN- $\gamma$  (starting from 10 ng/mL) or various VPgs (VPg<sup>EMCV</sup>, VPg<sup>TMEV</sup>, VPg<sup>SAFV</sup>, and VPg<sup>PV</sup>; starting from 2 ng/mL) were prepared and applied to cells overnight, including non-treated controls. The next day, after removal of supernatants, cells were infected with VSV at MOI 0.1 for 24 hours (see Section 2-3). Cell survival was quantified using Amido Black 10B elution (see Section 2-8).

Plaque assay: HeLa cells were seeded at  $2 \times 10^5$  cells/mL overnight. Cells were treated overnight with VPg<sup>EMCV</sup>, VPg<sup>TMEV</sup>, VPg<sup>SAFV</sup>, VPg<sup>PV</sup>, VPg<sup>PV-EMCV</sup>, VPg<sup>EMCV-Y3F</sup>, or VPg<sup>EMCV-8AA</sup> (1 ng/mL), including non-treated controls. The next day, supernatants were removed, and cells were infected with VSV at MOI 0.1 for 24 hours (see Section 2-3). Supernatants were collected, and viral titers were quantified by plaque assay using L929 cells (see Section 2-18).

### **2-32 *In vivo* infection models**

EMCV infection and iNOS inhibition experiment:

Female C57BL/6 mice (CLEA Japan), 12 weeks old, were used (see Section 2-2). Groups were divided into Mock + PBS, Mock + 1400W, EMCV + PBS, and EMCV + 1400W, with 5 mice per group ( $n = 5$ ). One day before infection, mice were injected intraperitoneally (100  $\mu$ L) with either PBS or 1400W (10 mg/kg). On day 0 post-

infection, mice were mock-infected (PBS) or infected with EMCV (1000 PFU/body, intraperitoneal, 100  $\mu$ L). The following day, mice were injected again with PBS or 1400W. On day 2 post-infection, blood was collected from the tails, and serum was isolated for further analyses (see Sections 2-19, 2-20, 2-23). On day 4 post-infection, all surviving mice were euthanized using isoflurane (Pfizer). Body weight and clinical symptoms were recorded daily.

IAV infection and intranasal VPg<sup>EMCV</sup> administration:

Female C57BL/6 mice (CLEA Japan), 12 weeks old, were used (see Section 2-2). Groups were divided into IAV + PBS and IAV + VPgEMCV, with 5 mice per group ( $n = 5$ ). One day before infection, mice were anesthetized with pentobarbital (50  $\mu$ g/g, intraperitoneal) and administered intranasally with PBS or VPg<sup>EMCV</sup> (10  $\mu$ g/mL, 30  $\mu$ L). On day 0 post-infection, mice were infected intranasally with IAV (500 PFU/body, non-lethal dose) under anesthesia. The following day, mice received a 2nd intranasal administration of PBS or VPg<sup>EMCV</sup>. Body weight was recorded daily for 17 days, after which all mice were euthanized using isoflurane (Pfizer).

## 2-33 Statistical analyses

GraphPad Prism 10 was used for figure generation and statistical analyses. All data in this study are presented as the mean (SD), and statistical comparisons were performed using biological replicates. For comparisons involving three or more groups, statistical significance was determined using one-way ANOVA (one independent variable) or two-way ANOVA (two independent variables), followed by Tukey's HSD post hoc test. Significance labels: \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ , \*\*\*\* $p \leq 0.0001$ , ns: not significant ( $p > 0.05$ ).

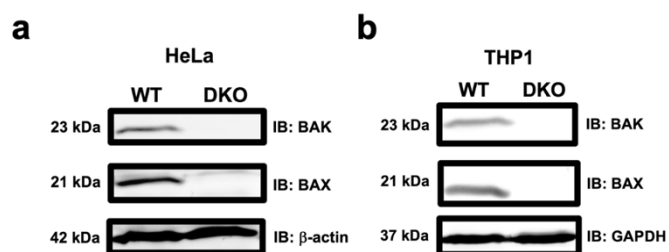
## **Chapter 3**

### **Results**

### 3-1 EMCV induces a distinct form of Caspase 8-dependent cell death pathway in host cells

This project was initiated based on unpublished preliminary observations in the laboratory, suggesting that several viruses trigger rapid cell death in host cells. At the same time, past reports showed the critical roles of the well-known pro-apoptotic proteins BAK and BAX (described in Section 1-1), both localized to mitochondria, in regulating virus-induced apoptosis following host cell infection. Specifically, the vesicular stomatitis virus (VSV) is known to induce cell death in infected host cells. Previous reports [108], together with unpublished accumulating data in the laboratory, suggested that infection of BAK- and/or BAX-deficient cells with VSV abrogated virus-induced cell death while simultaneously increasing viral titers.

To validate this hypothesis and to further investigate the mechanisms of cell death triggered by VSV and other RNA viruses, CRISPR/Cas9 plasmids were designed to generate knockout cell lines. Using these plasmids, BAK/BAX double-knockout (DKO) HeLa (cervical cancer) and THP-1 (monocyte) cell lines were successfully generated, and loss of protein expression was confirmed by Western blotting (**Figure 3-1a**, **Figure 3-1b**). The loss of function of these cell lines was also pre-confirmed by abrogation of cell death by the transcription inhibitor actinomycin D.



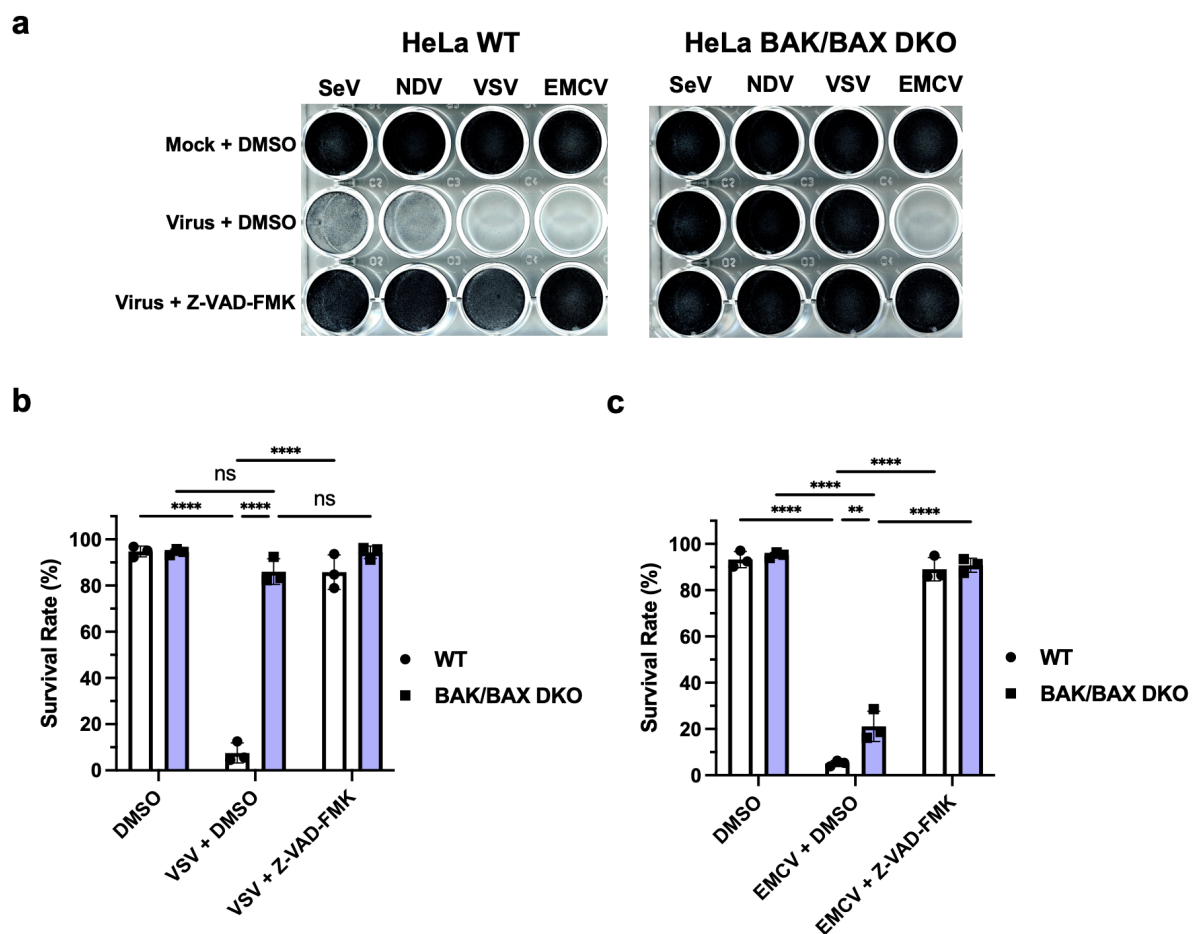
**Figure 3-1. Confirmation of BAK/BAX DKO by Western blotting.**

BAK/BAX DKO candidate clones of (a) HeLa or (b) THP-1 cells were lysed, and cell lysates were subjected to Western blotting using anti-BAK, anti-BAX, and anti-GAPDH antibodies.

After generating the BAK/BAX DKO HeLa cell line, virus-induced cell death was examined in detail. In addition to VSV, several other RNA viruses known to induce cell death in host cells were selected as candidates, including the Sendai virus (SeV), Newcastle disease virus (NDV), and the encephalomyocarditis virus (EMCV). Cell survival was quantified 24 hours post-infection in both WT HeLa cells and BAK/BAX DKO HeLa cells (**Figure 3-2a**).

All tested viruses induced significant cell death in WT HeLa cells. Notably, SeV, NDV, and VSV exhibited a strong dependency on both BAK and BAX, as BAK/BAX

DKO cells showed complete survival following infection. In contrast, EMCV-infected cells were eliminated within 24 hours post-infection even in the absence of BAK and BAX. However, pre-treatment with Z-VAD-FMK, a pan-caspase inhibitor, rescued EMCV-induced cell death. Furthermore, experiments using THP-1 cells revealed that comparison of VSV (**Figure 3-2b**) and EMCV (**Figure 3-2c**) infection showed cell death patterns similar to those observed in HeLa cells. These findings indicated that EMCV-induced cell death is highly caspase-dependent but mostly BAK and BAX-independent. At this stage, it was speculated that this form of cell death resembles apoptosis based on its dependence on caspase activity.



**Figure 3-2. Assessment of virus-induced cell death in WT vs. BAK/BAX DKO cells.**

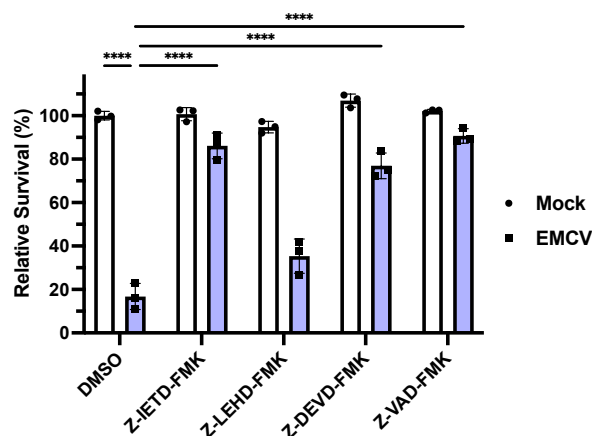
(a) WT or BAK/BAX DKO HeLa cells were infected with SeV, NDV, VSV, or EMCV (MOI 1.0) for 24 h, then fixed and stained with Amido Black 10B to assess cell survival. Mock-infected cells were used as negative controls. Cells were treated with either DMSO (control) or the pan-caspase inhibitor Z-VAD-FMK (10  $\mu$ M) 2 h prior to infection.

(b) WT or BAK/BAX DKO THP-1 cells were infected with VSV (MOI 1.0) for 24 h, and cell survival was assessed by Trypan Blue staining. Cells were treated with DMSO or Z-VAD-FMK as in (a).

(c) The same experiment as in (b) was performed using EMCV (MOI 1.0).

For (b, c): Data are presented as the mean (SD), and individual data points are biological replicates ( $n = 3$ ). Statistical significance was determined by two-way ANOVA, followed by Tukey's HSD post-hoc test. Labels: \*\* $p \leq 0.01$ , \*\*\*\* $p \leq 0.0001$ , ns: not significant ( $p > 0.05$ ).

Further investigation into the caspases required for this cell death pathway revealed that Caspase 8 was specifically essential for triggering EMCV-induced cell death, along with its downstream, Caspase 3 (**Figure 3-3**). In contrast, inhibition of Caspase 9, which functions downstream of the mitochondrial BAK/BAX-dependent pathway, had negligible effects on rescuing cell death. Results obtained using pharmacological inhibition were consistent with those observed using BAK/BAX DKO cells. Taken together, these results suggest that EMCV-induced cell death represents a Caspase 8-dependent extrinsic apoptotic pathway.



**Figure 3-3. Effect of caspase inhibitors on EMCV-induced cell death.**

HeLa cells were mock-infected or infected with EMCV (MOI 0.1) for 24 h. Cells were fixed and stained using Amido Black 10B, then eluted for relative survival quantification. Cells were treated with DMSO (control), Caspase 8 inhibitor Z-IETD-FMK (10  $\mu$ M), Caspase 9 inhibitor Z-LEHD-FMK (10  $\mu$ M), Caspase 3 inhibitor Z-DEVD-FMK (10  $\mu$ M), or pan-caspase inhibitor Z-VAD-FMK (10  $\mu$ M) 2 h prior to infection.

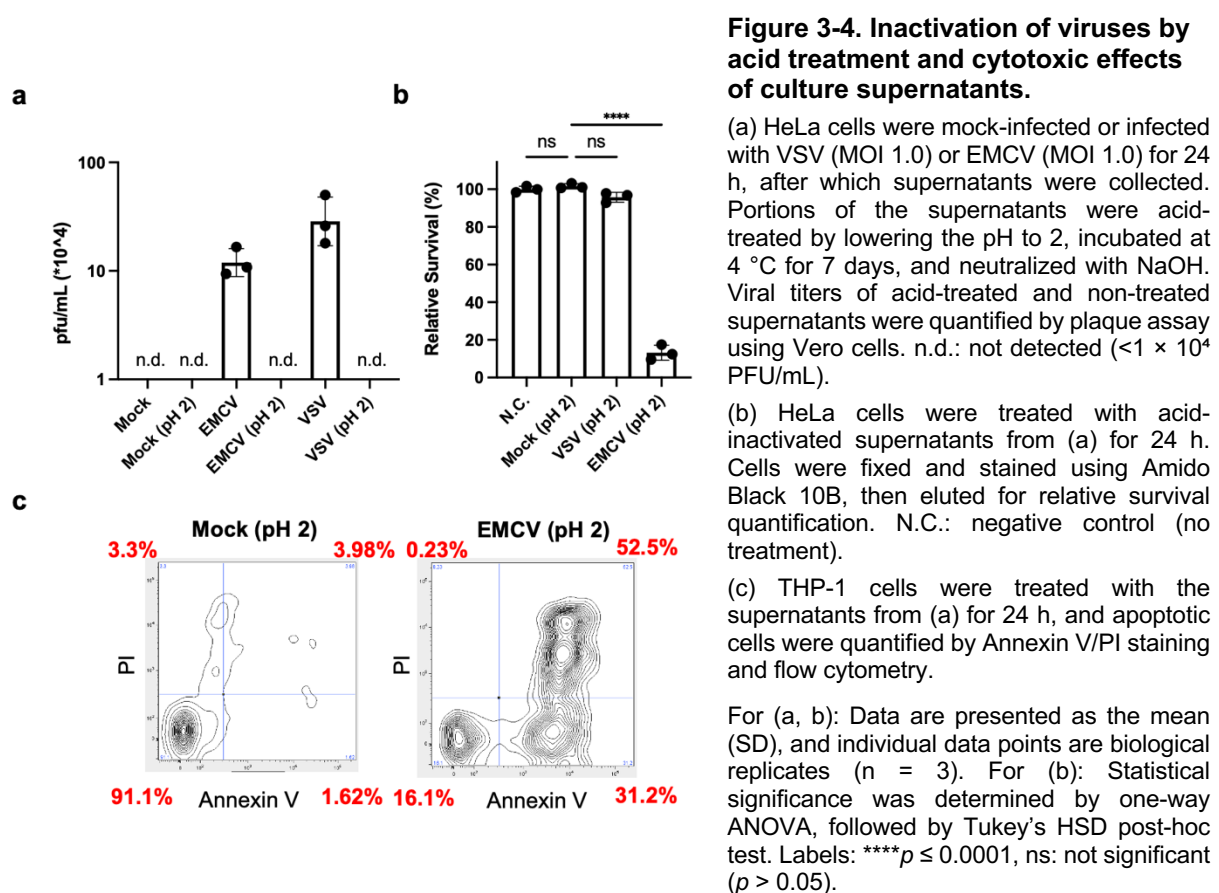
Data are presented as the mean (SD), and individual data points are biological replicates ( $n = 3$ ). Statistical significance was determined by two-way ANOVA, followed by Tukey's HSD post-hoc test. Label: \*\*\*\* $p \leq 0.0001$ .

### 3-2 Treatment with EMCV-inactivated supernatants induces potent cell death

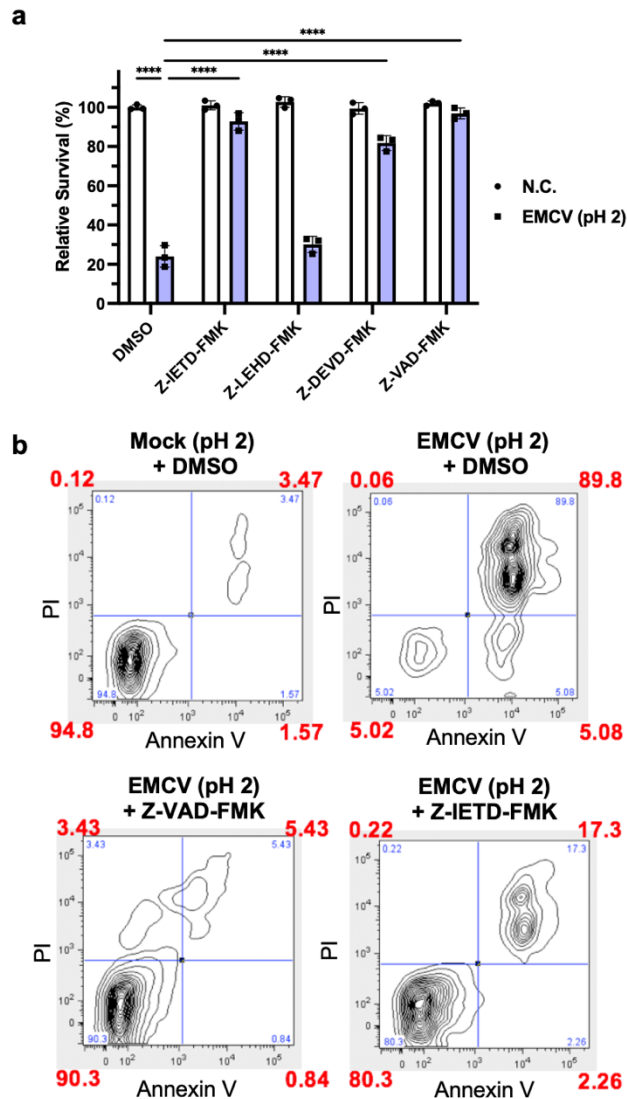
Given the strong involvement of Caspase 8 in EMCV-induced cell death and the possibility that this could represent extrinsic apoptosis, the next question was whether cytotoxic factors may be released into the culture supernatant during EMCV infection that can induce cell death in the absence of active viral infection.

To test this, several methods for viral inactivation were evaluated. Within these methods, one successful method was treatment with strong acid (HCl) to generate a low-pH condition (pH 2.0). This method is well-established for viral inactivation [109, 110] and is also known to preserve the biological activity of certain cytokines, such as type I IFNs [109, 111, 112]. Supernatants collected 24 hours post-infection from VSV- and EMCV-infected cultures were incubated at pH 2.0 for 7 days at 4°C and then neutralized using NaOH.

Complete viral inactivation of both VSV and EMCV in the treated supernatants was confirmed by plaque assay (**Figure 3-4a**). These inactivated supernatants were then applied to fresh cells for 24 hours to assess their cytotoxic effects. Treatment with inactivated VSV supernatant had no detectable effect on cell viability. In contrast, treatment with inactivated EMCV supernatant induced potent cell death in HeLa cells (**Figure 3-4b**). Similarly, treatment of THP-1 cells with the inactivated EMCV supernatant resulted in a marked increase in apoptotic cells, as assessed by Annexin V/PI staining (**Figure 3-4c**). Together, these results indicate that factors released during EMCV infection are sufficient to induce detectable cell death.



Consistent with the effects observed during EMCV infection (Figure 3-3), treatment with Z-IETD-FMK, a Caspase 8 inhibitor, or Z-VAD-FMK, a pan-caspase inhibitor, strongly suppressed cell death induced by the EMCV-inactivated supernatant in both HeLa cells (**Figure 3-5a**) and THP-1 cells (**Figure 3-5b**). These findings suggest that the Caspase 8-dependent cell death observed is mediated by components released during EMCV infection in the supernatant, rather than by the viral infection process itself.



**Figure 3-5. Effect of caspase inhibitors on cell death induced by acid-inactivated EMCV supernatant.**

(a) HeLa cells were untreated (N.C.) or treated with acid-inactivated EMCV supernatant (Figure 3-4a) for 24 h. Cells were fixed and stained using Amido Black 10B, then eluted for relative survival quantification. Cells were pre-treated with DMSO (control), Caspase 8 inhibitor Z-IETD-FMK (10  $\mu$ M), Caspase 9 inhibitor Z-LEHD-FMK (10  $\mu$ M), Caspase 3 inhibitor Z-DEVD-FMK (10  $\mu$ M), or pan-caspase inhibitor Z-VAD-FMK (10  $\mu$ M) 2 h prior to treatment.

(b) THP-1 cells were treated with the supernatants from Figure 3-4a for 24 h, and apoptotic cells were quantified by Annexin V/PI staining and flow cytometry. Cells were pre-treated with DMSO, Z-VAD-FMK, or Z-IETD-FMK in the same manner as in (a).

For (a): Data are presented as the mean (SD), and individual data points are biological replicates ( $n = 3$ ). Statistical significance was determined by two-way ANOVA, followed by Tukey's HSD post-hoc test. Label: \*\*\*\* $p \leq 0.0001$ .

### 3-3 TNF- $\alpha$ is critical for triggering cell death during EMCV infection

Having established that components released during EMCV infection induce rapid Caspase 8-dependent cell death, the critical factor responsible for triggering this pathway was investigated in detail. One group of candidates considered were host-derived inflammatory cytokines belonging to the TNF superfamily, several of which are known to induce Caspase 8-dependent cell death through well-established signaling pathways [62]. Among these candidates, the role of the most well-characterized cytokine in this family, TNF- $\alpha$  (described in detail in Section 1-3), was examined.

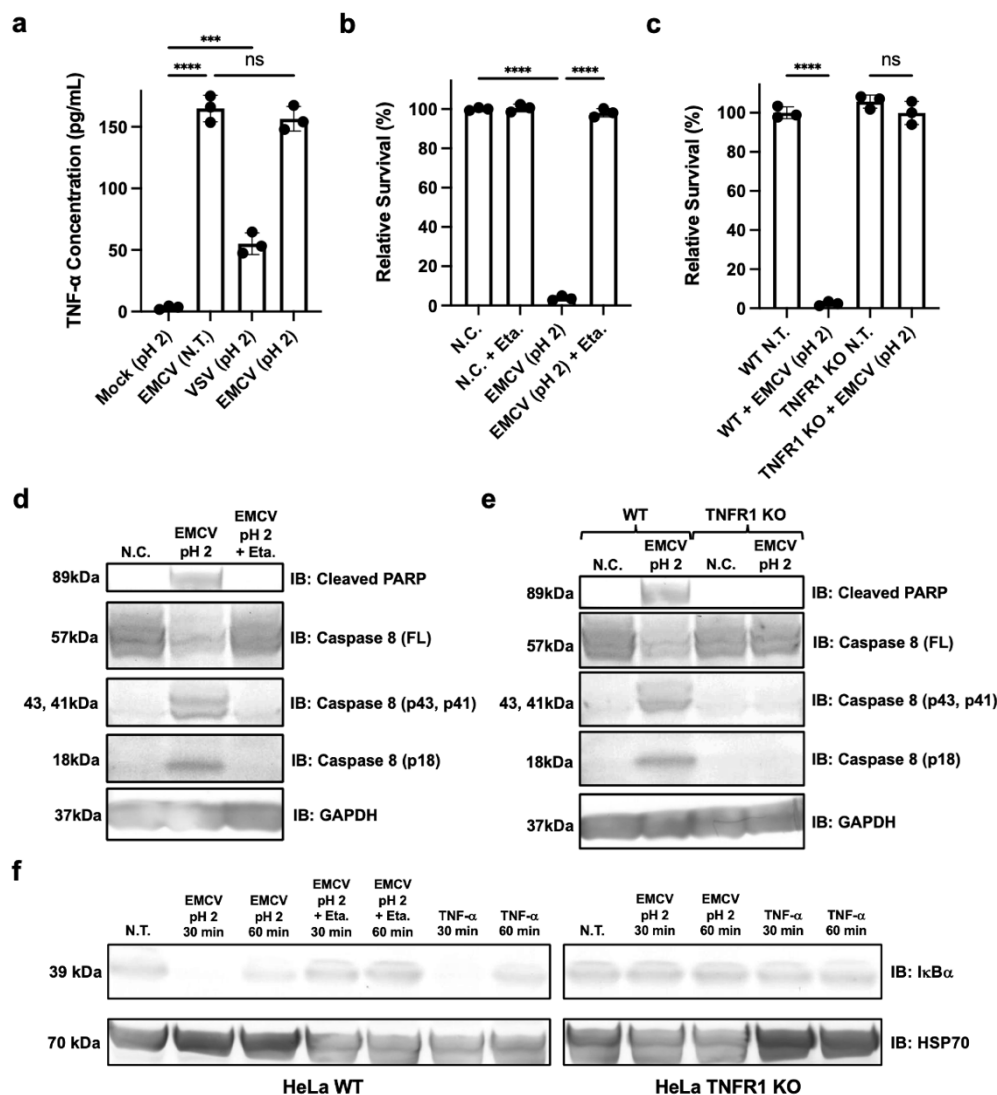
Using ELISA, TNF- $\alpha$  levels were measured in EMCV-infected culture supernatants before and after acid inactivation (**Figure 3-6a**). TNF- $\alpha$  was detected in

the supernatants, and its levels were not significantly altered by acid treatment, indicating that TNF- $\alpha$  remained stable under these conditions. As a reference, TNF- $\alpha$  induction was also observed during VSV infection, although at lower levels than those induced by EMCV.

To directly test the hypothesis that TNF- $\alpha$  is responsible for triggering this form of cell death, etanercept (ENBREL) was used to neutralize TNF- $\alpha$  activity in the post-infected supernatants (as described in Section 1-3). Etanercept was incubated with the EMCV-inactivated culture supernatant for 2 hours, followed by pulldown/depletion using Protein G magnetic beads prior to treatment of cells. While treatment with the inactivated EMCV supernatant alone induced cell death, etanercept-treated supernatant completely lost its ability to induce cell death in fresh HeLa cells (**Figure 3-6b**). To further confirm the requirement for TNF- $\alpha$  signaling, TNFR1-deficient HeLa cells were generated. In these cells, cell death induced by the inactivated EMCV supernatant was also completely abrogated (**Figure 3-6c**), indicating that TNF- $\alpha$  signaling through TNFR1 is essential for the observed cytotoxicity.

Western blotting further confirmed that cleavage of Caspase 8 and its downstream poly(ADP-ribose) polymerase (PARP), one hallmark of apoptotic cell death [113], occurred following treatment with the EMCV-inactivated supernatant. In contrast, cleavage of Caspase 8 and PARP were completely abolished either by etanercept neutralization of TNF- $\alpha$  (**Figure 3-6d**) or in TNFR1-deficient cells (**Figure 3-6e**). Finally, to verify that TNF- $\alpha$  retained biological activity following acid inactivation, the effects of the EMCV-inactivated supernatant were compared with those of recombinant human TNF- $\alpha$  in HeLa cells. Degradation of I $\kappa$ B $\alpha$ , which indicates NF- $\kappa$ B activation [114], was observed following treatment with both the EMCV supernatant and recombinant TNF- $\alpha$  with similar kinetics (**Figure 3-6f**). In contrast, treatment with etanercept-neutralized supernatant or treatment of TNFR1-deficient cells did not result in detectable I $\kappa$ B $\alpha$  degradation.

Together, these results show that TNF- $\alpha$  is the factor responsible for both NF- $\kappa$ B activation and Caspase-8-dependent cell death observed in EMCV-inactivated culture supernatants, and that TNF- $\alpha$  retains its biological activity following acid inactivation.



**Figure 3-6. Blockade of TNF-α signaling abrogates cell death induced by the inactivated EMCV supernatant.**

(a) HeLa cells were mock-infected or infected with VSV (MOI 1.0) or EMCV (MOI 1.0) for 24 h. Portions of the supernatants were acid-inactivated as in Figure 3-4a. TNF-α levels were quantified by ELISA. EMCV (N.T.): non-acid-treated.

(b) Fresh DMEM (N.C.) or acid-inactivated EMCV supernatant from (a) was incubated with etanercept (Eta., 10 μg/mL) for 2 h (Eta.-untreated controls were included). Etanercept complexes were removed using Protein G beads. HeLa cells were treated with these supernatants for 24 h. Cells were fixed and stained using Amido Black 10B, then eluted for relative survival quantification.

(c) WT or TNFR1 KO HeLa cells were treated with inactivated EMCV supernatant from (a) for 24 h, including non-treated controls (N.T.), and survival was quantified as in (b).

(d) HeLa cells were treated with inactivated EMCV supernatant or Eta.-depleted supernatant from (b) for 9 h, then lysed for Western blotting using anti-cleaved PARP, anti-Caspase 8, and anti-GAPDH antibodies.

(e) WT or TNFR1 KO HeLa cells were treated with inactivated EMCV supernatant from (a), and Western blotting was performed as in (d).

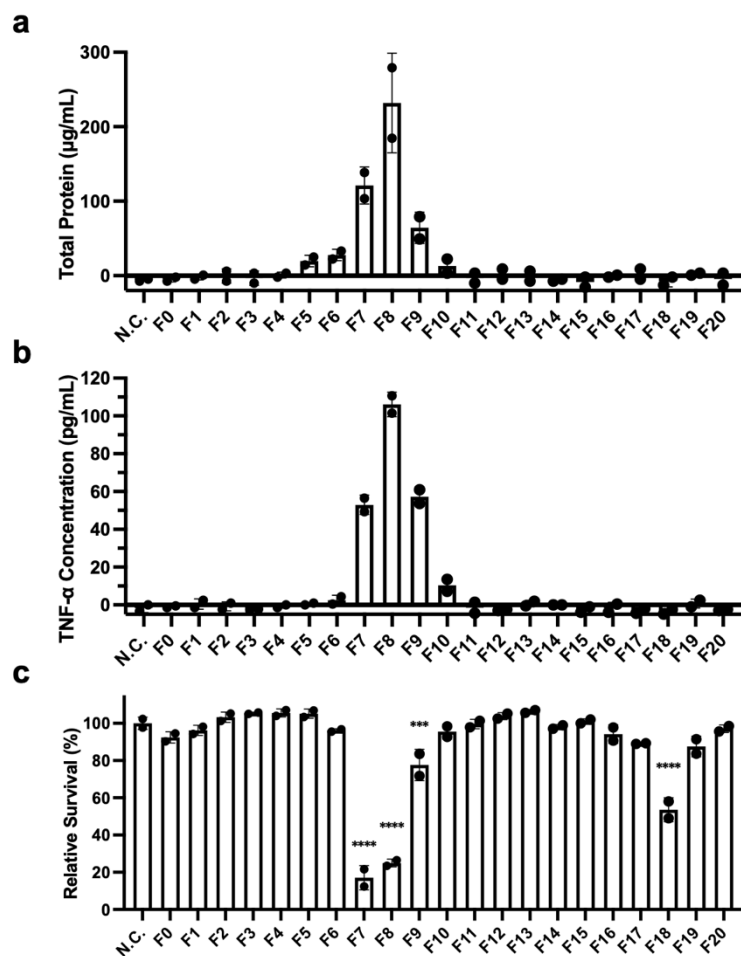
(f) WT or TNFR1 KO HeLa cells were treated with inactivated EMCV supernatant from (a), Eta.-depleted supernatant from (b), or recombinant human TNF-α (10 ng/mL) for the indicated time points, followed by Western blotting using anti-IκBα and anti-HSP70 antibodies.

For (a-c): Data are presented as the mean (SD), and individual data points are biological replicates (n = 3). Statistical significance was determined by one-way ANOVA, followed by Tukey's HSD post-hoc test. Labels: \*\*\* $p \leq 0.001$ , \*\*\*\* $p \leq 0.0001$ , ns: not significant ( $p > 0.05$ ).

### **3-4 A low molecular weight component smaller than 3 kDa released during EMCV infection synergizes with TNF- $\alpha$ to induce potent cell death**

Although TNF- $\alpha$  was confirmed to be induced during EMCV infection and identified as the critical factor triggering cell death in post-infected supernatants, one important question remained unresolved. As described in the Introduction (Sections 1-1 and 1-3), TNF- $\alpha$  alone cannot induce apoptosis in most cell types under physiological conditions, but instead primarily activates pathways such as NF- $\kappa$ B [61, 62]. For TNF- $\alpha$  signaling to result in Caspase-8-dependent apoptosis, cells must undergo sensitization through alteration of cell death regulatory mechanisms [61, 62]. Based on this well-established concept, it was hypothesized that possible additional factor(s) released into the supernatant during EMCV infection synergize with TNF- $\alpha$  to induce efficient cell death.

To investigate this possibility, EMCV-inactivated culture supernatants were fractionated by size-exclusion chromatography (SEC) using Sephadex G-25. The separated fractions were then treated onto HeLa cells to assess their cell death-inducing activities. Fractions 7–9 (F7-F9), corresponding to the void volume and containing most proteins as quantified by Bradford assay (**Figure 3-7a**), as well as TNF- $\alpha$  as measured by ELISA (**Figure 3-7b**), induced strong cell death following 24-hour treatment (**Figure 3-7c**). Notably, fraction 18 (F18), which should contain low-molecular-weight components, also exhibited weak but detectable cytotoxic activity when applied to cells alone. This observation suggested that more than one factor present in the EMCV-infected supernatant contributes to host cell death.



**Figure 3-7. Separation of supernatant components by size-exclusion chromatography.**

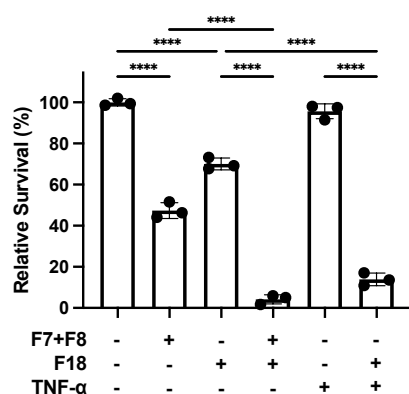
(a) HeLa cells were infected with EMCV (MOI 0.1) for 24 h, and the supernatant was acid-inactivated as in Figure 3-4a. The supernatant was separated into 20 fractions (F1–F20) using Sephadex G-25. F0 represents the initial flow-through. Total protein levels were quantified by Bradford assay.

(b) TNF-α levels in each fraction were quantified by ELISA.

(c) HeLa cells were treated with fractions from (a) diluted 1:1 with fresh DMEM for 24 h. Cells were fixed and stained using Amido Black 10B, then eluted for relative survival quantification.

Data are presented as the mean (SD), and individual data points are biological replicates (n = 2). For (c): Statistical significance was determined by one-way ANOVA, followed by Tukey's HSD post-hoc test. Asterisks indicate comparison with the N.C. group. Labels: \*\*\* $p \leq 0.001$ , \*\*\*\* $p \leq 0.0001$ .

To test whether these components actually act synergistically, the two peaks of active fractions, F7+F8 and F18, were combined and tested for cell death induction in HeLa cells (**Figure 3-8**). Treatment with either F7+F8 or F18 alone resulted in only partial cell death, while their combination induced potent synergistic cell death. Given that TNF-α had already been identified as the key trigger of cell death, F18 was next combined with recombinant human TNF-α. This combination resulted in robust cell death comparable to that observed with F7+F8 and F18 mixed together, indicating that the low-molecular-weight component in F18 synergizes with TNF-α.

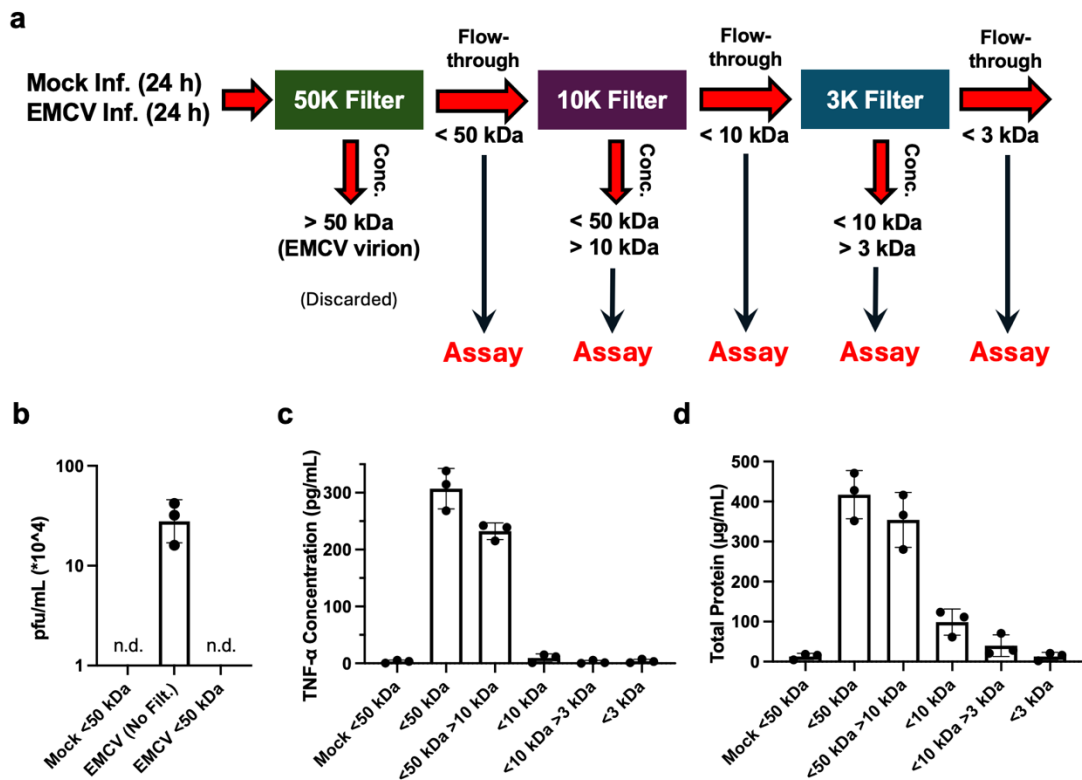


**Figure 3-8. Combination of active fractions results in potent cell death.**

HeLa cells were treated with the indicated combinations of fractions F7+F8 (diluted 4-fold with DMEM), fraction F18 (diluted 4-fold with DMEM), and the combination of F7+F8 and F18 at a final concentration of 1:1 total fractions:fresh DMEM), or recombinant human TNF-α (10 ng/mL) for 24 h. Fractions were obtained from Figure 3-7. Cells were fixed and stained with Amido Black 10B, and dye was eluted for relative survival quantification.

Data are presented as the mean (SD), and individual data points are biological replicates (n = 3). Statistical significance was determined by one-way ANOVA, followed by Tukey's HSD post-hoc test. Label: \*\*\*\* $p \leq 0.0001$ .

To precisely define the size of this synergistic low-molecular-weight factor, size-based centrifugal filtration was performed, as illustrated in **Figure 3-9a**. In this experiment, post-infected EMCV supernatants were not subjected to acid inactivation, but instead, infectious virions were first removed using a 50 kDa centrifugal filter. Complete removal of EMCV virions from the filtrate was confirmed (**Figure 3-9b**). The <50 kDa filtrate was then passed through 10 kDa and then 3 kDa centrifugal filters. The concentrates were adjusted back to their original volumes. Quantification of TNF- $\alpha$  and total protein levels revealed that TNF- $\alpha$  was contained in fractions larger than 10 kDa (<50 kDa and <50 kDa >10 kDa), consistent with its molecular size (**Figure 3-9c**), and that the majority of proteins were similarly present in the <50 kDa and <50 kDa >10 kDa fractions (**Figure 3-9d**).



**Figure 3-9. Separation of post-EMCV-infected supernatants by size filtration.**

(a) Schematic of the size-filtration experiment. HeLa cells were mock-infected or infected with EMCV (MOI 0.1) for 24 h. Supernatants were sequentially fractionated using centrifugal filtration membranes (Amicon Ultra). The 50 kDa cutoff filter retained EMCV virions (discarded). The <50 kDa flowthrough was further filtered using a 10 kDa cutoff filter to obtain the <50 kDa >10 kDa fraction and <10 kDa flowthrough. The <10 kDa flowthrough was further filtered using a 3 kDa cutoff filter to obtain the <10 kDa >3 kDa fraction and <3 kDa flowthrough. All concentrated fractions were adjusted to their original volumes.

(b) Viral titers of Mock <50 kDa flowthrough, original EMCV supernatant, and EMCV <50 kDa flow-through were quantified by plaque assay using Vero cells. n.d.: not detected ( $<1 \times 10^4$  PFU/mL).

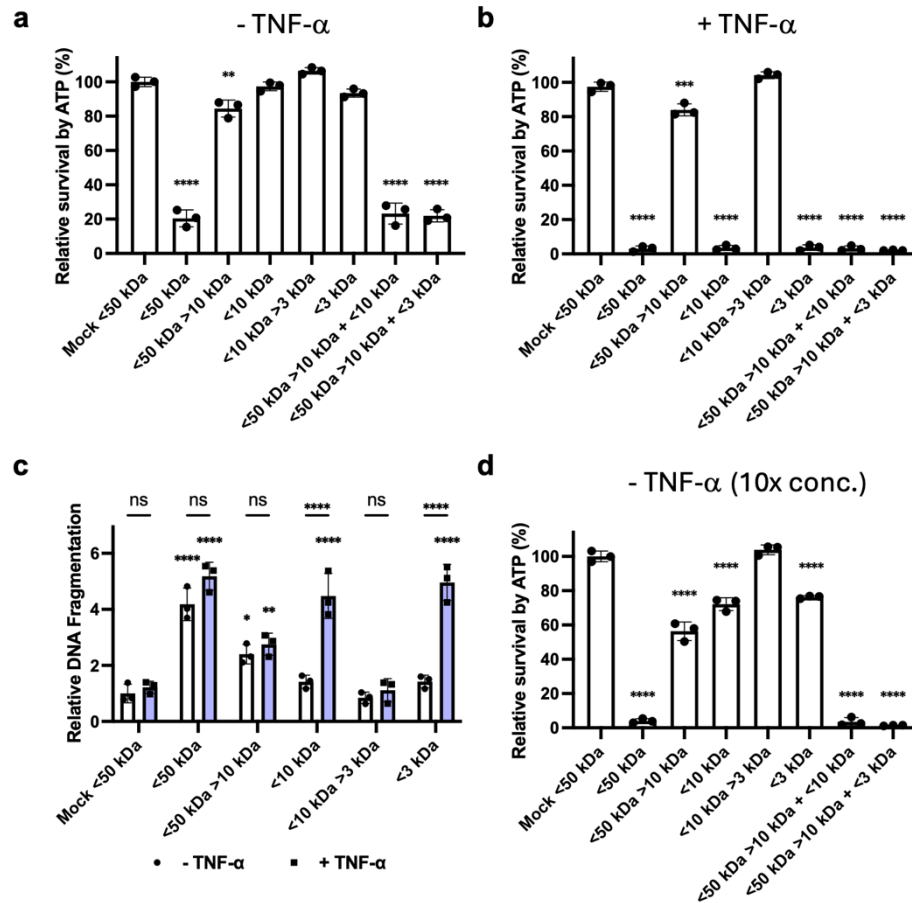
(c) TNF- $\alpha$  levels in fractions from (a) were quantified by ELISA.

(d) Total protein levels in fractions from (a) were quantified by Bradford assay.

For (b-d): Data are presented as the mean (SD), and individual data points are biological replicates ( $n = 3$ ).

All fractions obtained from this size-based separation were diluted 10-fold and then applied to HeLa cells to quantify cell death (**Figure 3-10a**). Treatment with the <50 kDa fraction alone induced rapid cell death, consistent with the fact that both TNF- $\alpha$  and the low-molecular-weight component should be contained. In contrast, treatment with the <50 kDa >10 kDa fraction alone, which contains TNF- $\alpha$  but lacks this low-molecular-weight component, resulted in only partial cell death. The <10 kDa and <3 kDa fractions alone did not induce significant cell death. However, when the <50 kDa >10 kDa fraction was combined with either the <10 kDa or <3 kDa fractions, rapid cell death was observed. These results indicated that a low-molecular-weight factor present in the <10 kDa and <3 kDa fractions synergizes with TNF- $\alpha$  to induce potent cell death. This conclusion was further supported by the observation that addition of recombinant human TNF- $\alpha$  to the <10 kDa or <3 kDa fractions showed the same cell death patterns (**Figure 3-10b**). Quantification of DNA fragmentation, which is a direct indicator of late-stage apoptosis, showed a consistent trend with or without recombinant TNF- $\alpha$  treatment (**Figure 3-10c**).

Interestingly, when fractions were applied at their original concentrations (10 $\times$  those used in **Figure 3-10a**), slight cytotoxicity was observed in the <50 kDa >10 kDa fraction as well as in the low-molecular-weight (<10 kDa and <3 kDa) fractions alone (**Figure 3-10d**). A similar phenomenon had been observed in the SEC experiments, where both F7-F8 and F18 individually exhibited some cytotoxicity (**Figures 3-8**). This suggests that the low-molecular-weight component itself may possess some mild cytotoxic activity at higher concentrations, and that additional factors larger than 10 kDa but smaller than 50 kDa could perhaps be contributing to overall cell death during EMCV infection. Nonetheless, these results clearly demonstrate that at least TNF- $\alpha$ , and a certain low-molecular-weight factor smaller than 3 kDa act synergistically to induce potent cell death.



**Figure 3-10. Quantification of cell death induced by size-filtered EMCV supernatants.**

(a) Size fractions from Figure 3-9 were diluted 10-fold with DMEM and applied to HeLa cells for 24 h, including indicated fraction combinations. Relative survival was quantified by ATP measurement using CellTiter-Glo.

(b) HeLa cells were treated as in (a) in the presence of recombinant human TNF- $\alpha$  (10 ng/mL).

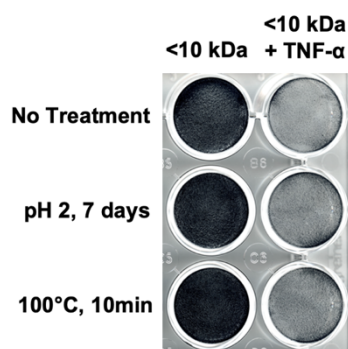
(c) HeLa cells were treated as in (a) (without TNF- $\alpha$ ) or (b) (with TNF- $\alpha$ ) for 9 h. Cells were lysed and DNA fragmentation was quantified by ELISA. Relative DNA fragmentation was calculated by setting the mean value of the mock <50 kDa, -TNF- $\alpha$  condition to 1.

(d) HeLa cells were treated as in (a) without dilution of the size fractions (original concentrations; 10 $\times$  of (a)), and relative survival was quantified as in (a).

For (a-d): Data are presented as the mean (SD), and individual data points are biological replicates ( $n = 3$ ). Statistical significance was determined by (a, b, d): one-way ANOVA, or (c): two-way ANOVA, followed by Tukey's HSD post-hoc test. (a, b, d): Asterisks indicate comparison with the Mock <50 kDa group. (c): Asterisks indicate comparison with the Mock <50 kDa, -TNF- $\alpha$  group. Labels: \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ , \*\*\*\* $p \leq 0.0001$ , ns: not significant ( $p > 0.05$ ).

To confirm that the activity of the <3 kDa factor was not some artifact generated from acid inactivation used earlier, post-EMCV-infected supernatants were first filtered through a 10 kDa centrifugal filter to remove both EMCV virions and TNF- $\alpha$ , followed by acid treatment (pH 2.0 for 7 days). In addition, to assess stability at high temperatures, the filtered supernatant was subjected to heat treatment (100°C for 10 minutes). These treated supernatants were then applied to fresh HeLa cells in the

presence or absence of recombinant TNF- $\alpha$  for 24 hours (**Figure 3-11**). No significant loss of cytotoxic activity was observed following either acid or heat treatment, indicating that this low-molecular-weight compound exhibits resistance to acid (low pH) and heat to a certain extent.



**Figure 3-11. Acid or heat treatment does not significantly affect cytotoxic activity of the EMCV supernatant.**

HeLa cells were infected with EMCV (MOI 0.1) for 24 h, and the supernatant was filtered through a 10 kDa cutoff membrane. A portion of the flowthrough was then treated with acid (pH 2, 7 days) or heat (100 °C, 10 min). These samples, including the non-treated condition, were applied to fresh HeLa cells for 24 h, with or without recombinant human TNF- $\alpha$  (10 ng/mL). Cells were fixed and stained with Amido Black 10B for survival quantification.

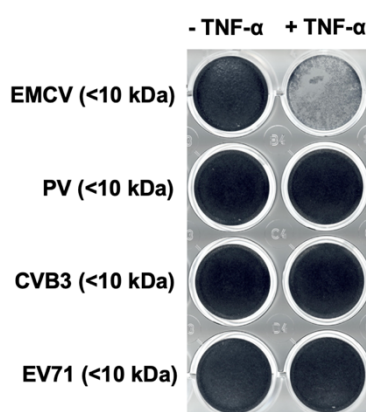
### **3-5 *Cardiovirus*-derived protein VPg induces synergistic cell death with TNF- $\alpha$ during EMCV infection**

At this stage, it had become clear that EMCV infection results in the release of cell death-inducing factors into the culture supernatant. TNF- $\alpha$  was identified as the critical trigger of apoptosis; however, additional factors were clearly required to synergize with TNF- $\alpha$  to induce potent cell death. The most intriguing component identified so far was this certain low-molecular-weight factor smaller than 3 kDa, isolated using both SEC and size-based centrifugal filtration. This factor also exhibited resistance to acid and heat treatment to a certain extent. Despite these findings, it remained unclear whether this component was something host-derived, or virus-derived. Importantly, this phenomenon was specific to EMCV infection and was clearly not observed after infection with other RNA viruses such as SeV, NDV, or VSV.

To determine whether this effect was unique to EMCV, or more broadly conserved among closely-related viruses, other members of the *Picornaviridae* family belonging to a different genus were examined. In contrast to EMCV, which belongs to the genus *Cardiovirus*, *Enterovirus* species were selected for comparison. With the assistance of a collaborator, poliovirus (PV, type I Mahoney strain), coxsackievirus B3 (CVB3, Nancy strain), and enterovirus 71 (EV71, strain Isehara VP1-145Q variant, selected for higher infectivity in HeLa cells [99]) were used to infect HeLa cells with

EMCV as the positive control. After 24 hours of infection, culture supernatants were filtered using a 10 kDa centrifugal filter to remove infectious virions and most cytokines, including TNF- $\alpha$ . The resulting filtrates were then treated to fresh HeLa cells, with or without the addition of recombinant human TNF- $\alpha$ .

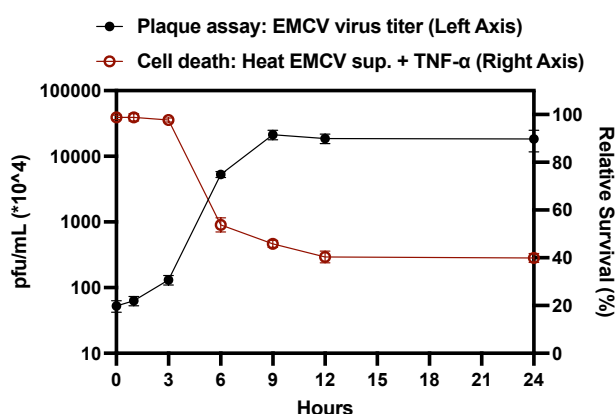
Interestingly, filtrates derived from all *Enterovirus* infections (PV, CVB3, and EV71) exhibited no cytotoxic activity, even when combined with recombinant TNF- $\alpha$ . In contrast, only the EMCV-derived supernatant showed clear TNF- $\alpha$ -dependent synergistic cytotoxicity (**Figure 3-12**). These results suggested that this synergistic factor is specific to EMCV infection, which means it is quite unlikely to represent some broadly induced host-derived factor such as IFNs or other inflammatory cytokines.



**Figure 3-12. *Enterovirus*-infected supernatant flowthroughs do not exhibit cytotoxicity with TNF- $\alpha$ .**

HeLa cells were infected with EMCV, PV, CVB3, or EV71 (MOI 1.0) for 24 h, and the supernatants were filtered through a 10 kDa cutoff membrane. The flowthroughs were applied to fresh HeLa cells for 24 h, with or without recombinant human TNF- $\alpha$  (10 ng/mL). Cells were fixed and stained with Amido Black 10B for survival quantification.

Further investigation revealed that the kinetics of EMCV replication was in parallel with the appearance of cytotoxic activity in the culture supernatant. Specifically, both EMCV titers and the release of the cytotoxic component significantly increased approximately 6 hours post-infection (**Figure 3-13**), a time point that corresponds with viral encapsidation and host cell lysis during the EMCV replication cycle [19].



**Figure 3-13. Increase of EMCV titer parallels the appearance of cytotoxic compounds in the supernatant.**

HeLa cells were infected with EMCV (MOI 0.1). Left axis: EMCV titers in the supernatant at the indicated time points were measured by plaque assay in Vero cells. Right axis: Supernatants collected at the indicated time points post-infection were heat-inactivated (100 °C, 10 min) to inactivate both EMCV and TNF- $\alpha$ . After 2-fold dilution with DMEM, samples were applied to fresh HeLa cells together with recombinant human TNF- $\alpha$  (10 ng/mL) for 24 h. Cells were fixed and stained with Amido Black 10B, then dye was eluted for relative survival quantification.

For both axes: Data are presented as the mean (SD), and data are biological replicates (n = 3).

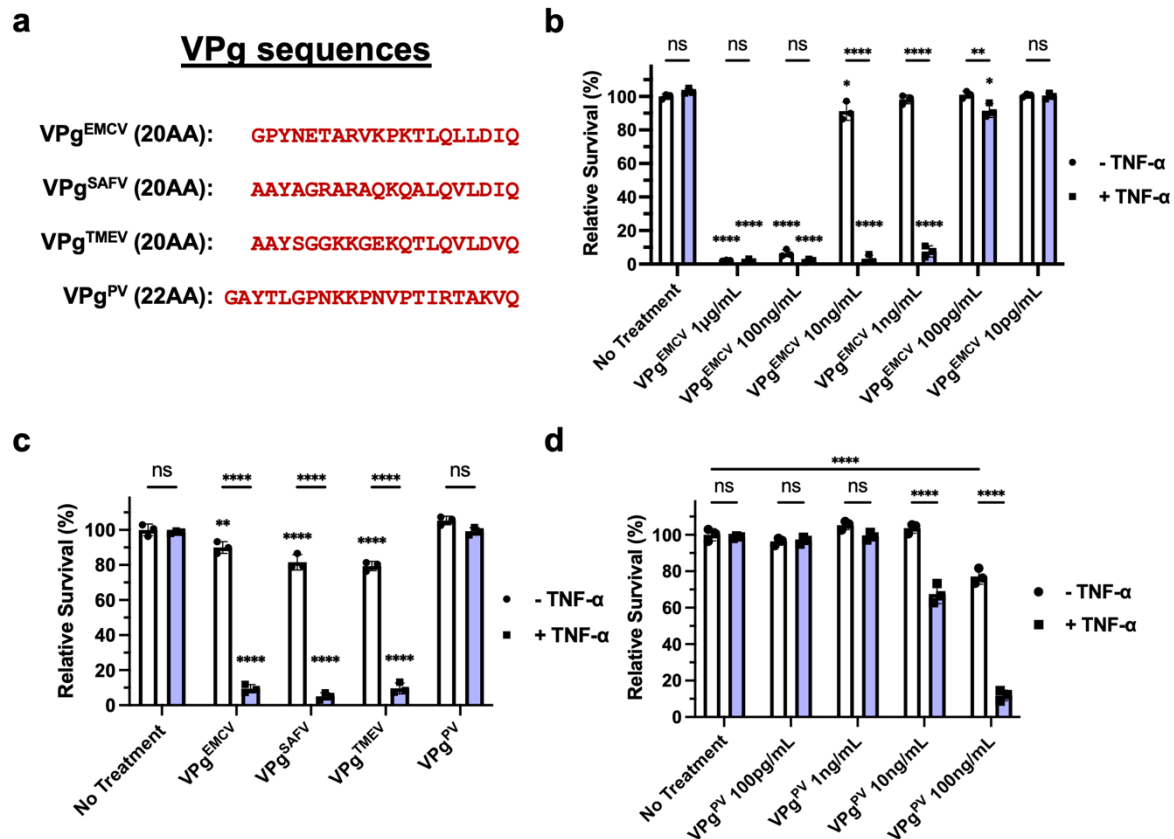
Based on these observations, the focus turned to potential EMCV-derived components. One candidate in mind was the smallest protein (of peptide size) encoded by EMCV, which was viral protein genome-linked (VPg/3B), as introduced previously (Sections 1-2 and 1-5). Curiously, one past report demonstrated that knockout of tyrosyl-DNA phosphodiesterase 2 (TDP2), the host enzyme that functions as VPg unlinkase during the EMCV life cycle (described also in Section 1-2-2), severely impairs viral growth and reduces viral titers [29, 30]. The same group also reported that TDP2 is cleaved approximately 6 hours post-EMCV infection (although the biological significance is yet unknown), and this phenomenon was not observed during *Enterovirus* infections such as PV or CVB3 [30].

Although the primary focus of these past studies was not regarding VPg itself, the temporal overlap between TDP2 cleavage, EMCV replication kinetics, and the appearance of this cytotoxic factor in the supernatant, together with the known fact that VPg of EMCV (referred to as VPg<sup>EMCV</sup> thereafter) is the only viral protein smaller than 3 kDa (~2.3 kDa [19], also see Section 1-2-3), led to the hypothesis that VPg<sup>EMCV</sup> may actually be this low-molecular-weight factor responsible for synergistic apoptosis in combination with TNF- $\alpha$ .

VPg<sup>EMCV</sup> consists of only 20 amino acids (**Figure 3-14a**), making it amenable to peptide synthesis. A synthetic 20-amino-acid VPg<sup>EMCV</sup> peptide was therefore generated and tested for its ability to induce cell death in HeLa cells with TNF- $\alpha$ . Treatment with VPg<sup>EMCV</sup> in combination with recombinant human TNF- $\alpha$  resulted in potent synergistic cell death within 24 hours, closely resembling the effects observed with the EMCV-inactivated supernatants earlier. This synergy was observed at concentrations below 1 ng/mL (**Figure 3-14b**). Interestingly, treatment with VPg<sup>EMCV</sup> alone at higher concentrations (~100 ng/mL) also induced cell death in the absence of recombinant TNF- $\alpha$ .

To assess whether this activity was conserved among VPgs from other *Picornaviridae*, VPgs of *Cardiovirus* members and from *Enterovirus* (**Figure 3-14a**) were synthesized and examined. In addition to VPg<sup>EMCV</sup>, VPg from Saffold virus (VPg<sup>SAFV</sup>), VPg from TMEV (VPg<sup>TMEV</sup>), and VPg from poliovirus (VPg<sup>PV</sup>) were synthesized and tested (**Figure 3-14c**). VPg<sup>EMCV</sup>, VPg<sup>SAFV</sup>, and VPg<sup>TMEV</sup>, all derived from *Cardiovirus*, exhibited strong synergistic activity with TNF- $\alpha$  in inducing cell death. In contrast, VPg<sup>PV</sup> did not show comparable activity with VPg<sup>EMCV</sup>. However, at higher concentrations ( $\geq 100$  ng/mL), VPg<sup>PV</sup> did exhibit weak cytotoxic effects,

although its activity was approximately 100-fold lower than that of *Cardiovirus* VPgs (Figure 3-14d). These findings are consistent with the infection/filtration experiments and indicate that VPgs derived from *Cardiovirus* possibly possess a unique ability to sensitize cells to TNF- $\alpha$ -mediated apoptosis.



**Figure 3-14. *Cardiovirus* VPgs induce dose-dependent cell death with TNF- $\alpha$ .**

(a) VPg sequences for four viruses in the *Picornaviridae* family are compared.

(b) HeLa cells were treated at indicated concentrations of VPg<sup>EMCV</sup>, with or without recombinant TNF- $\alpha$  (10 ng/mL). Cells were fixed and stained using Amido Black 10B, then eluted for relative survival quantification.

(c) HeLa cells were treated with 1 ng/mL of VPg<sup>EMCV</sup>, VPg<sup>SAFV</sup>, VPg<sup>TMEV</sup>, and VPg<sup>PV</sup>, with or without recombinant TNF- $\alpha$  (10 ng/mL), and survival quantification was performed in the same manner as (b).

(d) HeLa cells were treated at indicated concentrations of VPg<sup>PV</sup>, with or without recombinant TNF- $\alpha$  (10 ng/mL), and survival quantification was performed in the same manner as (b).

For (b-d): Data are presented as the mean (SD), and individual data points are biological replicates ( $n = 3$ ). Statistical significance was determined by two-way ANOVA, followed by Tukey's HSD post-hoc test. For (b-c): Asterisks indicate comparison with the No Treatment, -TNF- $\alpha$  group. Labels: \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\*\* $p \leq 0.0001$ , ns: not significant ( $p > 0.05$ ).

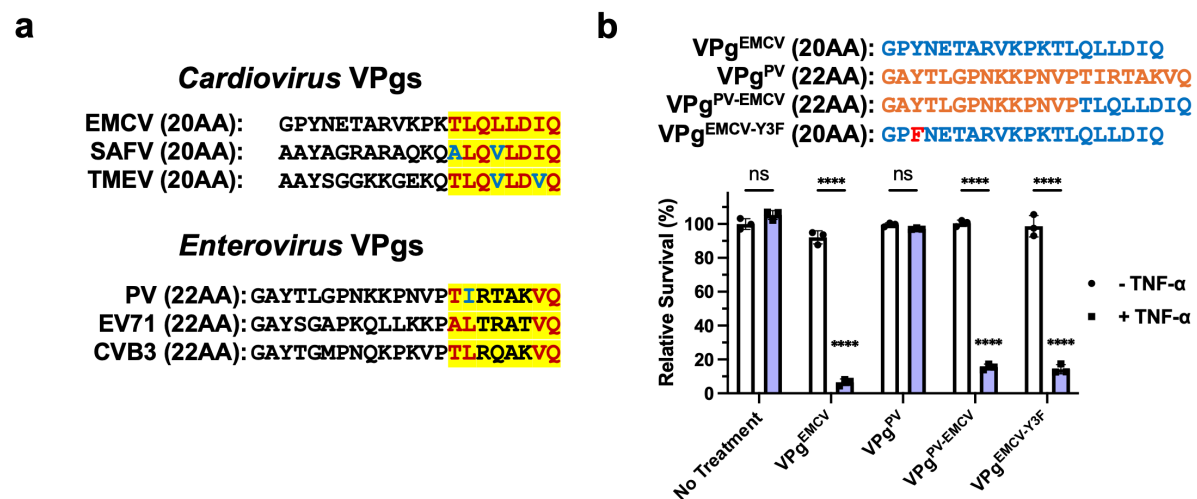
### 3-6 Identification of critical amino acid residues required for VPg<sup>EMCV</sup> activity

To further investigate the amino acid residues required for VPg-mediated cell death sensitization, the amino acid sequences of VPgs derived from *Cardiovirus* and *Enterovirus* were compared in detail (Figure 3-15a). As shown in the figure, VPgs

from *Cardiovirus* share eight highly conserved amino acids at the C-terminus, a feature that is absent in *Enterovirus* VPgs. Based on this observation, it was hypothesized that these conserved C-terminal residues are important for full VPg<sup>EMCV</sup> activity.

To test this hypothesis, a chimeric VPg peptide was synthesized (VPg<sup>PV-EMCV</sup>), in which the C-terminal eight amino acids were derived from VPg<sup>EMCV</sup>, while the N-terminal region come from VPg<sup>PV</sup>. The activity of this hybrid peptide was compared with that of VPg<sup>EMCV</sup> and VPg<sup>PV</sup>. Notably, this VPg<sup>PV-EMCV</sup> hybrid exhibited synergistic activity with TNF- $\alpha$  comparable to that of VPg<sup>EMCV</sup>, demonstrating that the eight C-terminal residues should be critical for VPg<sup>EMCV</sup> activity (**Figure 3-15b**).

To determine whether the cell death-sensitizing function of VPg<sup>EMCV</sup> may be independent of its canonical role as a primer for viral RNA synthesis, the third tyrosine residue (Y3) was substituted with phenylalanine (VPg<sup>EMCV-Y3F</sup>). This tyrosine residue is known to be essential for VPg uridylation and viral replication, and mutation at this site abolishes its canonical function ([31], also briefly explained in Section 1-2-2). This VPg<sup>EMCV-Y3F</sup> mutant still retained cell death-sensitizing activity comparable to that of VPg<sup>EMCV</sup> in the presence of TNF- $\alpha$  (**Figure 3-15b**), indicating that this cell death-related function of VPg<sup>EMCV</sup> is possibly completely independent of its role in viral RNA synthesis.



**Figure 3-15. The 8 C-terminal amino acids are critical for VPg activity.**

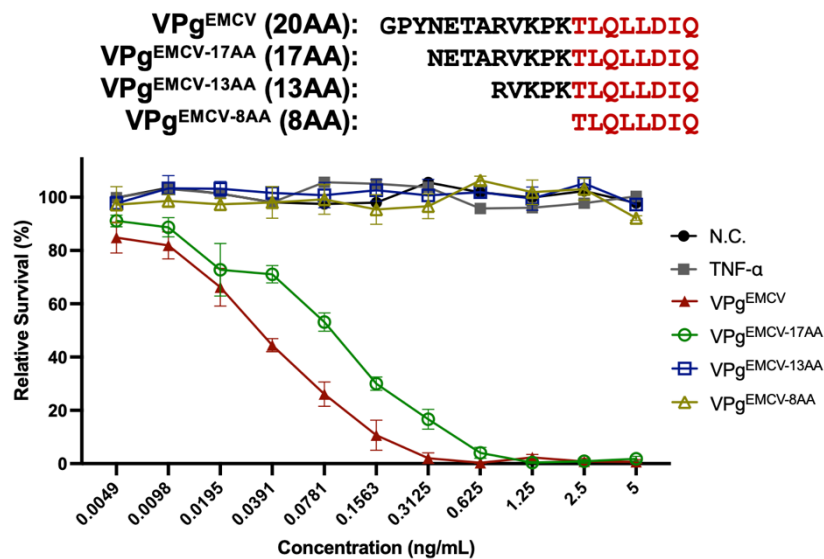
(a) VPg sequences of 3 *Cardiovirus* (EMCV, SAFV, TMEV), as well as 3 *Enterovirus* (PV, EV71, CVB3) are compared. The 8 C-terminal conserved amino acid region is highlighted in yellow. Identical amino acid residues to EMCV are in red, and closely-related amino acids are in blue.

(b) HeLa cells were treated with 1 ng/mL of VPg<sup>EMCV</sup>, VPg<sup>PV</sup>, VPg<sup>PV-EMCV</sup>, and VPg<sup>EMCV-Y3F</sup>, with or without recombinant TNF- $\alpha$  (10 ng/mL). Cells were fixed and stained using Amido Black 10B, then eluted for relative survival quantification.

For (b): Data are presented as the mean (SD), and individual data points are biological replicates ( $n = 3$ ). Statistical significance was determined by two-way ANOVA, followed by Tukey's HSD post-hoc test. Asterisks indicate comparison with the No Treatment, -TNF- $\alpha$  group. Labels: \*\*\*\* $p \leq 0.0001$ , ns: not significant ( $p > 0.05$ ).

Given that the C-terminal residues were shown to be critical, it was next hypothesized that the C-terminal region alone might be sufficient for VPg<sup>EMCV</sup> activity. To test this, N-terminally truncated VPg<sup>EMCV</sup> peptides were synthesized. These included VPg<sup>EMCV</sup> comprised of 17 amino acids (VPg<sup>EMCV-17AA</sup>), 13 amino acids (VPg<sup>EMCV-13AA</sup>), and 8 amino acids (VPg<sup>EMCV-8AA</sup>), which were evaluated for their ability to sensitize cells to TNF- $\alpha$ -induced cell death.

Unexpectedly, truncation of the N-terminal residues resulted in a marked loss of activity (**Figure 3-16**). While VPg<sup>EMCV-17AA</sup> retained partial activity, its cell death-sensitizing ability was markedly reduced compared with the full-length 20AA VPg<sup>EMCV</sup>. Curiously, VPg<sup>EMCV-13AA</sup> and VPg<sup>EMCV-8AA</sup> completely lost any detectable activity whatsoever. These findings indicate that although the conserved C-terminal residues in *Cardiovirus* are essential, the N-terminal region and overall peptide length are also critical for maintaining VPg<sup>EMCV</sup> activity.



**Figure 3-16. Truncation of N-terminal amino acids of VPg lose functional activity.**

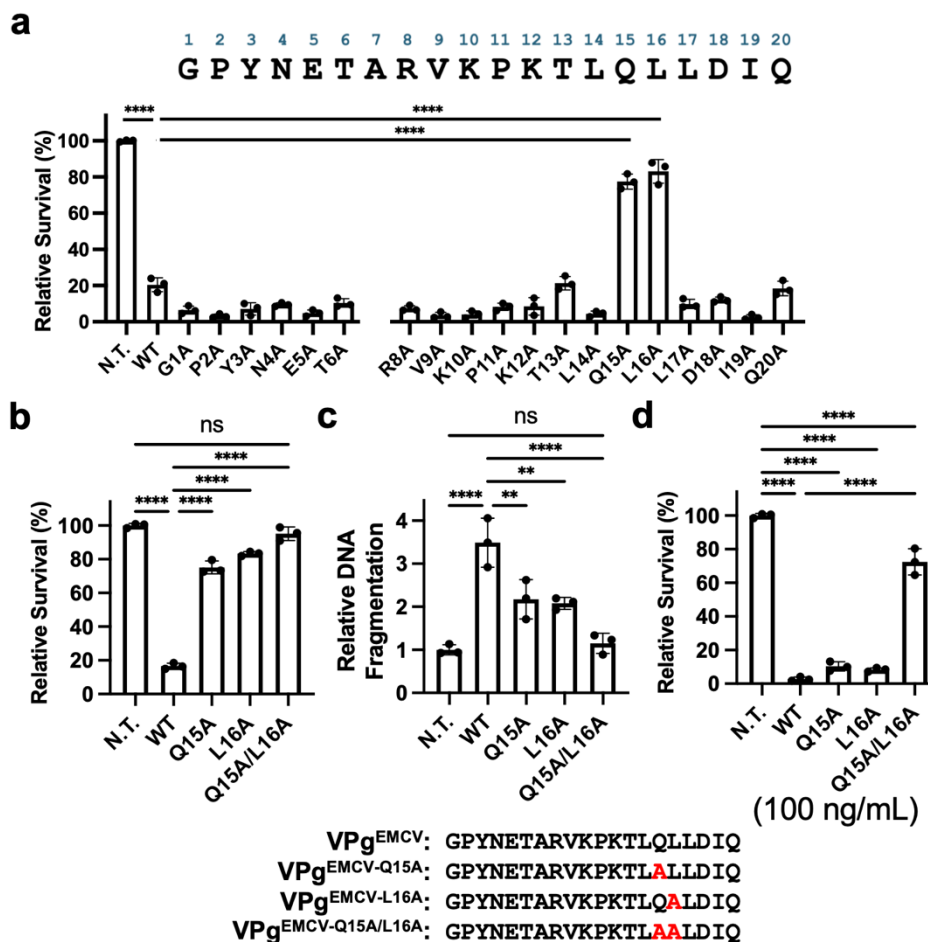
HeLa cells were treated for 24 h with 10 ng/mL recombinant human TNF- $\alpha$ , together with 2-fold serial dilutions (starting from 5 ng/mL) of VPg<sup>EMCV</sup>, VPg<sup>EMCV-17AA</sup>, VPg<sup>EMCV-13AA</sup>, and VPg<sup>EMCV-8AA</sup>. Conditions without the addition of TNF- $\alpha$  were also prepared (N.C.). Cells were fixed and stained using Amido Black 10B, then eluted for relative survival quantification.

Data are presented as the mean (SD), and data are biological replicates (n = 3).

To further identify specific amino acid residues required for VPg<sup>EMCV</sup> activity, alanine-scanning mutants were generated for each of the 20 amino acids. These mutant peptides were tested at a concentration of 10 ng/mL in HeLa cells in the presence of recombinant TNF- $\alpha$ . Substitution of the 15th glutamine (Q) residue with alanine (VPg<sup>EMCV-Q15A</sup>) or the 16th leucine (L) residue with alanine (VPg<sup>EMCV-L16A</sup>)

resulted in a significant reduction in cell death-sensitizing activity (**Figure 3-17a**). A double mutant combining both substitutions (VPg<sup>EMCV-Q15A/L16A</sup>) was also synthesized, and this peptide exhibited an even greater loss of activity (**Figure 3-17b**).

Quantification of DNA fragmentation further confirmed that late-stage apoptosis was markedly reduced in cells treated with these mutant peptides (**Figure 3-17c**). When tested at a 10-fold higher concentration (100 ng/mL), the two single mutants induced significant cell death, while the double mutant remained mostly inactive, although minimal cytotoxicity was observed (**Figure 3-17d**). Altogether, these results demonstrate that the C-terminal residues, particularly the 15th glutamine (Q) and 16th leucine (L), are critical for VPg<sup>EMCV</sup> activity, while the N-terminal region and overall peptide length also play essential roles in maintaining full biological function.



**Figure 3-17. Amino acid residues Q15 and L16 are crucial for VPg<sup>EMCV</sup> activity.**

(a) VPg<sup>EMCV</sup> with point mutant alanine substitutions in each residue (except A7) were generated, and these were tested for their ability to induce cell death (10 ng/mL), treated together with recombinant TNF- $\alpha$  (10 ng/mL) for 24 h. Cells were fixed and stained using Amido Black 10B, then eluted for relative survival quantification.

(b) HeLa cells were treated with 10 ng/mL of VPg<sup>EMCV</sup>, VPg<sup>EMCV-Q15A</sup>, VPg<sup>EMCV-L16A</sup>, and VPg<sup>EMCV-Q15A/L16A</sup>, with recombinant TNF- $\alpha$  (10 ng/mL) for 24 h. Survival quantification was performed in the same manner as (a).

(c) HeLa cells were treated with identical synthetic peptides and recombinant TNF- $\alpha$  with (b) for 9 h. Cells were lysed and DNA fragmentation levels were measured by ELISA. Relative DNA fragmentation values were calculated by setting the mean values of N.T. as 1.

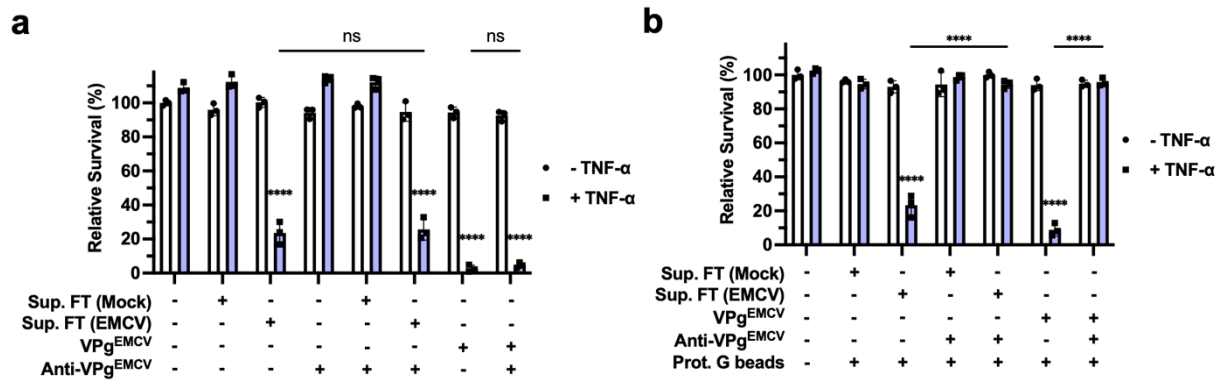
(d) HeLa cells were treated with identical synthetic peptides as in (b) but at 100 ng/mL, together with recombinant TNF- $\alpha$  (10 ng/mL) for 24 h. Survival quantification was performed in the same manner as (a).

Data are presented as the mean (SD), and individual data points are biological replicates ( $n = 3$ ). Statistical significance was determined by one-way ANOVA, followed by Tukey's HSD post-hoc test. Labels: \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.0001$ , ns: not significant ( $p > 0.05$ ).

### 3-7 Confirmation of VPg<sup>EMCV</sup> as the component released during EMCV infection

Although the amino acid residues required for VPg<sup>EMCV</sup> activity were identified, it had not yet been directly clarified that the low-molecular-weight component released into the culture supernatant during EMCV infection was indeed VPg<sup>EMCV</sup>. To address this question, a polyclonal rabbit antibody against VPg<sup>EMCV</sup> was generated (anti-3B/VPg<sup>EMCV</sup>). This antibody was first tested for its ability to directly neutralize the activity of VPg<sup>EMCV</sup>. The anti-VPg<sup>EMCV</sup> antibody was incubated with either EMCV-infected culture supernatant or synthetic VPg<sup>EMCV</sup> as a control, followed by treatment of HeLa cells in the presence or absence of recombinant TNF- $\alpha$  to assess cell death. Curiously, the anti-VPg<sup>EMCV</sup> antibody failed to neutralize the cell death-inducing activity of the EMCV supernatant. However, this result was consistent with the observation that the antibody was also unable to neutralize the activity of synthetic VPg<sup>EMCV</sup> itself, indicating that direct neutralization by the antibody was ineffective (**Figure 3-18a**).

Because of this limitation, an alternative approach was used, which was immunoprecipitation and depletion of VPg<sup>EMCV</sup> from the supernatant. The post-infected EMCV supernatant, as well as synthetic VPg<sup>EMCV</sup>, was subjected to pulldown using the anti-VPg<sup>EMCV</sup> antibody in combination with Protein G beads. The depleted samples were then treated to HeLa cells with or without recombinant TNF- $\alpha$ . Under these conditions, cell death-inducing activity was completely abrogated for both the EMCV supernatant and synthetic VPg<sup>EMCV</sup>, indicating that the low-molecular-weight component acting synergistically with TNF- $\alpha$  in the supernatant is indeed VPg<sup>EMCV</sup> (**Figure 3-18b**).



**Figure 3-18. Neutralization and depletion attempt of VPg<sup>EMCV</sup> in the supernatant.**

HeLa cells were Mock-infected or infected with EMCV (MOI 0.1) for 24 h, then the supernatants were heated (100 °C, 10 min) for virus and cytokine inactivation.

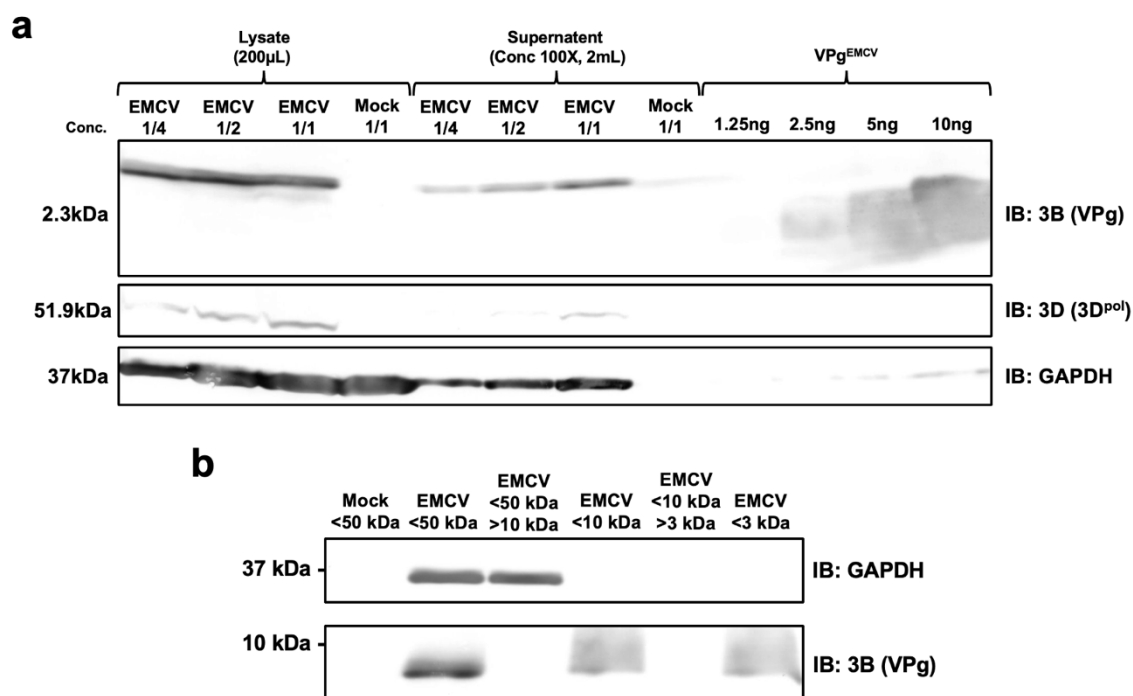
(a) The Mock and EMCV supernatants, as well as 2 ng/mL synthetic VPg<sup>EMCV</sup> in DMEM were mixed with anti-VPg<sup>EMCV</sup> antibody overnight for neutralization attempt. Fresh HeLa cells were treated with these samples (2-fold dilution) for 24 h, with or without the addition of recombinant TNF-α (10 ng/mL). Cells were fixed and stained using Amido Black 10B, then eluted for relative survival quantification.

(b) The Mock and EMCV supernatants, as well as 2 ng/mL synthetic VPg<sup>EMCV</sup> in DMEM were immunoprecipitated overnight using Protein G beads, with or without the pre-binding of anti-VPg<sup>EMCV</sup> antibody with beads. These samples were diluted and treated with or without recombinant TNF-α similarly as (a), and survival quantification was performed in the same manner as (a).

For (a, b): Data are presented as the mean (SD), and individual data points are biological replicates (n = 3). Statistical significance was determined by two-way ANOVA, followed by Tukey's HSD post-hoc test. Asterisks indicate comparison with the negative control (- all treatments), -TNF-α group. Labels: \*\*\*\* $p \leq 0.0001$ , ns: not significant ( $p > 0.05$ ).

To further confirm the presence of VPg<sup>EMCV</sup> in EMCV-infected samples, Western blot analysis was performed to detect VPg<sup>EMCV</sup> in both infected cell lysates and post-infected culture supernatants. Known concentrations of synthetic VPg<sup>EMCV</sup> were included as references. The culture supernatants were concentrated using a centrifugal evaporator to increase protein yield before analysis. VPg<sup>EMCV</sup> was detected in both infected cell lysates and culture supernatants, along with the viral 3D protein (3D<sup>Pol</sup>) (**Figure 3-19a**). Cytoplasmic glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was also detected in the post-infected supernatant, indicating that viral infection possibly resulted in cell lysis, following release of its cytosolic contents. Interestingly, the migration patterns of natural and synthetic VPg<sup>EMCV</sup> differed slightly: natural VPg<sup>EMCV</sup> appeared as a sharp band, while synthetic VPg<sup>EMCV</sup> appeared as a smeared band with slightly faster migration.

To determine whether VPg<sup>EMCV</sup> was present in the size-based fractions previously shown to exhibit activity, Western blotting was performed on fractions obtained by centrifugal filtration (<50 kDa, <50 kDa >10 kDa, <10 kDa, <10 kDa >3 kDa, and <3 kDa). VPg<sup>EMCV</sup> was detected in the <50 kDa, <10 kDa, and <3 kDa fractions (**Figure 3-19b**), consistent with its predicted molecular size and confirming that VPg<sup>EMCV</sup> corresponds to the <3 kDa component responsible for synergistic cell death with TNF- $\alpha$ . GAPDH, which is approximately 37 kDa in size and likely released due to cytosolic leakage during EMCV-induced cell lysis, was detected only in the <50 kDa and <50 kDa >10 kDa fractions, indicating that size-based fractionation was performed appropriately.



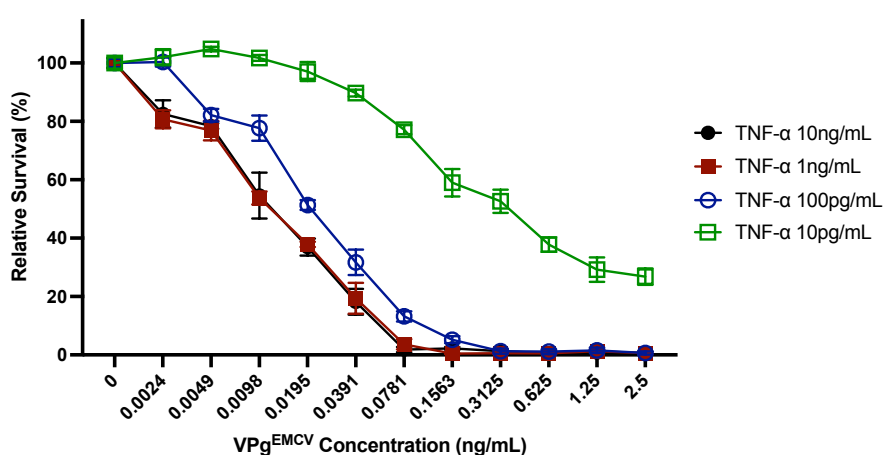
**Figure 3-19. VPg<sup>EMCV</sup> is detected in the post-infected supernatants or fractions.**

(a) HeLa cells were Mock-infected or infected with EMCV (MOI 0.1) for 9 h. The supernatants and cell lysates (in 200  $\mu$ L lysis buffer) were then collected. The supernatants were concentrated 100x of the original concentration using centrifugal concentration. The supernatants and lysates were subjected to SDS-PAGE (20  $\mu$ L/lane) without dilution (1/1), diluted 2-fold (1/2) and 4-fold (1/4). As reference, 2-fold serial dilutions of known concentrations of synthetic VPg<sup>EMCV</sup> (from 10 ng) were similarly loaded as indicated. Western blot analysis was performed using anti-VPg<sup>EMCV</sup>, anti-Mengo 3D, and anti-GAPDH antibodies.

(b) The fractions obtained from using size filtration from Figure 3-9 were concentrated 100x of the original concentration using centrifugal evaporation, then subjected to Western blotting (10  $\mu$ L/lane) using anti-VPg<sup>EMCV</sup> and anti-GAPDH.

Next, it was examined whether the concentration of TNF- $\alpha$  secreted during EMCV infection was sufficient to induce cell death in combination with VPg<sup>EMCV</sup>. In most experiments involving recombinant TNF- $\alpha$ , a concentration of 10 ng/mL was used, as this is the manufacturer's recommended dose and is commonly used as a saturating concentration in many literatures [115, 116]. However, ELISA quantification from previous experiments (**Figures 3-6a, 3-7b, and 3-9c**) indicated that approximately 100-350 pg/mL of TNF- $\alpha$  is released into the culture supernatant during EMCV infection. To address this question, recombinant human TNF- $\alpha$  at concentrations of 10 ng/mL, 1 ng/mL, 100 pg/mL, and 10 pg/mL was combined with 2-fold serial dilutions of VPg<sup>EMCV</sup> (starting at 2.5 ng/mL) and applied to HeLa cells for 24 hours to assess cell death.

These experiments demonstrated that 1 ng/mL TNF- $\alpha$  was sufficient to achieve near-maximal efficacy, with no significant difference compared to 10 ng/mL (**Figure 3-20**). VPg<sup>EMCV</sup> exhibited strong cell death-sensitizing activity even at very low concentrations. At 100 pg/mL TNF- $\alpha$ , the synergistic effect with VPg<sup>EMCV</sup> was slightly reduced at lower VPg<sup>EMCV</sup> concentrations (<0.1 ng/mL) but remained sufficient to induce potent cell death. At 10 pg/mL TNF- $\alpha$ , cell death was still detectable, although significantly attenuated. These results indicate that the 100–350 pg/mL TNF- $\alpha$  levels detected in EMCV-infected supernatants are sufficient to induce detectable cell death under the tested conditions.



**Figure 3-20. TNF- $\alpha$  induced during EMCV infection is sufficient to induce cell death with VPg<sup>EMCV</sup>.**

HeLa cells were treated with 2-fold serially diluted VPg<sup>EMCV</sup> (starting from 2.5 ng/mL), together with varying concentrations of recombinant human TNF- $\alpha$  (10 ng/mL, 1 ng/mL, 100 pg/mL, and 10 pg/mL) for 24 h. The condition without adding VPg<sup>EMCV</sup> (0 ng/mL) was placed as negative control. Cells were fixed and stained using Amido Black 10B, then eluted for relative survival quantification.

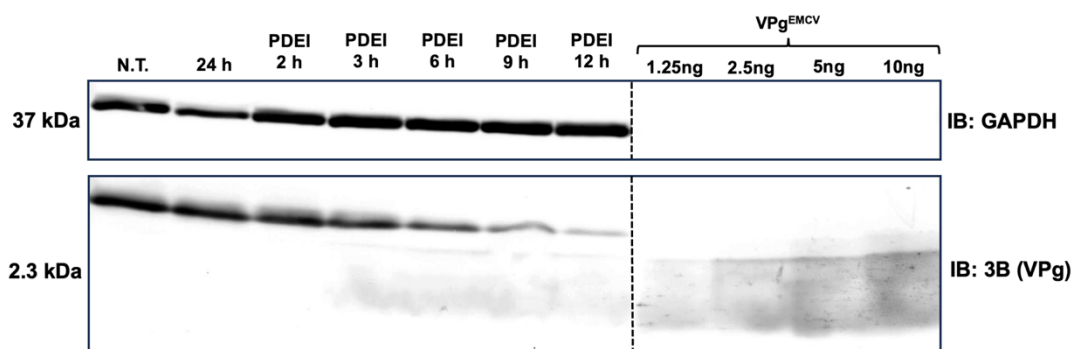
Data are presented as the mean (SD), and data are biological replicates (n = 3).

### 3-8 Identification of the modification status and quantification of natural VPg<sup>EMCV</sup>, and comparison of its activity with synthetic VPg<sup>EMCV</sup>

An important observation in the previous section was the difference in electrophoretic mobility and immunoblot band patterns between natural and synthetic VPg<sup>EMCV</sup>. It has been already established for a long time that VPg can exist in a uridylated form, commonly referred to as VPg-pUpU ([31, 117]; also briefly mentioned in Section 1-2-2). One previous report [117] also showed that the immunoblotting patterns of free VPg and uridylated VPg (VPg-pUpU) are distinct: VPg-pUpU appears as a sharp band, whereas free VPg migrates slightly faster and often appears as a smeared band. These characteristics from past literature closely resembled the band patterns observed in this study, where natural VPg<sup>EMCV</sup> appeared as a sharp band and synthetic VPg<sup>EMCV</sup> appeared as a smear.

It is also known that phosphodiesterase I (PDEI) digestion can remove the covalently attached uridine residues from VPg-pUpU, converting uridylated VPg into its free form [118]. Based on these reports, it was hypothesized that the observed difference in band patterns between natural and synthetic VPg<sup>EMCV</sup> was due to uridylation of natural VPg<sup>EMCV</sup>, and that PDEI treatment should convert the sharp band of natural VPg<sup>EMCV</sup> into a smear-like pattern similar to that of synthetic VPg<sup>EMCV</sup>.

To test this hypothesis, snake venom-derived PDEI was obtained and applied to EMCV-infected culture supernatants following a previously reported protocol [118]. As expected, PDEI treatment converted the sharp band of natural VPg<sup>EMCV</sup> into a smeared band, similar to that of synthetic VPg<sup>EMCV</sup> (**Figure 3-21**). Prolonged PDEI treatment, however, resulted in a reduction of overall band intensity. Although the exact cause of this reduction remains unclear, it is possible that trace impurities in the snake venom-derived PDEI, such as residual protease activity may have contributed to degradation. Nevertheless, these results support the conclusion that the difference in band patterns between natural and synthetic VPg<sup>EMCV</sup> are due to uridylation of VPg<sup>EMCV</sup>, consistent with previous reports.



**Figure 3-21. Natural VPg<sup>EMCV</sup> in the supernatant is uridylated, and band patterns change upon PDEI treatment.**

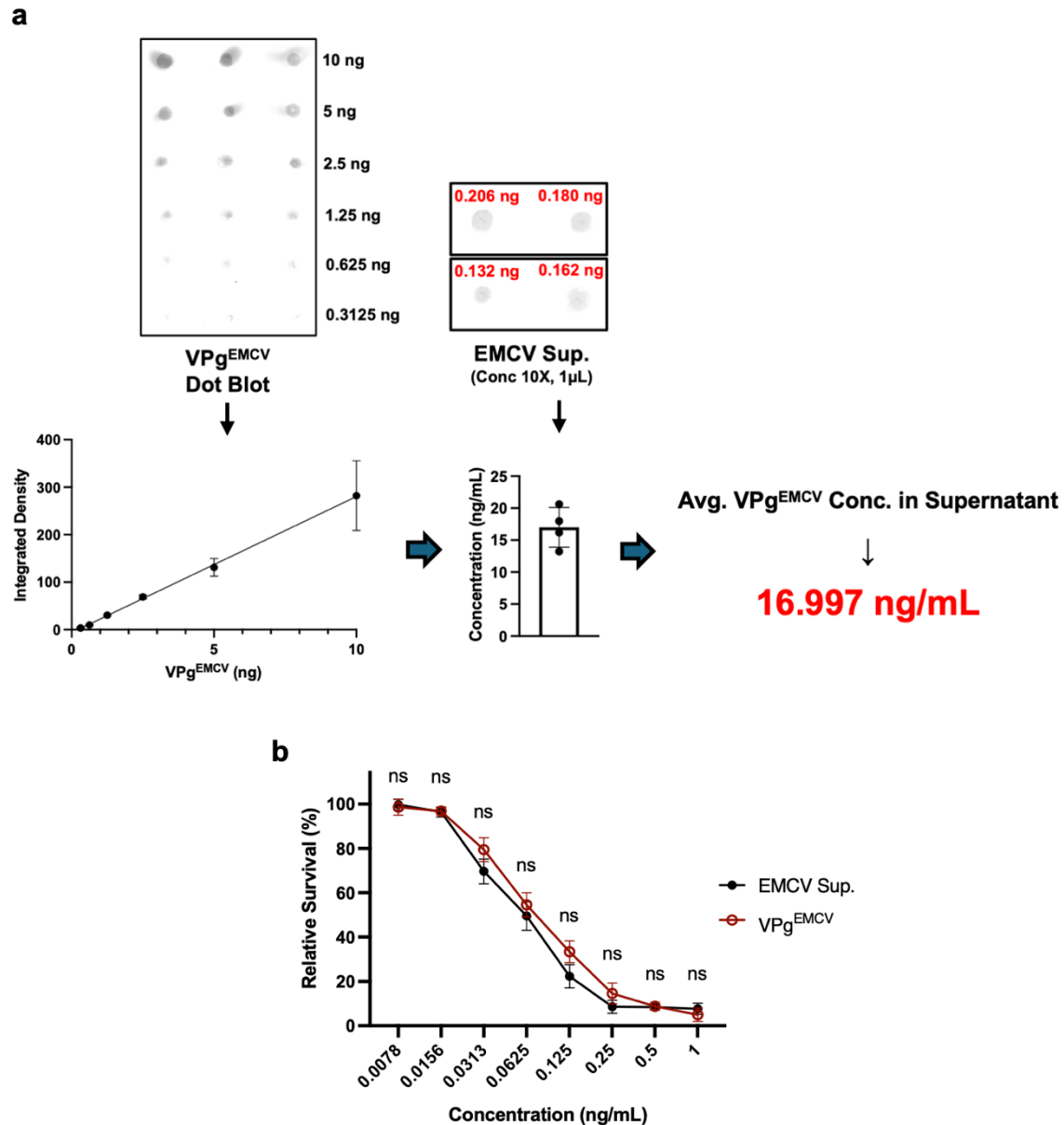
HeLa cells were infected with EMCV (MOI 0.1) for 24 h, and the supernatants were filtered using a 50 kDa cutoff membrane. Portions of the flowthrough were treated with PDEI for the indicated times at 37 °C (including a 24 h incubation without PDEI). The supernatants were then heated at 100 °C for 10 min to inactivate PDEI, concentrated 100× using centrifugal concentration, and subjected to SDS-PAGE (20 µL/lane). As references, two-fold serial dilutions of known concentrations of synthetic VPg<sup>EMCV</sup> (starting from 10 ng) were loaded as indicated. Western blotting was performed using anti-VPg<sup>EMCV</sup> and anti-GAPDH antibodies.

Next, the concentration of natural VPg<sup>EMCV</sup> present in the culture supernatant was quantified using the generated anti-VPg<sup>EMCV</sup> antibody. Direct quantification by Western blotting was challenging because the modification status of natural VPg<sup>EMCV</sup> affects band migration, and PDEI treatment also resulted in partial loss of band intensity. Therefore, an alternative approach, dot blotting was employed, as total signal intensity in dot blots should not be theoretically influenced by size or band shape.

A standard curve was generated using serial dilutions of known concentrations of synthetic VPg<sup>EMCV</sup>, and the natural VPg<sup>EMCV</sup> concentration in EMCV-infected culture supernatants was quantified accordingly (**Figure 3-22a**). Integrated density values were calculated using the software ImageJ, and the average concentration of natural VPg<sup>EMCV</sup> in the supernatant was estimated to be approximately 17 ng/mL. This concentration is definitely within the range required to induce cell death in combination with TNF-α, as demonstrated in previous experiments using synthetic VPg<sup>EMCV</sup>.

To directly compare the biological activity of natural and synthetic VPg<sup>EMCV</sup>, their concentrations were adjusted to equivalent levels (using **Figure 3-22** as reference), starting at 1 ng/mL followed by 2-fold serial dilution, and then applied to HeLa cells together with TNF-α. No significant differences were observed in cell death-sensitizing activity between natural and synthetic VPg<sup>EMCV</sup> (**Figure 3-22b**). These results indicate that uridylation of VPg<sup>EMCV</sup> should not affect its ability to sensitize cells

to TNF- $\alpha$ -induced apoptosis. Furthermore, the comparable activities of natural and synthetic VPg<sup>EMCV</sup> support the accuracy of the dot blot-based quantification used to estimate VPg<sup>EMCV</sup> concentrations in the supernatant.



**Figure 3-22. Natural and synthetic VPg<sup>EMCV</sup> have comparable cell death-inducing activities.**

(a) Dot blotting was performed using two-fold serial dilutions of known concentrations of synthetic VPg<sup>EMCV</sup> starting from 10 ng ( $n = 3$ ) (top left). HeLa cells were infected with EMCV (MOI 0.1) for 24 h, and supernatants were filtered using a 3 kDa cutoff membrane, concentrated 10X using a centrifugal evaporator, and blotted ( $n = 4$ ) (top right). Blots were detected using anti-VPg<sup>EMCV</sup>. A reference curve was generated from synthetic VPg<sup>EMCV</sup> dot blots (bottom left), and the average VPg<sup>EMCV</sup> concentration in the supernatants was calculated (bottom right). Data are presented as the mean (SD). Synthetic VPg<sup>EMCV</sup>:  $n = 3$ ; EMCV Sup.:  $n = 4$ .

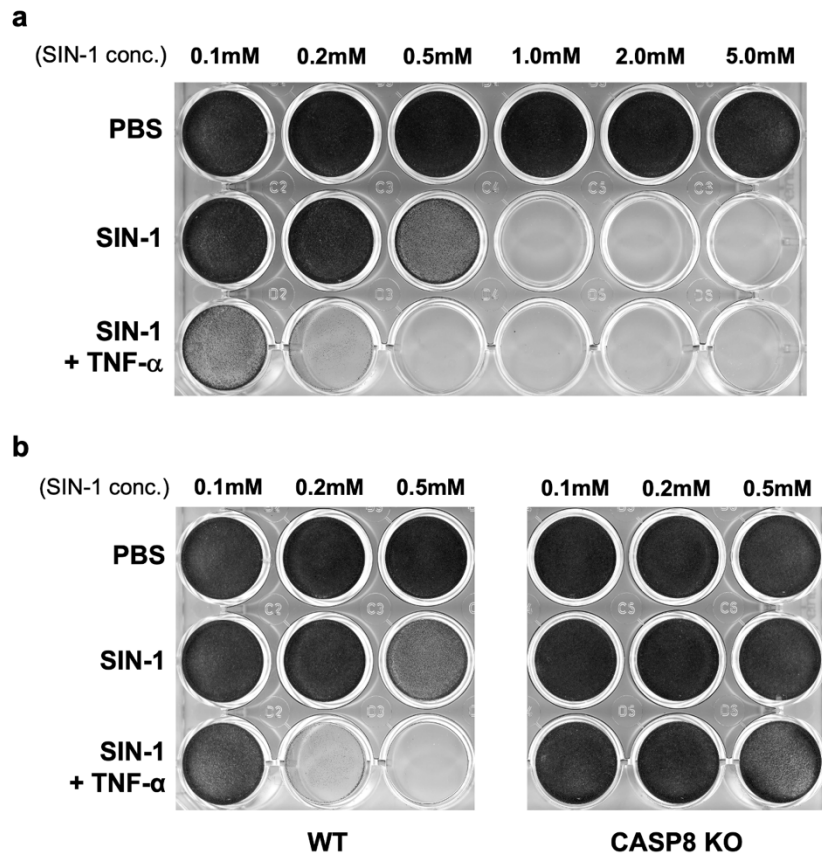
(b) The cell death-inducing activities of natural VPg<sup>EMCV</sup> in the supernatant (concentration determined in (a)) and synthetic VPg<sup>EMCV</sup> were compared. HeLa cells were treated with two-fold serial dilutions of either natural or synthetic VPg<sup>EMCV</sup> (starting from 1 ng/mL) for 24 h, together with recombinant human TNF- $\alpha$  (10 ng/mL). Data are presented as the mean (SD), and data are biological replicates ( $n = 3$ ). Statistical significance was determined by two-way ANOVA, followed by Tukey's HSD post-hoc test. Statistical comparisons were performed between the EMCV Sup. and synthetic VPg<sup>EMCV</sup> groups. Label: not significant ( $p > 0.05$ ).

### **3-9 VPg<sup>EMCV</sup> induces iNOS/NO through the JAK–STAT1-dependent pathway to sensitize cells to TNF- $\alpha$ -triggered cell death**

Simultaneously while identifying the low-molecular-weight component working synergistically with TNF- $\alpha$ , the pathway responsible for sensitization to Caspase-8-dependent apoptosis induced by EMCV-inactivated supernatants was also investigated. While going through the literature on TNF- $\alpha$ -dependent cell death, multiple reports from the late 2010s to early 2020s describing synergistic effects between TNF- $\alpha$  and IFN- $\gamma$  signaling [78, 79, 119] brought interest. One major study published in 2021 ([78]; also briefly referenced in Section 1-4-1) reported that during SARS-CoV-2 infection, synergistic TNF- $\alpha$  and IFN- $\gamma$  signaling induces iNOS expression, leading to nitric oxide (NO) accumulation and sensitization to Caspase-8-dependent cell death. This synergy was proposed to contribute to cytokine storm-associated pathology in COVID-19.

Although the SARS-CoV-2 context is not directly related to this study, the central concept that NO accumulation can synergize with TNF- $\alpha$  to induce cell death raised the possibility that iNOS/NO may also contribute to the sensitization mechanism observed in this study. To test whether NO itself could enhance TNF- $\alpha$ -mediated cytotoxicity, 3-Morpholinosydnonimine hydrochloride (SIN-1), a spontaneous NO donor, was used to assess NO-dependent cytotoxicity in HeLa cells. Treatment with  $\geq 0.5$  mM SIN-1 for 24 hours induced cell death, and  $\geq 1.0$  mM SIN-1 resulted in near-complete loss of viable cells within 24 hours (**Figure 3-23a**). Co-treatment of SIN-1 with TNF- $\alpha$  (10 ng/mL) resulted in clear synergy, inducing partial cell death even at lower SIN-1 concentrations (0.1 mM) and potent cell death at concentrations  $\geq 0.2$  mM (**Figure 3-23a**).

Next, to validate the Caspase 8-dependency of this NO + TNF- $\alpha$ -induced cell death, the same experiment was performed using Caspase 8 knockout (CASP8 KO) cells. Cell death was almost completely abrogated in CASP8 KO cells (**Figure 3-23b**), confirming that Caspase 8 plays an essential role in this pathway.



**Figure 3-23. NO donor SIN-1 and TNF-α induce synergistic cell death.**

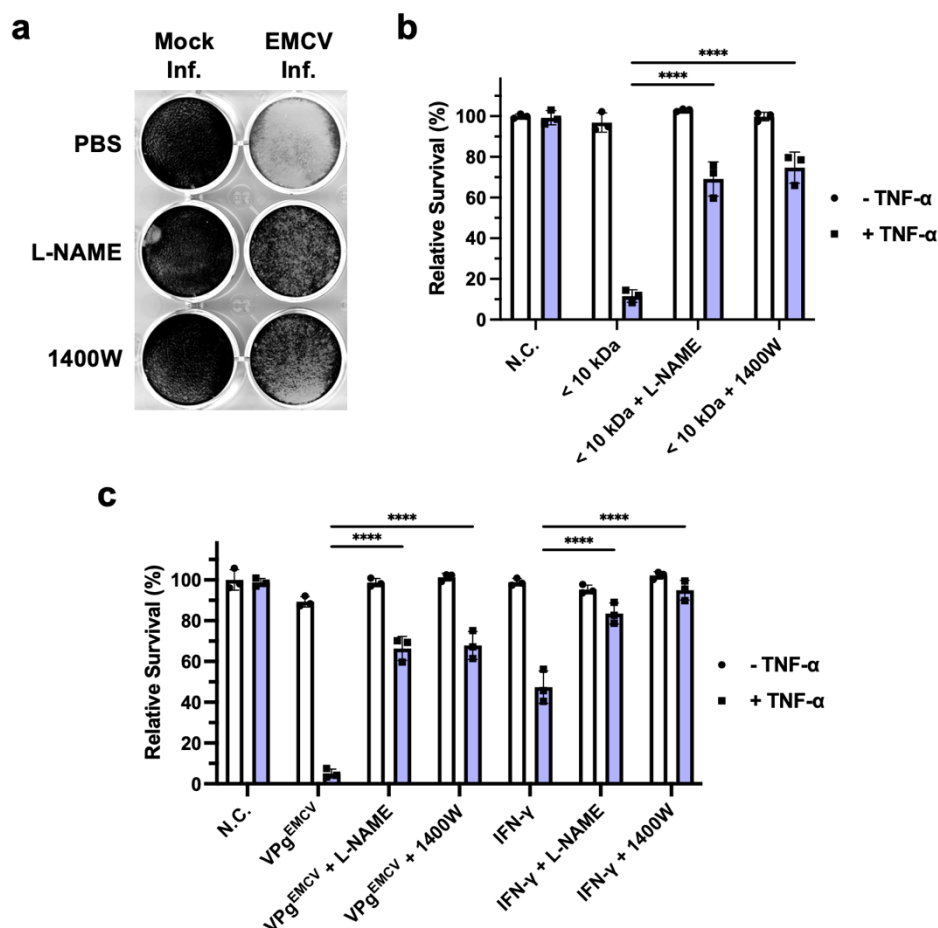
(a) HeLa cells were treated with the indicated concentrations of SIN-1, with or without recombinant TNF-α (10 ng/mL) for 24 h, then fixed and stained with Amido Black 10B to assess cell survival. PBS-treated cells were used as negative controls.

(b) WT or CASP8 KO HeLa cells were treated with SIN-1 or recombinant TNF-α under identical conditions as in (a), then fixed and stained as in (a).

After confirming that NO accumulation and TNF-α co-treatment can synergistically induce caspase-8-dependent cell death, it was next tested whether EMCV infection and cell death induced by post-infected supernatants are iNOS-dependent. To address this, NG-Nitro-L-arginine Methyl Ester (L-NAME), a pan-NOS inhibitor, and N-(3-(Aminomethyl)benzyl)acetamidine (1400W), a selective iNOS inhibitor, were used. HeLa cells were treated with L-NAME or 1400W 2 hours prior to EMCV infection (MOI 0.01). Both inhibitors significantly reduced cell death, indicating that iNOS contributes to EMCV-induced cell death (**Figure 3-24a**).

Next, iNOS dependency was tested using the <10 kDa flowthrough from EMCV-infected supernatants or synthetic VPg<sup>EMCV</sup> in combination with recombinant TNF-α. Both L-NAME and 1400W significantly inhibited cell death induced by the <10 kDa flowthrough (**Figure 3-24b**) and by synthetic VPg<sup>EMCV</sup> (**Figure 3-24c**), showing

that cell death induced by both natural and synthetic VPg<sup>EMCV</sup> is iNOS-dependent. This also suggests that the iNOS dependency observed during EMCV-induced cell death is largely due to VPg<sup>EMCV</sup>. As reference, co-treatment with IFN- $\gamma$  and TNF- $\alpha$  also induced cell death in HeLa cells (**Figure 3-24c**), and this effect was iNOS-dependent, consistent with previous reports (reference). Together, these results suggest that IFN- $\gamma$  and VPg<sup>EMCV</sup> engage similar cell death-sensitizing pathways.



**Figure 3-24. iNOS inhibition suppresses cell death induced by EMCV infection or VPg<sup>EMCV</sup> + TNF- $\alpha$ , similarly to IFN- $\gamma$  + TNF- $\alpha$ .**

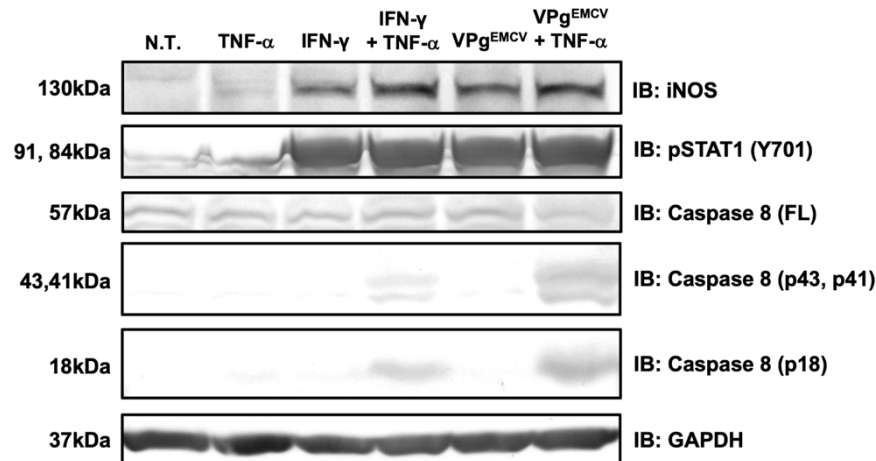
(a) HeLa cells were pre-treated with PBS, pan-NOS inhibitor L-NAME (1 mM), or iNOS inhibitor 1400W (100  $\mu$ M) for 2 h, then infected with EMCV (MOI 0.01) for 24 h. Cells were fixed and stained using Amido Black 10B.

(b) HeLa cells were treated with the EMCV <10 kDa flowthrough (from Figure 3-9a) at a 1:10 dilution (fraction:DMEM), with or without recombinant human TNF- $\alpha$  (10 ng/mL) for 24 h. L-NAME (1 mM) or 1400W (100  $\mu$ M) was added 2 h before treatment. Non-treated conditions (N.C.) were included as controls. Cells were fixed and stained using Amido Black 10B, then eluted for relative survival quantification.

(c) HeLa cells were treated with VPg<sup>EMCV</sup> (1 ng/mL) or recombinant human IFN- $\gamma$  (10 ng/mL), with or without recombinant human TNF- $\alpha$  (10 ng/mL) for 24 h. L-NAME or 1400W pre-treatment and N.C. conditions were the same as in (b). Relative survival was quantified as in (b).

For (b, c): Data are presented as the mean (SD), and individual data points are biological replicates (n = 3). Statistical significance was determined by two-way ANOVA, followed by Tukey's HSD post-hoc test. Label: \*\*\*\*p  $\leq$  0.0001.

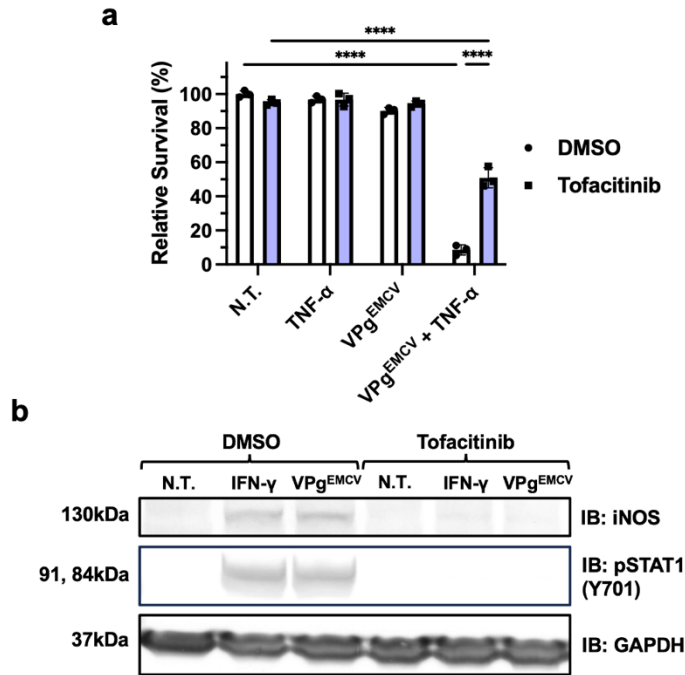
Western blotting further demonstrated that both VPg<sup>EMCV</sup> and IFN- $\gamma$  induce iNOS expression (**Figure 3-25**). When combined with recombinant TNF- $\alpha$ , this promotes cleavage of Caspase 8, consistent with apoptosis execution. Notably, VPg<sup>EMCV</sup> also induced STAT1 phosphorylation at tyrosine 701, suggesting activation of a signaling axis similar to IFN- $\gamma$ .



**Figure 3-25. VPg<sup>EMCV</sup> and IFN- $\gamma$  induce iNOS, and Caspase 8 cleavage is induced by TNF- $\alpha$  co-treatment.**

HeLa cells were treated with TNF- $\alpha$  (10 ng/mL), IFN- $\gamma$  (10 ng/mL), VPg<sup>EMCV</sup> (1 ng/mL), or the indicated combinations for 9 h. Cell lysates were subjected to Western blotting using anti-iNOS, anti-pSTAT1 (Y701), anti-Caspase 8, and anti-GAPDH antibodies.

To test whether JAK-STAT signaling is required, HeLa cells were pre-treated with Tofacitinib, a JAK inhibitor, 2 h prior to stimulation with VPg<sup>EMCV</sup> and TNF- $\alpha$ . Tofacitinib significantly increased survival following VPg<sup>EMCV</sup> and TNF- $\alpha$  treatment (**Figure 3-26b**), supporting a critical role for JAK-dependent signaling in this pathway. Consistently, JAK inhibition completely abrogated iNOS induction and STAT1 phosphorylation induced by both IFN- $\gamma$  and VPg<sup>EMCV</sup> (**Figure 3-26c**), further indicating that VPg<sup>EMCV</sup> activates a JAK–STAT1-dependent pathway similar to IFN- $\gamma$  signaling.



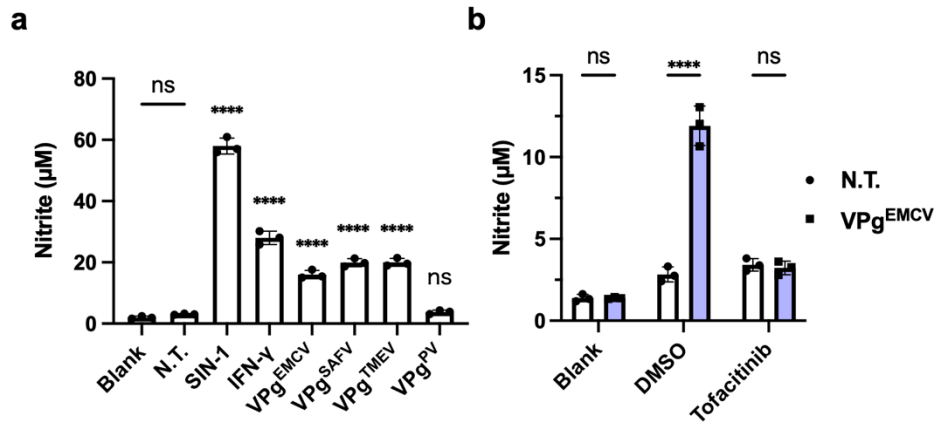
**Figure 3-26. JAK inhibition rescues cell death induced by VPg<sup>EMCV</sup> + TNF-α and abrogates STAT1 phosphorylation by VPg<sup>EMCV</sup> or IFN-γ.**

(a) HeLa cells were pretreated with DMSO (control) or the JAK inhibitor Tofacitinib (500 nM) for 2 h, then treated with recombinant TNF-α (10 ng/mL), VPg<sup>EMCV</sup> (1 ng/mL), or their combination for 24 h. Cells were fixed and stained using Amido Black 10B, then eluted for relative survival quantification.

(b) HeLa cells were pre-treated as in (a), then treated with IFN-γ (10 ng/mL) or VPg<sup>EMCV</sup> (1 ng/mL) for 9 h. Cell lysates were subjected to Western blotting using anti-iNOS, anti-pSTAT1 (Y701), and anti-GAPDH antibodies.

For (a): Data are presented as the mean (SD), and individual data points are biological replicates (n = 3). Statistical significance was determined by two-way ANOVA, followed by Tukey's HSD post-hoc test. Label: \*\*\*\*p ≤ 0.0001.

To confirm downstream NO production resulting from JAK–STAT activation and iNOS induction, total nitrite levels in culture supernatants were measured by Griess quantification 24 hours after stimulation. HeLa cells were treated with SIN-1 (positive control), IFN-γ, VPg<sup>EMCV</sup>, VPg<sup>SAFV</sup>, VPg<sup>TMEV</sup>, and VPg<sup>PV</sup>. Compared to untreated controls, nitrite levels were significantly increased following all treatments except VPg<sup>PV</sup> (**Figure 3-27a**). This finding is consistent with the cell death assays earlier, in which VPg<sup>PV</sup> did not exhibit strong activity at lower concentrations (~1 ng/mL). Pre-treatment with Tofacitinib reduced nitrite levels to near-basal levels (**Figure 3-27b**), indicating that VPg<sup>EMCV</sup> induces NO production via a JAK-dependent pathway, consistent with the observed STAT1 phosphorylation, iNOS induction, and cell death.



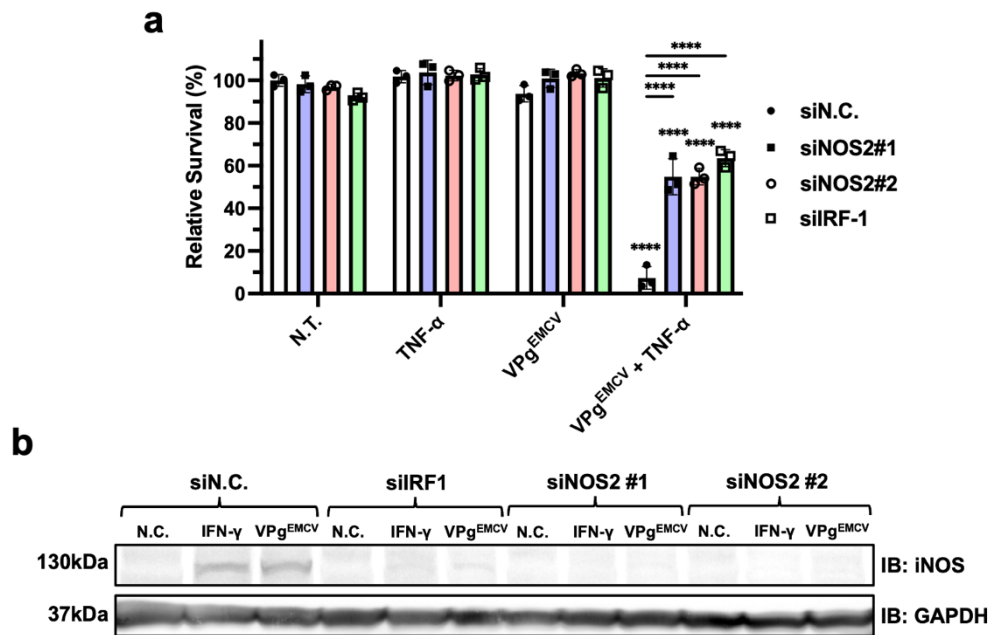
**Figure 3-27. *Cardiovirus* VPgs induce nitric oxide similarly to IFN-γ in a JAK-dependent manner.**

(a) HeLa cells were treated for 24 h with SIN-1 (0.1 mM), IFN-γ (10 ng/mL), or various VPgs (1 ng/mL). Total nitrite levels in the supernatants were quantified using Griess assay. Blank: DMEM only.

(b) HeLa cells were pre-treated with DMSO (control) or Tofacitinib (500 nM) for 2 h, then treated with VPg<sup>EMCV</sup> (1 ng/mL) for 24 h. Total nitrite levels were quantified as in (a).

For (a, b): Data are presented as the mean (SD), and individual data points are biological replicates (n = 3). Statistical significance was determined by (a): one-way ANOVA, or (b): two-way ANOVA, followed by Tukey's HSD post-hoc test. For (a): Asterisks and "ns" indicate comparisons with the N.T. group. Labels: \*\*\*\* $p \leq 0.0001$ , ns: not significant ( $p > 0.05$ ).

Previous literature suggests that IRF-1 is induced upstream of iNOS and contributes to NOS2 transcription [76], as also described in the Introduction (Section 1-4-1). To test this, RNAi silencing was performed using siRNAs targeting IRF-1, as well as 2 independent siRNAs targeting NOS2 (iNOS). These cells were then assessed for cell death following VPg<sup>EMCV</sup> and recombinant TNF-α treatment. Silencing of either IRF-1 or NOS2 significantly inhibited cell death (**Figure 3-28a**). Western blotting further confirmed that iNOS induction by IFN-γ or VPg<sup>EMCV</sup> was abrogated by iNOS silencing as expected, and also by IRF-1 silencing (**Figure 3-28b**). Together, these results support the model in which VPg<sup>EMCV</sup> induces a JAK-STAT1-IRF-1-iNOS pathway leading to NO production, sensitizing cells to TNF-α-triggered, Caspase-8-dependent apoptosis.



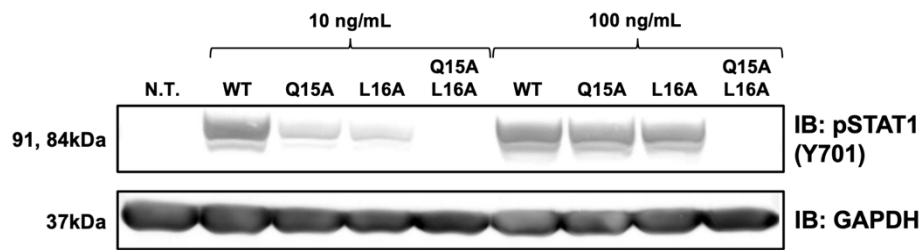
**Figure 3-28. IRF-1 silencing upstream of iNOS suppresses cell death induced by VPg<sup>EMCV</sup> and TNF-α.**

(a) HeLa cells were transfected with three different siRNAs (10 nM) for 48 h, then treated with recombinant TNF-α (10 ng/mL), VPg<sup>EMCV</sup> (1 ng/mL), or their combination for 24 h. Cells were fixed and stained using Amido Black 10B, then eluted for relative survival quantification.

(b) HeLa cells transfected with siRNAs as in (a) were treated with recombinant IFN-γ (10 ng/mL) or VPg<sup>EMCV</sup> (1 ng/mL) for 9 h. Cell lysates were subjected to Western blotting using anti-iNOS and anti-GAPDH antibodies.

For (a): Data are presented as the mean (SD), and individual data points are biological replicates (n = 3). Statistical significance was determined by two-way ANOVA, followed by Tukey's HSD post-hoc test. Asterisks indicate comparisons between the N.T. groups of the identical siRNA. Label: \*\*\*\*p ≤ 0.0001.

Finally, VPg<sup>EMCV</sup> alanine-scan mutants previously shown to exhibit reduced cell death activity (**Figure 3-17**) were tested for their ability to induce STAT1 phosphorylation at Y701. HeLa cells were treated with the Q15A, L16A, or Q15A/L16A mutant peptides, followed by Western blot analysis. At 10 ng/mL, both Q15A and L16A markedly reduced STAT1 phosphorylation, while the double mutant completely lost any detectable activity (**Figure 3-29**). At 100 ng/mL, the concentration at which VPg<sup>EMCV</sup> alone induced strong cytotoxicity, STAT1 phosphorylation for the single mutants was comparable to WT, but was still completely abolished for the double mutant (**Figure 3-28**). These results indicate that the ability of the VPg<sup>EMCV</sup> point mutants to phosphorylate STAT1 strongly correlates with their cell death-inducing activity.



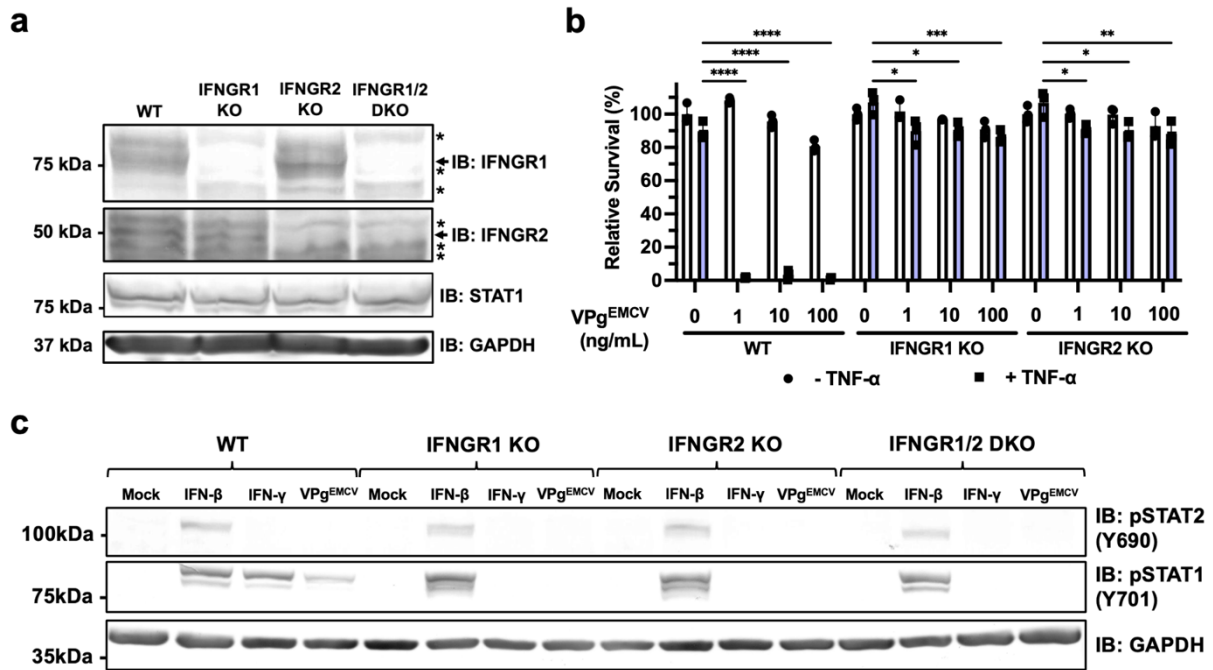
**Figure 3-29. Residues Q15 and L16 are critical for downstream signaling by VPg<sup>EMCV</sup>.**

HeLa cells were treated with 10 ng/mL or 100 ng/mL of VPg<sup>EMCV</sup> (WT), VPg<sup>EMCV</sup>-Q15A (Q15A), VPg<sup>EMCV</sup>-L16A (L16A), or VPg<sup>EMCV</sup>-Q15A/L16A (Q15A/L16A) for 1 h. Cell lysates were subjected to Western blotting using anti-pSTAT1 (Y701) and anti-GAPDH antibodies. An N.T. (non-treated) lane was included.

### 3-10 VPg<sup>EMCV</sup> functions as an IFNGR agonist and binds directly to IFNGR

Given the strong similarity between VPg<sup>EMCV</sup>-induced signaling and IFN- $\gamma$  signaling, the next hypothesis was that VPg<sup>EMCV</sup> may perhaps utilize the same receptor. To test this, CRISPR-Cas9 knockout plasmids targeting the interferon gamma receptor (IFNGR) subunits IFNGR1 and IFNGR2 (described in Section 1-4-2) were generated. HeLa cell lines lacking IFNGR1, IFNGR2, or both IFNGR1 and IFNGR2 were successfully generated and validated (**Figure 3-30a**).

Following confirmation of generating successful knockouts, these cells were tested for susceptibility to VPg<sup>EMCV</sup>- and TNF- $\alpha$ -induced cell death. Knockout of either IFNGR1 or IFNGR2 resulted in significant abrogation of cell death induced by VPg<sup>EMCV</sup> in combination with recombinant TNF- $\alpha$ , even at higher concentrations of VPg<sup>EMCV</sup> (100 ng/mL) (**Figure 3-30b**). Next, the effect of IFNGR deficiency on STAT phosphorylation was examined. WT, IFNGR1 KO, IFNGR2 KO, and IFNGR1/2 DKO cells were treated with IFN- $\beta$ , IFN- $\gamma$ , or VPg<sup>EMCV</sup>, and phosphorylation of STAT1 and STAT2 were assessed (**Figure 3-30c**). As expected, IFN- $\beta$  induced phosphorylation of both STAT1 and STAT2 in all cell lines, consistent with signaling through IFNAR1/2, a different receptor (details in Section 1-4-1). In contrast, IFN- $\gamma$  and VPg<sup>EMCV</sup> induced STAT1 phosphorylation but failed to phosphorylate STAT2 in WT cells. Importantly, STAT1 phosphorylation induced by IFN- $\gamma$  and VPg<sup>EMCV</sup> was completely abrogated in all IFNGR-deficient cell lines, demonstrating that VPg<sup>EMCV</sup> activates STAT1 via IFNGR1/2 in a manner identical to IFN- $\gamma$ .



**Figure 3-30. Cell death induced by VPg<sup>EMCV</sup> + TNF-α and downstream signaling by VPg<sup>EMCV</sup> are abolished by IFNGR1/2 knockout.**

(a) IFNGR1 KO, IFNGR2 KO, and IFNGR1/2 DKO HeLa cells were generated. Lysates from WT, IFNGR1 KO, IFNGR2 KO, and IFNGR1/2 DKO cells were subjected to Western blotting using anti-IFNGR1, anti-IFNGR2, anti-STAT1, and anti-GAPDH antibodies to confirm knockout efficiency. Asterisks indicate non-specific bands.

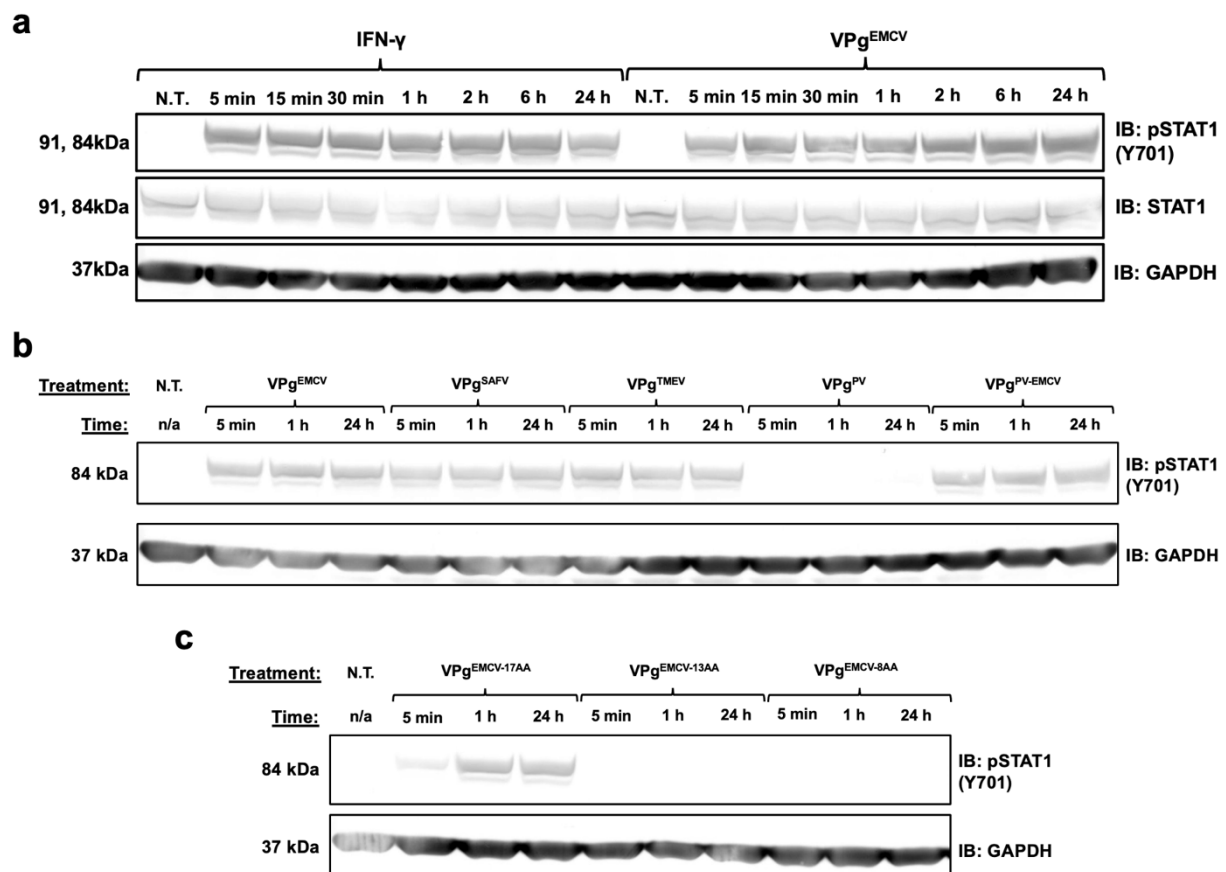
(b) WT, IFNGR1 KO, or IFNGR2 KO HeLa cells were treated with the indicated concentrations of VPg<sup>EMCV</sup>, with or without recombinant human TNF-α (10 ng/mL) for 24 h. Cells were fixed and stained using Amido Black 10B, then eluted for relative survival quantification.

(c) WT, IFNGR1 KO, IFNGR2 KO, or IFNGR1/2 DKO HeLa cells were treated with recombinant human IFN-β (1000 U/mL), recombinant human IFN-γ (10 ng/mL), or VPg<sup>EMCV</sup> (1 ng/mL) for 9 h. Cell lysates were subjected to Western blotting using anti-pSTAT2 (Y690), anti-pSTAT1 (Y701), and anti-GAPDH antibodies.

For (b): Data are presented as the mean (SD), and individual data points are biological replicates (n = 3). Statistical significance was determined by two-way ANOVA, followed by Tukey's HSD post-hoc test. Labels: \**p* ≤ 0.05, \*\**p* ≤ 0.01, \*\*\**p* ≤ 0.001, \*\*\*\**p* ≤ 0.0001.

To exclude the possibility that VPg<sup>EMCV</sup> induces STAT1 phosphorylation indirectly through autocrine/paracrine IFN-γ production, several experiments were performed. First, the kinetics of STAT1 phosphorylation at Y701 were compared between IFN-γ and VPg<sup>EMCV</sup> treatments (**Figure 3-31a**). STAT1 phosphorylation was detectable as early as 5 minutes following IFN-γ treatment, as expected. VPg<sup>EMCV</sup> similarly induced STAT1 phosphorylation within 5 minutes, closely matching IFN-γ kinetics.

To determine whether this rapid STAT1 activation was similar among other VPgs, HeLa cells were treated with 1 ng/mL VPg<sup>EMCV</sup>, VPg<sup>SAFV</sup>, VPg<sup>TMEV</sup>, VPg<sup>PV</sup>, and the polio-EMCV hybrid VPg (VPg<sup>PV-EMCV</sup>) for 5 minutes, 1 hour, or 24 hours (**Figure 3-31b**). All tested *Cardiovirus*-derived VPgs induced robust STAT1 phosphorylation from early to late time points, while VPg<sup>PV</sup> failed to induce STAT1 phosphorylation. Notably, STAT1 phosphorylation was restored with VPg<sup>PV-EMCV</sup>, consistent with its restored biological activity in cell death. Truncated VPg<sup>EMCV</sup> variants showed consistent results with cell death: VPg<sup>EMCV-17AA</sup> induced weak early STAT1 phosphorylation, which increased at later time points (1 h, 24 h), while VPg<sup>EMCV-13AA</sup> and VPg<sup>EMCV-8AA</sup> exhibited no detectable STAT1 activation whatsoever (**Figure 3-31c**).



**Figure 3-31. Kinetics of IFN- $\gamma$  and VPg<sup>EMCV</sup> signaling are comparable, and STAT1 phosphorylation is induced by various VPgs.**

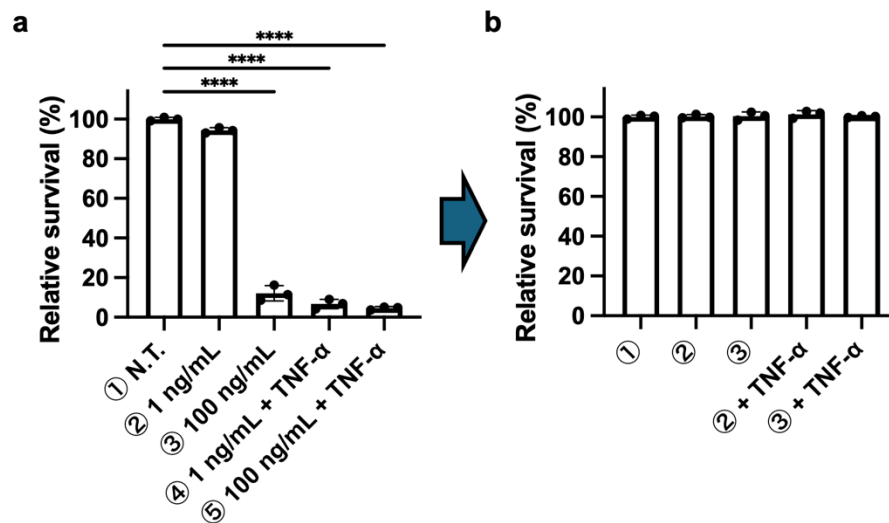
(a) HeLa cells were treated with either IFN- $\gamma$  (10 ng/mL) or VPg<sup>EMCV</sup> (1 ng/mL) for the indicated time points, after which cell lysates were subjected to Western blotting using anti-pSTAT1 (Y701), anti-STAT1, and anti-GAPDH antibodies.

(b) HeLa cells were treated with 1 ng/mL of various VPgs for 5 min, 1 h, or 24 h, then cell lysates were subjected to Western blotting using anti-pSTAT1 (Y701) and anti-GAPDH antibodies.

(c) HeLa cells were treated with 1 ng/mL of the indicated N-terminal truncated VPgs in the same manner as in (b), and cell lysates were subjected to Western blotting as in (b).

A washout experiment was next performed to further assess whether VPg<sup>EMCV</sup> acts directly on IFNGR. HeLa cells were treated with 1 ng/mL or 100 ng/mL VPg<sup>EMCV</sup> for 10 minutes, with or without recombinant TNF- $\alpha$ , after which the supernatant was removed and cells were washed twice with PBS to remove any residual VPg<sup>EMCV</sup>. Despite this brief treatment, potent cell death was triggered in cells treated with VPg<sup>EMCV</sup> alone at 100 ng/mL or with VPg<sup>EMCV</sup> + TNF- $\alpha$  (**Figure 3-32a**), indicating that VPg<sup>EMCV</sup>-triggered signaling occurs rapidly.

To test whether any soluble factors were released in the supernatant during VPg<sup>EMCV</sup> stimulation, supernatants collected 24 hours after the initial VPg<sup>EMCV</sup> treatment were applied to fresh HeLa cells in the presence or absence of recombinant TNF- $\alpha$ . These supernatants failed to induce cell death under all tested conditions (**Figure 3-32b**), demonstrating that VPg<sup>EMCV</sup> does not trigger secretion of IFN- $\gamma$  or other cytotoxic factors responsible for autocrine/paracrine signaling. Together, these results strongly support the conclusion that VPg<sup>EMCV</sup> and other *Cardiovirus* VPgs function directly via IFNGR1/2, rather than indirectly activating IFNGR signaling through cytokine induction.



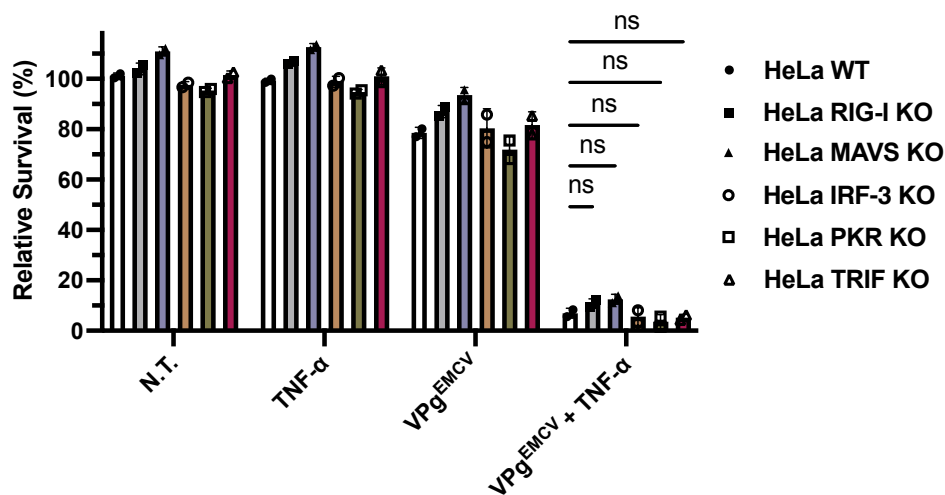
**Figure 3-32. Cell death induced by VPg<sup>EMCV</sup> + TNF- $\alpha$  is not mediated by autocrine/paracrine IFN- $\gamma$  or other cytotoxic factors.**

(a) HeLa cells were treated for 10 min with one of the following conditions: ① non-treated control (N.T.), ② VPg<sup>EMCV</sup> (1 ng/mL), ③ VPg<sup>EMCV</sup> (100 ng/mL), ④ VPg<sup>EMCV</sup> (1 ng/mL) + recombinant TNF- $\alpha$  (10 ng/mL), or ⑤ VPg<sup>EMCV</sup> (100 ng/mL) + recombinant TNF- $\alpha$  (10 ng/mL). After treatment, cells were washed twice with PBS and cultured in fresh DMEM (10% FBS). After 24 h, cells were fixed and stained using Amido Black 10B, then eluted for relative survival quantification.

(b) Supernatants from conditions ①, ②, and ③ in (a) were collected, centrifuged to remove debris, and then applied to fresh HeLa cells for 24 h at a 1:1 ratio (fresh DMEM:collected supernatant). Recombinant TNF- $\alpha$  (10 ng/mL) was added as indicated. Relative survival was quantified as in (a).

For (a, b): Data are presented as the mean (SD), and individual data points are biological replicates (n = 3). Statistical significance was determined by one-way ANOVA, followed by Tukey's HSD post-hoc test. Label: \*\*\*\* $p \leq 0.0001$ .

To further examine whether other innate immune pathways may contribute to VPg<sup>EMCV</sup>-induced cell death, HeLa cells deficient in key pattern recognition receptor (PRR) signaling components, including RIG-I, MAVS, IRF-3, PKR, and TRIF (knockout cell lines from [94]), were tested for their response to VPg<sup>EMCV</sup> and TNF- $\alpha$ . All knockout cell lines exhibited levels of cell death comparable to WT cells (**Figure 3-33**), indicating that these innate immune signaling pathways should be dispensable for VPg<sup>EMCV</sup> + TNF- $\alpha$  mediated cell death.



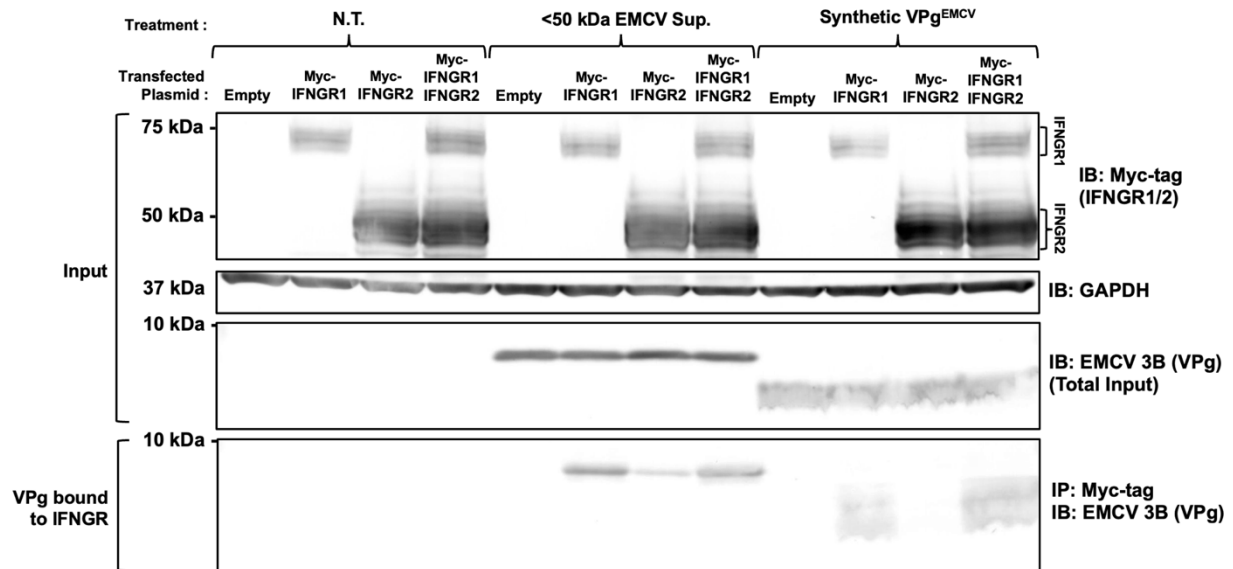
**Figure 3-33. Major innate immune PRR signaling components are dispensable for cell death induced by VPg<sup>EMCV</sup> + TNF- $\alpha$ .**

WT, RIG-I KO, MAVS KO, IRF-3 KO, PKR KO, or TRIF KO HeLa cells were treated with recombinant TNF- $\alpha$  (10 ng/mL), VPg<sup>EMCV</sup> (1 ng/mL), or their combination for 24 h. Cells were fixed and stained using Amido Black 10B, then eluted for relative survival quantification.

Data are presented as the mean (SD), and individual data points are biological replicates ( $n = 3$ ). Statistical significance was determined by two-way ANOVA, followed by Tukey's HSD post-hoc test. Label: ns, not significant ( $p > 0.05$ ).

Given the strong functional evidence that VPg<sup>EMCV</sup> acts as an IFNGR agonist, direct physical interaction between VPg<sup>EMCV</sup> and IFNGR was next investigated. Because detection of VPg<sup>EMCV</sup> bound to endogenous IFNGR was technically challenging, Myc-tagged IFNGR1 and IFNGR2 were overexpressed in HEK293T cells. Cells expressing Myc-IFNGR1, Myc-IFNGR2, or both were treated for 1 hour with either natural VPg<sup>EMCV</sup> derived from 50 kDa-filtered EMCV-infected supernatants or synthetic VPg<sup>EMCV</sup>, followed by co-immunoprecipitation (co-IP) using an anti-Myc-tag antibody.

Co-IP analysis revealed that both natural and synthetic VPg<sup>EMCV</sup> were specifically pulled down from cells expressing Myc-IFNGR1 or Myc-IFNGR2 (**Figure 3-34**). Notably, stronger VPg<sup>EMCV</sup> bands were observed in IFNGR1-expressing cells compared to IFNGR2-expressing cells, suggesting stronger or possibly preferential binding of VPg<sup>EMCV</sup> to IFNGR1.

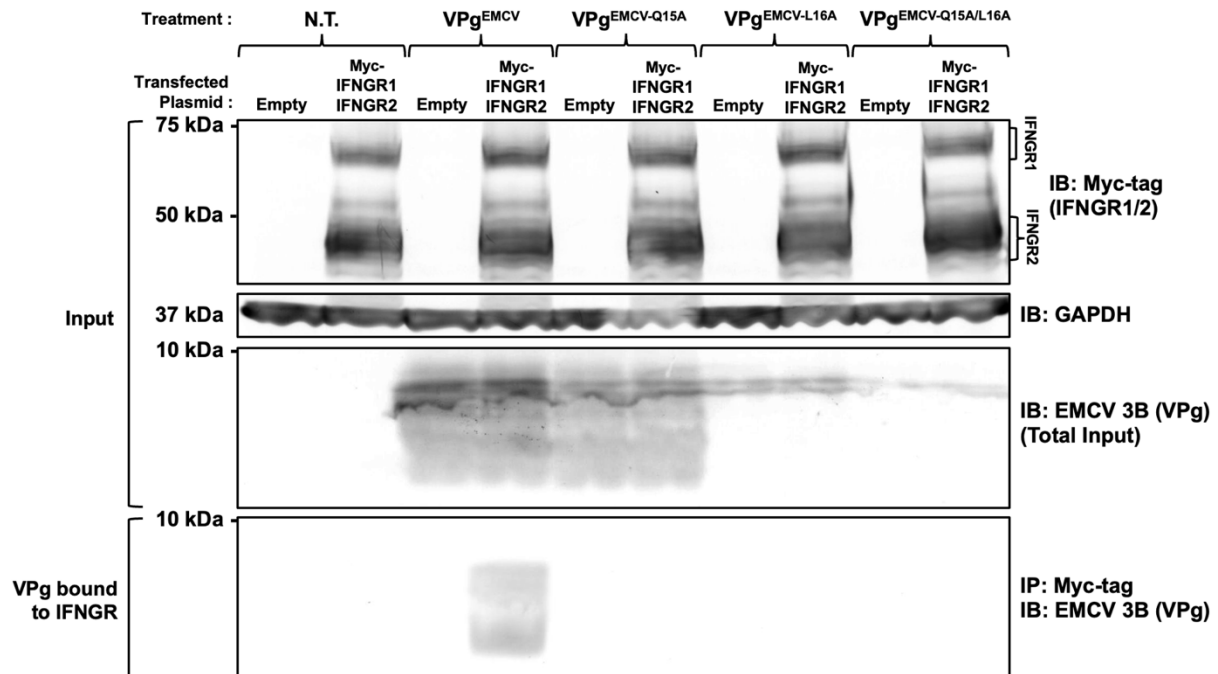


**Figure 3-34. Natural and synthetic VPg<sup>EMCV</sup> physically interact with IFNGR1/2.**

HEK293T cells were transfected with expression vectors encoding Myc-IFNGR1 (6 µg), Myc-IFNGR2 (6 µg), or both receptors (total 12 µg) for 48 h. Empty transfection controls (Lipofectamine only) were included. Cells were then treated with either the 50 kDa cutoff filter flowthrough of 10×-concentrated EMCV-infected supernatant (HeLa cells infected for 24 h at MOI 1.0) or synthetic VPg<sup>EMCV</sup> (100 ng/mL) for 1 h. Non-treated (N.T.) conditions were also prepared. Cells were lysed in 200 µL lysis buffer, and 100 µL of each lysate was subjected to immunoprecipitation using Protein G–anti-Myc tag conjugate. Immunocomplexes were eluted with 40 µL elution buffer. Eluates (20 µL; 1/2), input lysates (1/5), and 1/5 of the total VPg input (from either supernatant or synthetic VPg<sup>EMCV</sup>) were analyzed by Western blotting using anti-Myc-tag, anti-VPg<sup>EMCV</sup>, and anti-GAPDH antibodies.

Finally, it was examined whether the VPg<sup>EMCV</sup> mutants that failed to induce cell death (**Figure 3-17**) and STAT1 phosphorylation (**Figure 3-29**) also lost the ability to physically interact with IFNGR. HEK293T cells co-expressing Myc-IFNGR1 and Myc-IFNGR2 were treated with WT VPg<sup>EMCV</sup>, VPg<sup>EMCV-Q15A</sup>, VPg<sup>EMCV-L16A</sup>, or VPg<sup>EMCV-Q15A/L16A</sup> for 1 hour, followed by co-IP. VPg<sup>EMCV-Q15A</sup> bands were completely abolished from anti-Myc-tag pulldown despite the comparable input levels, strongly indicating a loss of IFNGR binding (**Figure 3-35**). Unfortunately, however, due to limitations of the anti-VPg<sup>EMCV</sup> antibody, VPg<sup>EMCV-L16A</sup> and the double mutant could not be properly detected in the input, preventing any definitive conclusions regarding their binding.

Nevertheless, the loss of IFNGR binding by VPg<sup>EMCV-Q15A</sup> strongly supports a model in which VPg<sup>EMCV</sup> physically interacts with IFNGR, and that disruption of this interaction site explains the loss of function observed in this mutant.



**Figure 3-35. The VPg<sup>EMCV</sup> Q15A mutant, defective in cell death sensitization and downstream signaling, shows reduced binding to IFNGR1/2.**

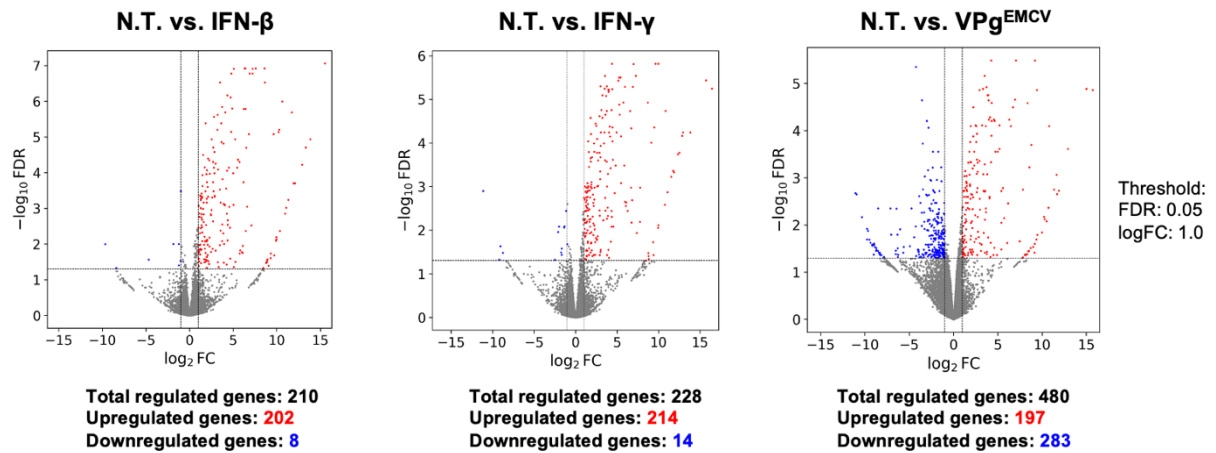
HEK293T cells were co-transfected with expression vectors encoding Myc-IFNGR1 (6 µg) and Myc-IFNGR2 (6 µg) for 48 h. Empty transfection controls (Lipofectamine only) were included. Cells were then treated with VPg<sup>EMCV</sup>, VPg<sup>EMCV-Q15A</sup>, VPg<sup>EMCV-L16A</sup>, or VPg<sup>EMCV-Q15A/L16A</sup> (100 ng/mL) for 1 h. Non-treated (N.T.) conditions were also prepared. Cells were lysed in 200 µL lysis buffer, and 100 µL of each lysate was subjected to immunoprecipitation using Protein G–anti-Myc tag conjugate. Immunocomplexes were eluted with 40 µL elution buffer. Eluates (20 µL; 1/2), input lysates (1/5), and 1/5 of the total VPg input were analyzed by Western blotting using anti-Myc-tag, anti-VPg<sup>EMCV</sup>, and anti-GAPDH antibodies.

(Important note: input bands for VPg<sup>EMCV-L16A</sup> and VPg<sup>EMCV-Q15A/L16A</sup> were not properly detected using the anti-VPg<sup>EMCV</sup> antibody, likely due to altered epitope recognition. Therefore, binding comparisons were limited to WT and Q15A. The whole Western blot data is included for transparency.)

### 3-11 VPg<sup>EMCV</sup> upregulates ISGs similar to IFN-γ signaling and downregulates a distinct subset of genes

Having established now that VPg<sup>EMCV</sup> functions as an IFNGR agonist and physically interacts with the receptor, downstream signaling other than iNOS induction were investigated. HeLa cells were treated with IFN-β, IFN-γ, or VPg<sup>EMCV</sup> for 24 hours, after which total RNA was extracted and subjected to RNA-seq analysis. Differentially expressed genes were identified by comparison with non-treated (N.T.) control cells.

IFN- $\beta$ , IFN- $\gamma$ , and VPg<sup>EMCV</sup> treatment resulted in 202, 214, and 197 upregulated genes, respectively (**Figure 3-36**).



**Figure 3-36. Regulation of genes by IFN- $\beta$ , IFN- $\gamma$ , or VPg<sup>EMCV</sup> treatment.**

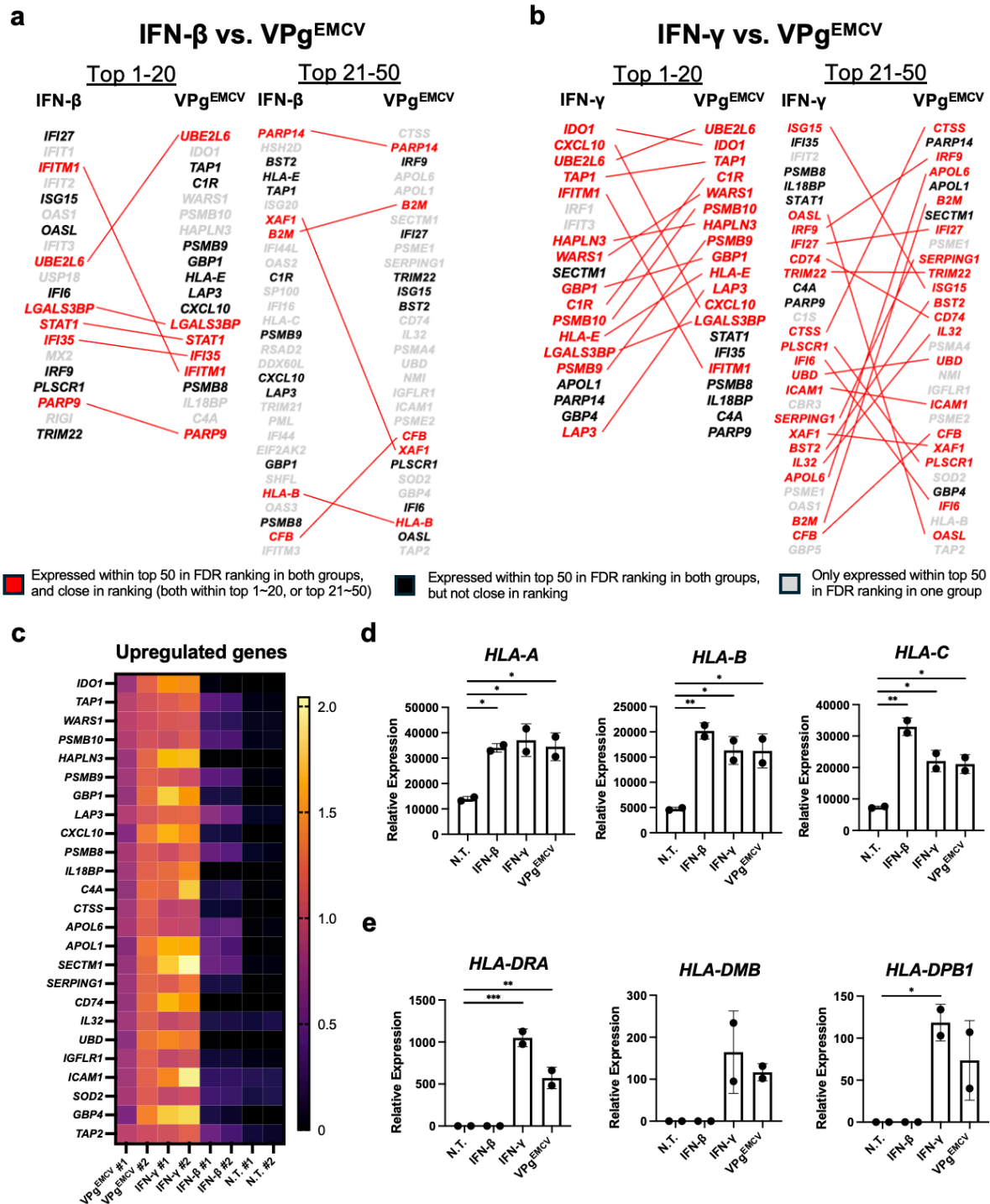
RNA sample preparation: HeLa cells ( $n = 2$ ) were treated with IFN- $\beta$  (1000 U/mL), IFN- $\gamma$  (10 ng/mL), or VPg<sup>EMCV</sup> (1 ng/mL) for 24 h. Non-treated (N.T.) conditions were also prepared. Total RNA was extracted from the cells, and gene expression profiles were analyzed using RNA-seq.

Volcano plots were generated, showing upregulated and downregulated genes compared to the control (N.T.) group. Threshold values for differentially expressed genes were set as follows: false discovery rate (FDR) = 0.05 and log fold change (logFC) = 1.0.

The top 50 upregulated genes induced by IFN- $\beta$ , IFN- $\gamma$ , and VPg<sup>EMCV</sup> were then compared across treatment groups. These genes were divided into the top 20 and the top 21–50 upregulated genes by VPg<sup>EMCV</sup>, and analyzed by comparing IFN- $\beta$  vs. VPg<sup>EMCV</sup>-induced genes and IFN- $\gamma$  vs. VPg<sup>EMCV</sup>-induced genes. Although some overlap was observed between IFN- $\beta$  and VPg<sup>EMCV</sup> (**Figure 3-37a**), a stronger similarity was observed between IFN- $\gamma$  and VPg<sup>EMCV</sup> gene expression profiles (**Figure 3-37b**). This result is consistent with earlier findings that VPg<sup>EMCV</sup> signals through IFNGR. Heatmap analysis further confirmed that 25 out of the top 50 genes upregulated by VPg<sup>EMCV</sup> were also highly induced by IFN- $\gamma$ , but not by IFN- $\beta$  (**Figure 3-37c**), indicating that VPg<sup>EMCV</sup> induces IFN- $\gamma$ -like ISG expression.

A clear distinction between IFN- $\beta$  and IFN- $\gamma$  signaling was observed in the regulation of Human Leukocyte Antigen (HLA) genes. It is well-known that both type I and type II IFNs induce class I HLA genes (*HLA-A*, *HLA-B*, *HLA-C*), which are involved in endogenous antigen presentation [120, 121, 122]. In contrast, induction of class II HLA genes (HLA-D family members such as *HLA-DRA*, *HLA-DMB*, and *HLA-DPB1*), which mediate exogenous antigen presentation, is predominantly driven by IFN- $\gamma$  [123, 124]. Analysis of VPg<sup>EMCV</sup>-treated cells revealed upregulation of both class I (**Figure 3-37d**) and class II HLAs (**Figure 3-37e**), representing the expression patterns

induced by IFN- $\gamma$ . These results further support the conclusion that VPg<sup>EMCV</sup> and IFN- $\gamma$  have similar transcriptional responses.



**Figure 3-37. Gene expression profiles of upregulated genes by IFN- $\beta$ , IFN- $\gamma$ , or VPg<sup>EMCV</sup> treatments.**

RNA-seq analysis data (n = 2) from Figure 3-36 were used for all figures.

(a) Top 50 genes upregulated by IFN- $\beta$  or VPg<sup>EMCV</sup>. Comparisons of the top 1-20 (left) and top 21-50 (right) upregulated genes, ranked by FDR (N.T. vs. IFN- $\beta$  and N.T. vs. VPg<sup>EMCV</sup>), are shown. Red genes indicate those upregulated by both IFN- $\gamma$  and VPg<sup>EMCV</sup> and closely ranked. Black genes indicate genes upregulated by both IFN- $\beta$  and VPg<sup>EMCV</sup> but not closely ranked (one in the top 1-20 and the other in the top 21-50). Gray genes indicate genes upregulated by either IFN- $\beta$  or VPg<sup>EMCV</sup> but not by both stimuli.

(b) Top 50 genes upregulated by IFN- $\gamma$  or VPg<sup>EMCV</sup>. All definitions and comparison methods are identical to those in (a).

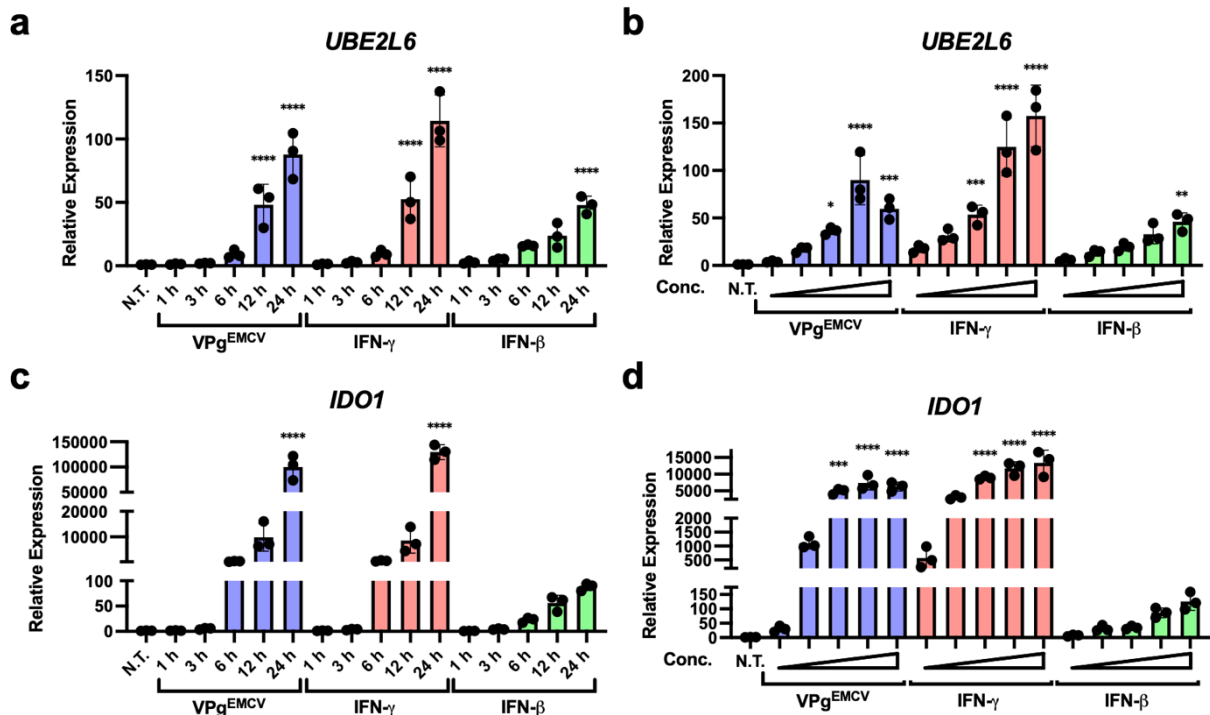
(c) Heatmap comparing relative expression levels of 25 top genes (among the top 50 upregulated genes by VPg<sup>EMCV</sup>) that are highly upregulated by IFN- $\gamma$  and VPg<sup>EMCV</sup>. Mean values of VPg<sup>EMCV</sup> were set as 1 for relative comparison.

(d) Comparison of relative expression levels of 3 class I HLAs (*HLA-A*, *HLA-B*, and *HLA-C*) induced by IFN- $\beta$ , IFN- $\gamma$ , or VPg<sup>EMCV</sup>.

(e) Comparison of relative expression levels of 3 class II HLAs (*HLA-DRA*, *HLA-DMB*, and *HLA-DPB1*) induced by IFN- $\beta$ , IFN- $\gamma$ , or VPg<sup>EMCV</sup>.

For (d, e): Data are presented as the mean (SD), and individual data points are biological replicates (n = 2). Statistical significance was determined by one-way ANOVA, followed by Tukey's HSD post-hoc test. Labels: \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ .

Two representative ISGs were selected for further validation by RT-qPCR to assess time- and concentration-dependent induction. *UBE2L6*, which was upregulated by IFN- $\beta$ , IFN- $\gamma$ , and VPg<sup>EMCV</sup> in RNA-seq analysis, showed increased expression beginning approximately 6 hours post-treatment for all three conditions (**Figure 3-38a**), as well as dose-dependent induction (**Figure 3-38b**). In contrast, *IDO1* which was selectively upregulated by IFN- $\gamma$  and VPg<sup>EMCV</sup> exhibited similar kinetics, with induction starting at approximately 6 hours (**Figure 3-38c**) and increasing in a concentration-dependent manner (**Figure 3-38d**).



**Figure 3-38. Time- and dose-dependent upregulation of 2 representative genes by IFN- $\beta$ , IFN- $\gamma$ , or VPg<sup>EMCV</sup> treatments.**

For (a, c): HeLa cells (n = 3) were treated with VPg<sup>EMCV</sup> (1 ng/mL), IFN- $\gamma$  (10 ng/mL), or IFN- $\beta$  (1000 U/mL) for indicated time points, after which RNA was extracted for RT-qPCR analysis.

For (b, d): HeLa cells (n = 3) were treated for 12 h with varying concentrations of VPg<sup>EMCV</sup> (0.01 ng/mL, 0.1 ng/mL, 1 ng/mL, 10 ng/mL, or 100 ng/mL), IFN- $\gamma$  (0.1 ng/mL, 1 ng/mL, 10 ng/mL, 100 ng/mL, or 1000 ng/mL), or IFN- $\beta$  (10 U/mL, 100 U/mL, 1000 U/mL, 10000 U/mL, or 100000 U/mL), followed by RNA extraction for RT-qPCR analysis.

(a) Time-dependent relative expression of *UBE2L6*.

(b) Dose-dependent relative expression of *UBE2L6*.

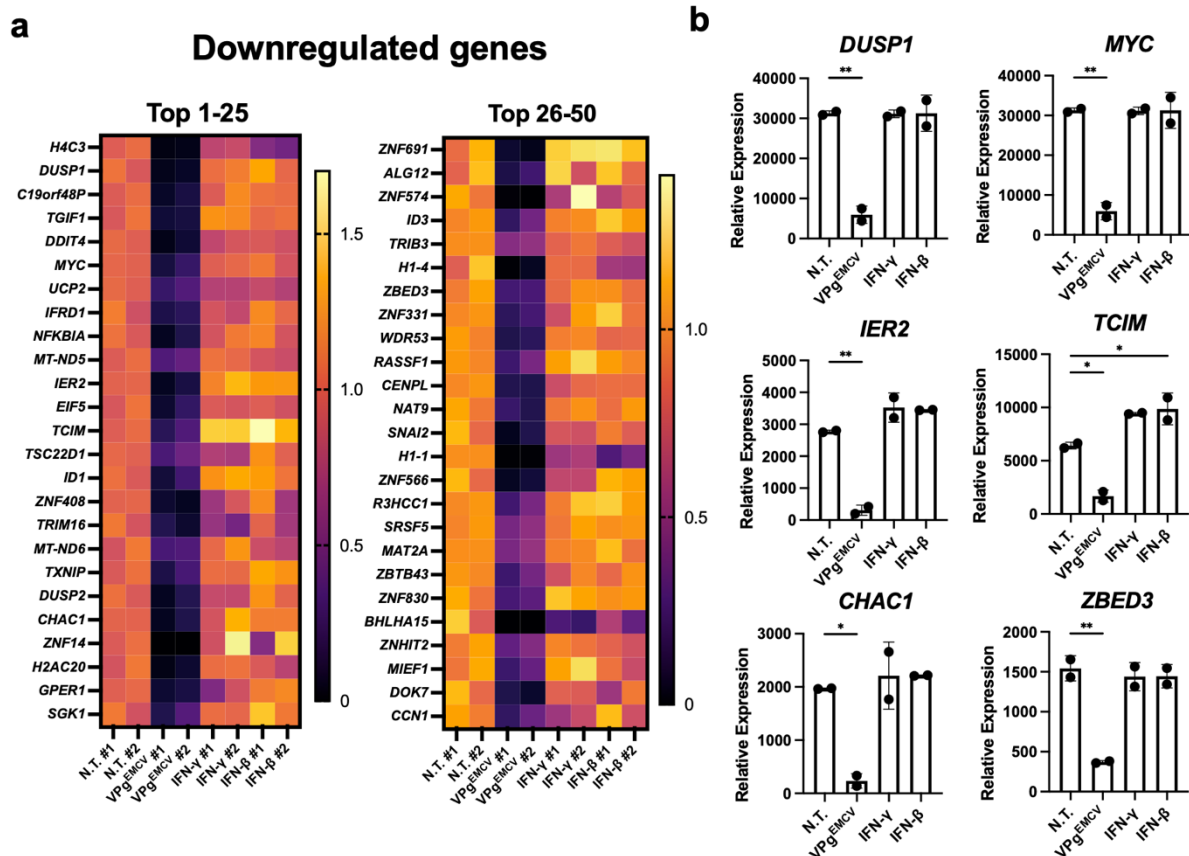
(c) Time-dependent relative expression of *IDO1*.

(d) Dose-dependent relative expression of *IDO1*.

For (a-d): Data are presented as the mean (SD), and individual data points are biological replicates (n = 3). Statistical significance was determined by one-way ANOVA, followed by Tukey's HSD post-hoc test. Asterisks indicate comparisons with the N.T. group. Labels: \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ , \*\*\*\* $p \leq 0.0001$ .

An unexpected observation from RNA-seq analysis was the presence of many downregulated genes associated specifically with VPg<sup>EMCV</sup> treatment. While only 8 and 14 genes were downregulated following IFN- $\beta$  and IFN- $\gamma$  treatment, respectively, VPg<sup>EMCV</sup> treatment resulted in downregulation of 283 genes (**Figure 3-36**). A heatmap of the top 50 downregulated genes revealed that most of these changes were specific to VPg<sup>EMCV</sup> and not shared with IFN- $\beta$  or IFN- $\gamma$  (**Figure 3-39a**), with only a small subset of genes (e.g., *H4C3*, *TRIM16*, *H1-1*, *BHLHA15*) showing some overlap. Even for these genes, downregulation was much more pronounced following VPg<sup>EMCV</sup> treatment.

Several downregulated VPg<sup>EMCV</sup>-specific genes included some known proto-oncogenes such as *DUSP1*, *MYC*, *IER2*, *TCIM*, *CHAC1*, and *ZBED3* (**Figure 3-39b**). These genes were not significantly downregulated by IFN- $\beta$  or IFN- $\gamma$ , suggesting that VPg<sup>EMCV</sup> possesses some ability for transcriptional regulation which is partially distinct from canonical IFN- $\gamma$  signaling.



**Figure 3-39. Gene expression profiles of VPg<sup>EMCV</sup>-downregulated genes and comparison with IFN treatments.**

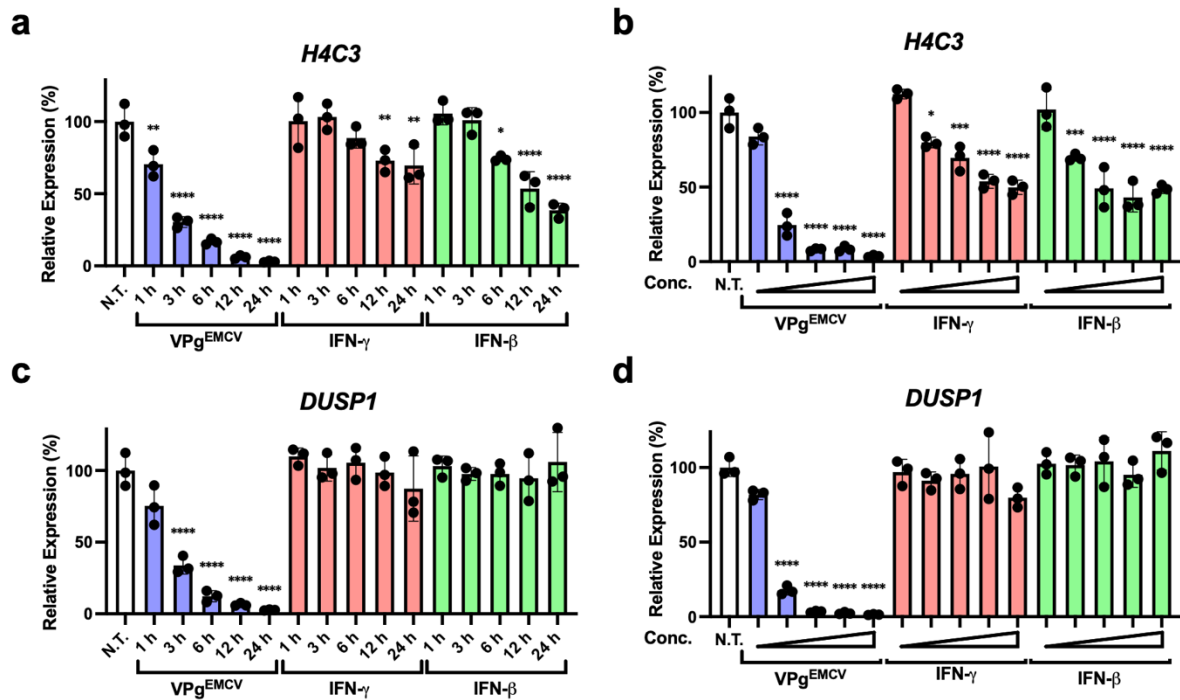
RNA-seq analysis data (n = 2) from Figure 3-36 were used for all figures.

(a) Heatmap comparing relative downregulation of the top 50 genes downregulated by VPg<sup>EMCV</sup>, comparing VPg<sup>EMCV</sup>, IFN-γ, and IFN-β treatments. Mean values of the N.T. group were set as 1 for relative comparison.

(b) Comparison of relative expression levels of 6 proto-oncogenes (*DUSP1*, *MYC*, *IER2*, *TCIM*, *CHAC1*, and *ZBED3*) following VPg<sup>EMCV</sup>, IFN-γ, or IFN-β treatment.

For (b): Data are presented as the mean (SD), and individual data points are biological replicates (n = 2). Statistical significance was determined by one-way ANOVA, followed by Tukey's HSD post-hoc test. Labels: \**p* ≤ 0.05, \*\**p* ≤ 0.01.

Two representative downregulated genes were further validated by RT-qPCR. *H4C3*, the most strongly downregulated gene identified by RNA-seq, showed rapid suppression by VPg<sup>EMCV</sup> as early as 3 hours post-treatment, while downregulation by IFN-β or IFN-γ was much weaker and delayed until approximately 6 hours (**Figure 3-40a**). Concentration-dependent analysis also revealed that VPg<sup>EMCV</sup> downregulated *H4C3* at low concentrations (~0.1 ng/mL) and with greater magnitude than either IFN treatments (**Figure 3-40b**). Similarly, *DUSP1* which was selectively downregulated by VPg<sup>EMCV</sup>, showed rapid downregulation beginning at 3 hours post-treatment, with no downregulation observed by IFN-β or IFN-γ treatment (**Figure 3-40c**, **Figure 3-40d**).



**Figure 3-40. Time- and dose-dependent downregulation of 2 representative genes by IFN- $\beta$ , IFN- $\gamma$ , or VPg<sup>EMCV</sup> treatments.**

For (a, c): HeLa cells ( $n = 3$ ) were treated with VPg<sup>EMCV</sup> (1 ng/mL), IFN- $\gamma$  (10 ng/mL), or IFN- $\beta$  (1000 U/mL) for indicated time points, after which RNA was extracted for RT-qPCR analysis.

For (b, d): HeLa cells ( $n = 3$ ) were treated for 12 h with varying concentrations of VPg<sup>EMCV</sup> (0.01 ng/mL, 0.1 ng/mL, 1 ng/mL, 10 ng/mL, or 100 ng/mL), IFN- $\gamma$  (0.1 ng/mL, 1 ng/mL, 10 ng/mL, 100 ng/mL, or 1000 ng/mL), or IFN- $\beta$  (10 U/mL, 100 U/mL, 1000 U/mL, 10000 U/mL, or 100000 U/mL), followed by RNA extraction for RT-qPCR analysis.

(a) Time-dependent relative expression of *H4C3*.

(b) Dose-dependent relative expression of *H4C3*.

(c) Time-dependent relative expression of *DUSP1*.

(d) Dose-dependent relative expression of *DUSP1*.

For (a-d): Data are presented as the mean (SD), and individual data points are biological replicates ( $n = 3$ ). Statistical significance was determined by one-way ANOVA, followed by Tukey's HSD post-hoc test. Asterisks indicate comparisons with the N.T. group. Labels: \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ , \*\*\*\* $p \leq 0.0001$ .

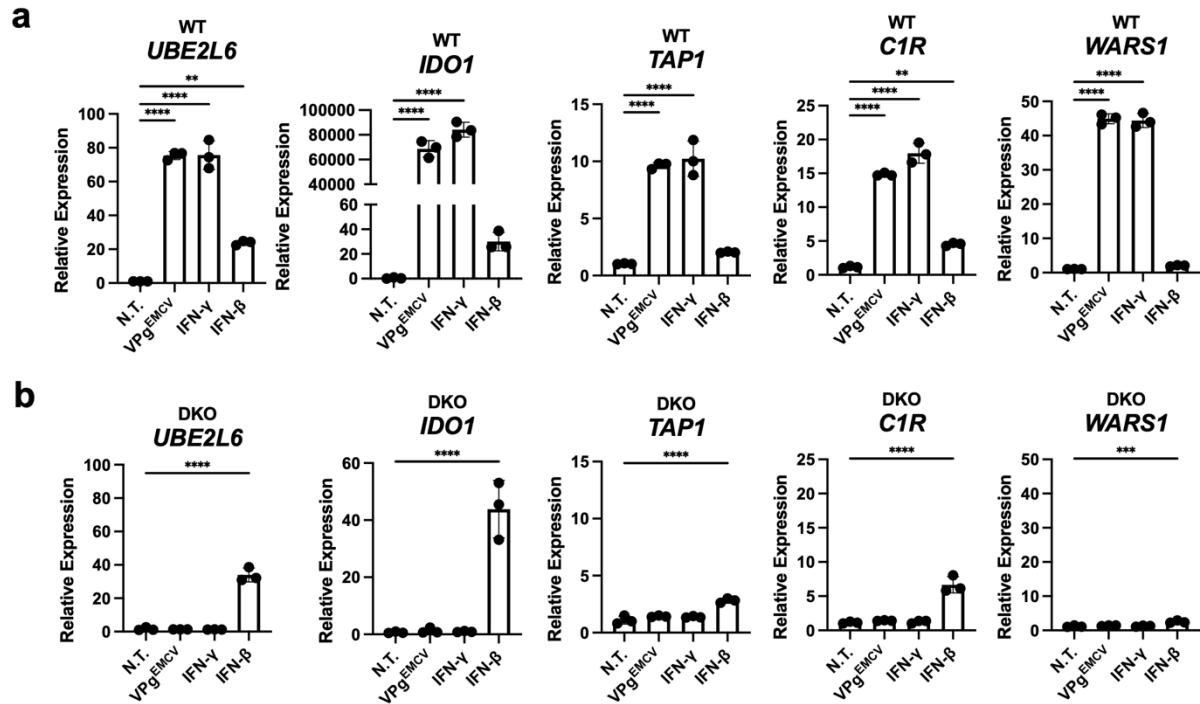
Altogether, these data show that although VPg<sup>EMCV</sup> upregulates genes (ISGs) very similar to IFN- $\gamma$  signaling, confirmed at least for many of the top genes upregulated by VPg<sup>EMCV</sup>, it also uniquely downregulates a distinct set of genes (non-ISGs). This selective downregulation suggests that VPg<sup>EMCV</sup> has additional regulatory effects beyond the canonical IFN- $\gamma$  signaling.

### 3-12 Downregulation of unique genes by VPg<sup>EMCV</sup> is partially independent of IFNGR

Two major hypotheses were considered to explain the observation that many genes were downregulated specifically by VPg<sup>EMCV</sup> but not by IFNs. First, structural studies have suggested that engineered or modified IFN- $\gamma$  variants can engage “noncanonical” IFNGR assemblies and could alter downstream transcriptional outputs [87]. In addition, type I IFN signaling is known to show differences in downstream gene expression patterns depending on the agonist subtype, even though these responses are mediated through the same IFNAR1/2 receptor complex [125, 126]. If VPg<sup>EMCV</sup> behaves similarly as these “modified agonists,” then transcriptional differences could occur while being fully dependent on IFNGR1 and IFNGR2. A second hypothesis was that the VPg<sup>EMCV</sup>-specific gene downregulation occurs completely independent of IFNGR1/2, implying that VPg<sup>EMCV</sup> may possess an additional target/activity beyond its function as an IFNGR agonist.

To test whether VPg<sup>EMCV</sup>-specific gene downregulation depends on IFNGR, IFNGR1/2 double-knockout (DKO) HeLa cells were utilized. WT and IFNGR1/2 DKO cells were treated with IFN- $\beta$ , IFN- $\gamma$ , or VPg<sup>EMCV</sup>, after which RNA was extracted for RT-qPCR analysis. Expression levels of the top five VPg<sup>EMCV</sup>-upregulated genes (*UBE2L6*, *IDO1*, *TAP1*, *C1R*, and *WARS1*) and the top five VPg<sup>EMCV</sup>-downregulated genes (*H4C3*, *DUSP1*, *C19orf48P*, *TGIF1*, and *DDIT4*) were quantified.

First, analysis of the five upregulated genes (ISGs) showed that these genes were induced by IFN- $\beta$ , IFN- $\gamma$ , and VPg<sup>EMCV</sup>, with stronger induction by IFN- $\gamma$  and VPg<sup>EMCV</sup>, consistent with the RNA-seq results (**Figure 3-41a**). Importantly, IFNGR1/2 knockout completely abrogated induction of ISGs by IFN- $\gamma$  and VPg<sup>EMCV</sup>, while IFN- $\beta$ -mediated induction of ISGs was unaffected, as expected (**Figure 3-41b**). These data confirm that IFNGR1/2 is essential for ISG upregulation by VPg<sup>EMCV</sup>.



**Figure 3-41. Top 5 genes upregulated by VPg<sup>EMCV</sup> in WT and IFNGR1/2 DKO cells.**

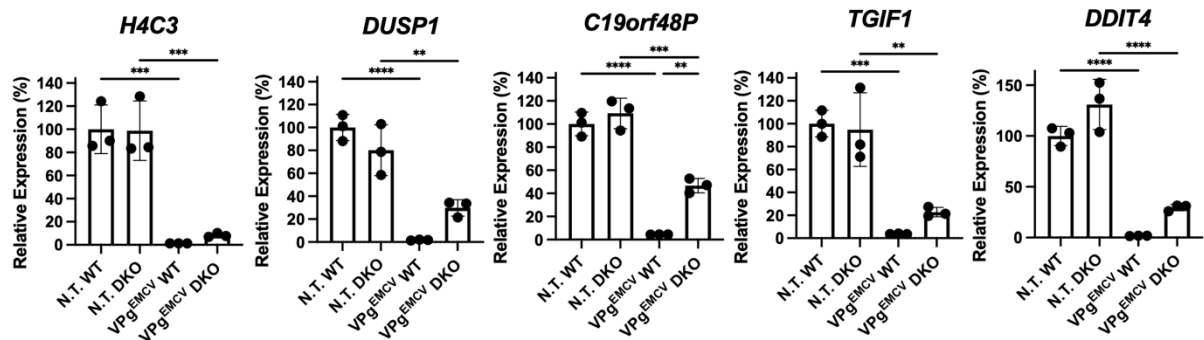
WT or IFNGR1/2 DKO HeLa cells ( $n = 3$ ) were treated with VPg<sup>EMCV</sup> (1 ng/mL), IFN- $\gamma$  (10 ng/mL), or IFN- $\beta$  (1000 U/mL) for 24 h, followed by RNA extraction and RT-qPCR analysis.

(a) Relative expression of *UBE2L6*, *IDO1*, *TAP1*, *C1R*, and *WARS1* in WT cells.

(b) Relative expression of the same genes in IFNGR1/2 DKO cells.

For (a, b): Data are presented as the mean (SD), and individual data points are biological replicates ( $n = 3$ ). Statistical significance was determined by one-way ANOVA, followed by Tukey's HSD post-hoc test. Asterisks indicate comparisons with the N.T. group. Labels: \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ , \*\*\*\* $p \leq 0.0001$ .

The more important question was regarding the VPg<sup>EMCV</sup>-specific downregulated genes. Interestingly, in both WT and IFNGR1/2 DKO cells, downregulation of all 5 genes still occurred following VPg<sup>EMCV</sup> treatment (**Figure 3-42**). However, the magnitude of downregulation was consistently weaker in IFNGR1/2 DKO cells. While VPg<sup>EMCV</sup>-treated WT cells showed <5% relative expression for all 5 genes relative to N.T. controls, DKO cells retained approximately 10–40% expression (depending on the gene). These results indicate that while ISG induction by VPg<sup>EMCV</sup> is strictly IFNGR1/2-dependent, downregulation of VPg<sup>EMCV</sup>-specific genes is at least partially IFNGR-independent. At the same time, because downregulation is somehow attenuated in IFNGR1/2 DKO cells, the contribution/synergy of IFNGR-dependent signaling to this process cannot be excluded.



**Figure 3-42. Top 5 genes downregulated by VPg<sup>EMCV</sup> in WT and IFNGR1/2 DKO cells.**

WT or IFNGR1/2 DKO HeLa cells (n = 3) were treated with VPg<sup>EMCV</sup> (1 ng/mL) for 24 h, followed by RNA extraction and RT-qPCR analysis. Relative expression levels of *H4C3*, *DUSP1*, *C19orf48P*, *TGIF1*, and *DDIT4* were measured.

Data are presented as the mean (SD), and individual data points are biological replicates (n = 3). Statistical significance was determined by one-way ANOVA, followed by Tukey's HSD post-hoc test. Asterisks indicate comparisons with the N.T. group. Labels: \*\**p* ≤ 0.01, \*\*\**p* ≤ 0.001, \*\*\*\**p* ≤ 0.0001.

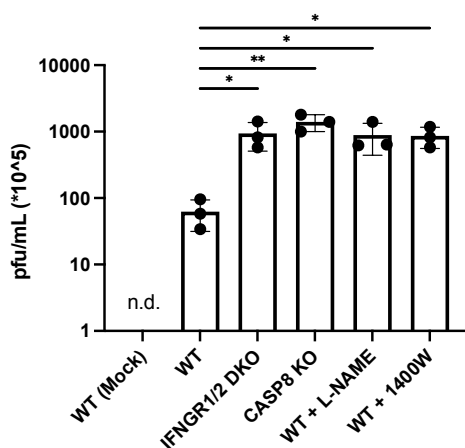
### 3-13 iNOS-dependent cell death during EMCV infection reduces viral load *in vitro* and *in vivo*

Data up to this point showed that VPg<sup>EMCV</sup>, which is released during EMCV infection, functions as an IFNGR agonist and induces iNOS/NO via the JAK-STAT1 signaling pathway. TNF-α, which is released as a host response, synergizes with VPg<sup>EMCV</sup> signaling to induce potent Caspase 8-dependent apoptosis. Next, the biological significance of this iNOS-dependent cell death response was investigated.

It was shown earlier that iNOS inhibition using 1400W suppresses cell death induced during EMCV infection (**Figure 3-24a**). To assess how this iNOS-dependent cell death affects viral replication and viral load, EMCV was infected into WT, IFNGR1/2 DKO, and CASP8 KO cells. In parallel, WT HeLa cells were pre-treated with either pan-NOS inhibitor L-NAME or iNOS-selective inhibitor 1400W for 2 hours prior to infection. Viral titers in the culture supernatants at 24 hours post-infection were quantified by plaque assay using Vero cells.

A significant increase in viral titer was observed in IFNGR1/2 DKO cells, CASP8 KO cells, and WT cells treated with either NOS inhibitors, with an approximate one log-fold increase (**Figure 3-43**). These results suggest that iNOS/NO-dependent, TNF-α-triggered apoptosis contributes to suppression of EMCV replication and a reduction in viral load. Because HeLa cells are highly unlikely to produce IFN-γ during

EMCV infection, and this was also shown from the results described previously (**Figure 3-31**, **Figure 3-32**), the effect observed in IFNGR1/2 DKO cells is most likely explained by loss of VPg<sup>EMCV</sup>-mediated IFNGR signaling. Taken together, the similar phenotype observed with IFNGR1/2 DKO, iNOS inhibition, and CASP8 knockout supports the model in which abrogation of VPg<sup>EMCV</sup>-IFNGR signaling inhibits iNOS induction and downstream Caspase 8-dependent apoptosis, allowing increased viral replication and increased EMCV load.



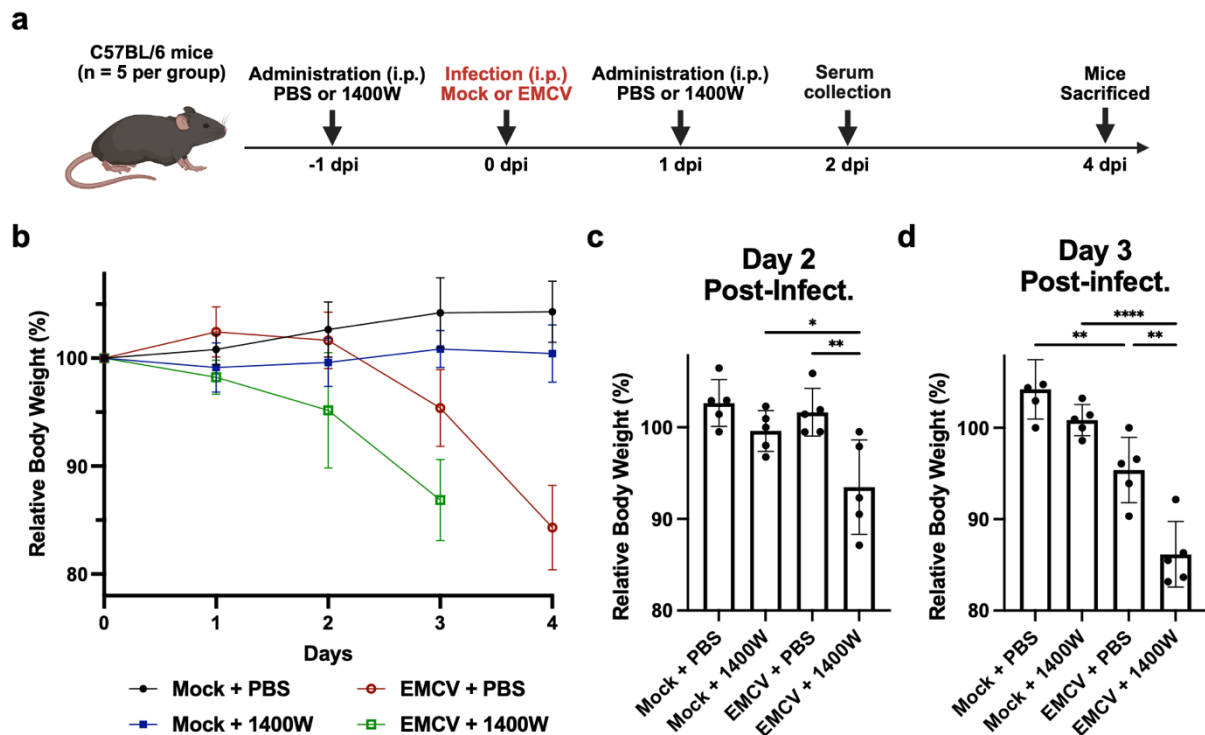
**Figure 3-43. Inhibition of downstream signaling components of VPg<sup>EMCV</sup> increases viral titer *in vitro*.**

WT, IFNGR1/2 DKO, or CASP 8 KO HeLa cells were infected with EMCV (MOI 0.1) for 24 h. For conditions of iNOS inhibition, WT HeLa cells were pre-treated with L-NAME (1 mM) or 1400W (100  $\mu$ M) for 2 h prior to infection. Mock infection conditions were also prepared as negative controls. Supernatants were collected, and viral titers were determined by plaque assay using Vero cells. Data are presented as the mean (SD), and individual data points are biological replicates (n = 3). Statistical significance was determined by one-way ANOVA, followed by Tukey's HSD post-hoc test. Labels: \* $p \leq 0.05$ , \*\* $p \leq 0.01$ . n.d.: not detected ( $< 1 \times 10^5$  pfu/mL).

Because the above experiments were restricted *in vitro*, the role of iNOS during EMCV infection was next investigated *in vivo*. C57BL/6 mice (n = 5 per group) were prepared for EMCV infection, with one group administered intraperitoneally (i.p.) with PBS (control) and the other group administered with 1400W to inhibit iNOS. Mock-infected groups were prepared in parallel (PBS or 1400W). As shown in the experimental scheme (**Figure 3-44a**), mice were administered PBS or 1400W one day before infection and again after EMCV infection. Serum was collected from all mice at day 2 post-infection, and all mice were sacrificed at day 4 post-infection following proper euthanasia guidelines. Body weight and clinical symptoms were recorded daily.

Comparison of body weight between groups revealed a clear difference in weight loss kinetics (**Figure 3-44b**). In mice without iNOS inhibition (PBS + EMCV group), weights remained relatively stable until day 2 post-infection and began to decline from day 3. In contrast, mice treated with 1400W (1400W + EMCV group) began to lose weight by day 2 post-infection, showing a statistically significant reduction compared to the PBS + EMCV group (**Figure 3-44c**). By day 3 post-infection,

iNOS-inhibited mice showed marked weight loss (average ~85%) compared with mice without iNOS inhibition (average ~95%) (**Figure 3-44d**).



**Figure 3-44. iNOS inhibition by 1400W accelerates weight loss caused by EMCV infection *in vivo*.**

(a) Schematic of the mouse infection experiment. C57BL/6 mice were divided into four groups: Mock + PBS, Mock + 1400W, EMCV + PBS, and EMCV + 1400W (n = 5 per group). Mice were injected intraperitoneally (i.p.) with either PBS (control) or 1400W (10 mg/kg) 1 day pre- and post-EMCV infection, and were infected i.p. with either Mock or EMCV (1000 PFU/body) on day 0. Sera were collected on day 2 post-infection, and all mice were sacrificed on day 4 post-infection.

(b) Body weights of mice were measured daily and plotted.

(c) Comparison of body weights between groups shown in (b) at day 2 post-infection.

(d) Comparison of body weights between groups shown in (b) at day 3 post-infection.

For (b-d): Data are presented as the mean (SD). In (b), data represent each mouse (n = 5); in (c, d), individual data points represent each mouse (n = 5). For (c, d): Statistical significance was determined by one-way ANOVA, followed by Tukey's HSD post-hoc test. Labels: \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\*\* $p \leq 0.0001$ .

To compare clinical severity, mouse conditions were scored on a 5-level scale: healthy (1), slightly weakened (2), very weakened (3), paralyzed/almost dead (4), and dead (5). A table summarizing individual mouse scores was used instead of a standard survival curve, as this clearly captures the differences between the groups (**Figure 3-45**). Mice without iNOS inhibition (PBS + EMCV group) remained relatively healthy through day 3. Several mice became severely weakened or paralyzed by day 4, although all mice were still alive at that time point. In contrast, mice treated with 1400W

began showing mild symptoms at day 2, progressed to severe weakness or paralysis by day 3, and all mice were dead by day 4. Together, the body weight loss and symptom progression indicate that iNOS inhibition definitely worsens the symptoms after EMCV infection *in vivo*, consistent with the increased EMCV titer observed upon suppression of iNOS-dependent apoptosis *in vitro*.

| EMCV<br>+ PBS | Day 1 | Day 2 | Day 3 | Day 4 |
|---------------|-------|-------|-------|-------|
| Mouse 1       | 1     | 1     | 1     | 3     |
| Mouse 2       | 1     | 1     | 2     | 4     |
| Mouse 3       | 1     | 1     | 1     | 3     |
| Mouse 4       | 1     | 1     | 1     | 4     |
| Mouse 5       | 1     | 1     | 1     | 2     |

| EMCV<br>+1400W | Day 1 | Day 2 | Day 3 | Day 4 |
|----------------|-------|-------|-------|-------|
| Mouse 1        | 1     | 2     | 4     | 5     |
| Mouse 2        | 1     | 1     | 3     | 5     |
| Mouse 3        | 1     | 1     | 3     | 5     |
| Mouse 4        | 1     | 2     | 4     | 5     |
| Mouse 5        | 1     | 1     | 2     | 5     |

**Condition Score:**

1: Healthy 2: Slightly weakened 3: Very weakened 4: Paralyzed/almost dead 5: Dead

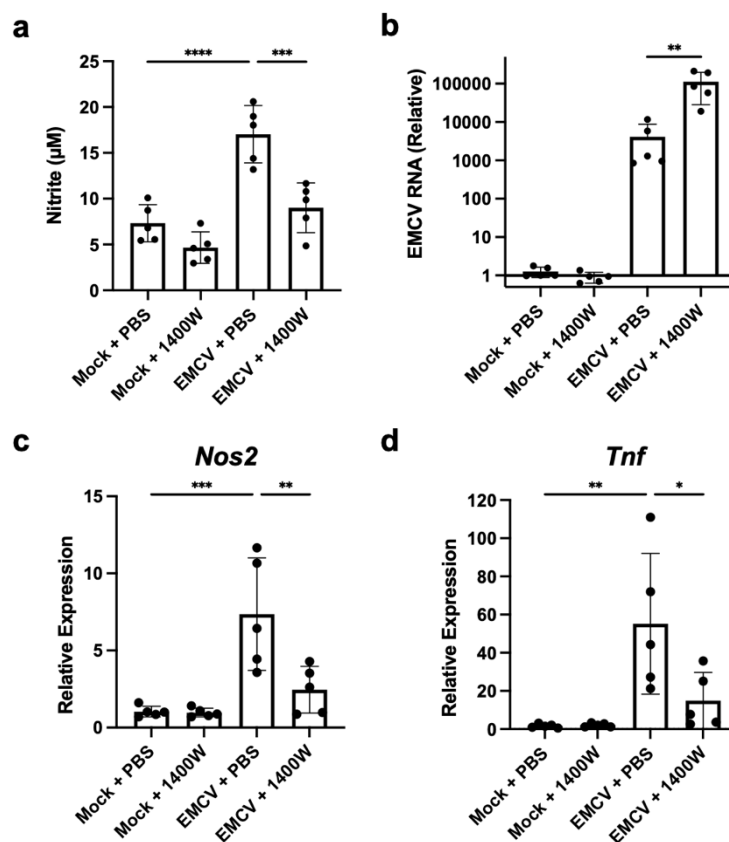
**Figure 3-45. Inhibition of iNOS by 1400W *in vivo* accelerates symptoms and death caused by EMCV infection.**

Daily symptoms/conditions of individual mice from Figure 3-44a were scored by visual assessment using the following criteria: healthy (1), slightly weakened (2), very weakened (3), paralyzed/almost dead (4), and dead (5).

Serum collected at day 2 post-infection was further analyzed to compare the groups. Total nitrite levels in the serum were quantified by Griess assay. Nitrite levels were significantly increased in EMCV-infected mice compared to Mock groups, and this increase was significantly reduced by 1400W treatment (**Figure 3-46a**), indicating that NO production occurs during EMCV infection *in vivo* and is strongly dependent on iNOS activity.

EMCV RNA in serum was quantified by qPCR as an indicator of circulating viral load. As expected, EMCV RNA was detected in serum at day 2 post-infection. iNOS inhibition resulted in a significant increase in EMCV RNA, exceeding one log-fold on average (**Figure 3-46b**), consistent with the *in vitro* results and supporting the interpretation that iNOS-dependent responses contribute to reduction of EMCV load.

Finally, expression levels of *Nos2* (iNOS) and *Tnf* (TNF- $\alpha$ ) were quantified. Both *Nos2* (**Figure 3-46c**) and *Tnf* (**Figure 3-46d**) were significantly increased after EMCV infection, supporting that induction of iNOS and TNF- $\alpha$  observed *in vitro* is also relevant *in vivo*. As expected, 1400W treatment was associated with reduced *Nos2* expression. *Tnf* expression was also reduced in the iNOS-inhibited group. Taken together, these results support that VPg<sup>EMCV</sup>-induced iNOS/NO induction and TNF- $\alpha$ -triggered Caspase 8-dependent cell death act to reduce EMCV load *in vitro*, and that inhibition of iNOS *in vivo* is associated with reduced nitrite levels, increased circulating EMCV RNA, and accelerated symptoms (including early death).



**Figure 3-46. iNOS inhibition during EMCV infection increases NO levels and EMCV load in circulating blood.**

All samples were obtained from mice shown in Figure 3-44a.

(a) Total nitrite levels in mouse sera at day 2 post-infection were quantified using Griess assay.

(b) Relative EMCV RNA levels in sera at day 2 post-infection were quantified by qPCR.

(c) Relative *Nos2* expression in sera at day 2 post-infection was quantified by qPCR.

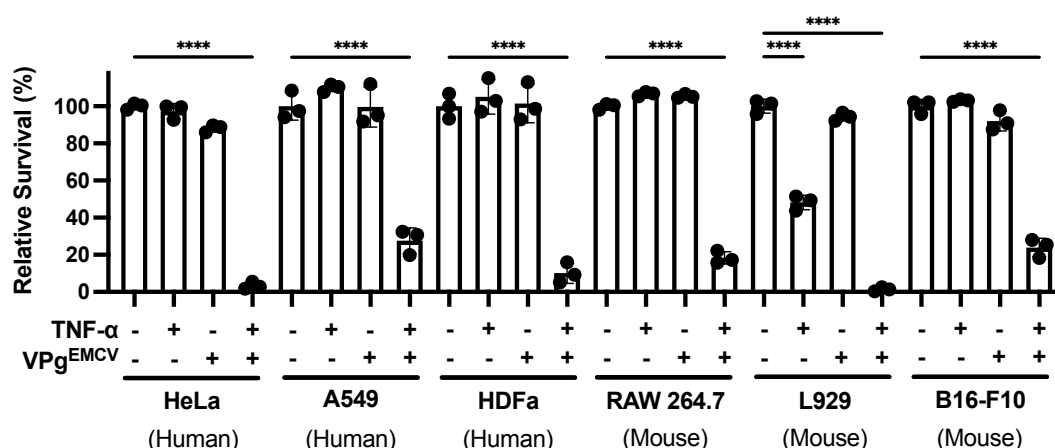
(d) Relative *Tnf* expression in sera at day 2 post-infection was quantified by qPCR.

For (a-d): Data are presented as the mean (SD), and individual data points represent each mouse ( $n = 5$ ). Statistical significance was determined by one-way ANOVA, followed by Tukey's HSD post-hoc test. Labels: \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$ , \*\*\*\* $p \leq 0.0001$ .

### 3.14 Assessing the potential efficacy of VPg<sup>EMCV</sup>

Some properties of VPg<sup>EMCV</sup> were investigated further, particularly regarding its potential efficacy for practical clinical use. First, the potent cell death induced by low concentrations of VPg<sup>EMCV</sup> in combination with TNF- $\alpha$  was tested in some additional cell lines (**Figure 3-47**). In addition to HeLa cells, A549 cells (human lung carcinoma) were tested, and cell death was induced in A549 cells similarly to HeLa cells. Cell death was also induced in primary human dermal fibroblasts, adult (HDFa) when treated with the combination of TNF- $\alpha$  and VPg<sup>EMCV</sup>.

Next, murine cell lines were tested for cell death induction by VPg<sup>EMCV</sup> together with recombinant murine TNF- $\alpha$ , including RAW 264.7 cells (mouse macrophage cells derived from a tumor), L929 cells (mouse fibroblasts), and B16-F10 cells (mouse melanoma). Surprisingly, potent cell death was induced not only in human cells but also in all 3 murine cell types (**Figure 3-47**). As reference, TNF- $\alpha$  alone can induce partial cell death in L929 cells, consistent with the known TNF- $\alpha$  sensitivity of this cell line (ref). Altogether, these results suggest that VPg<sup>EMCV</sup> can promote TNF- $\alpha$ -dependent cell death across diverse cell types and is active in both human and murine cells.

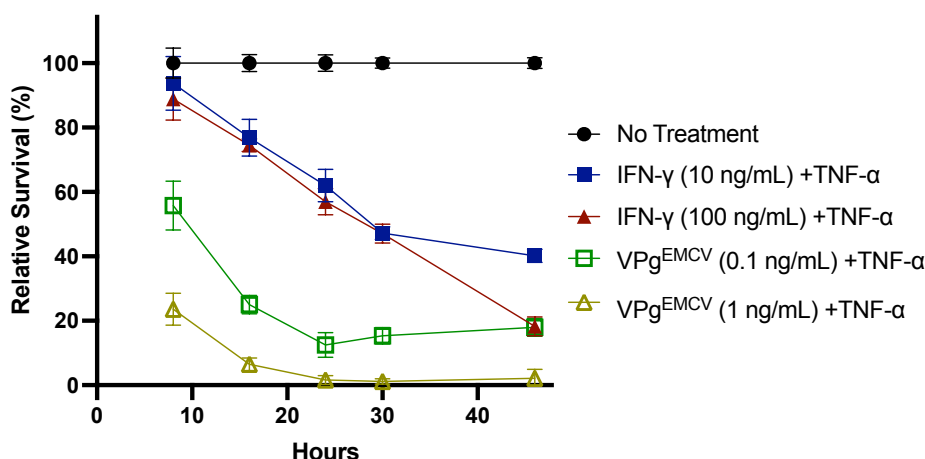


**Figure 3-47. VPg<sup>EMCV</sup> + TNF- $\alpha$  induces cell death in various human and murine cell lines.**

Three human cell types (HeLa, A549, and primary adult human dermal fibroblasts (HDFa)) and three murine cell types (RAW 264.7, L929, and B16-F10) were treated with recombinant TNF- $\alpha$  (10 ng/mL; human or murine TNF- $\alpha$  according to the cell type), VPg<sup>EMCV</sup> (1 ng/mL), or their combination for 24 h. Cells were fixed and stained using Amido Black 10B, then eluted for relative survival quantification.

Data are presented as the mean (SD), and individual data points are biological replicates (n = 3). Statistical significance was determined by one-way ANOVA, followed by Tukey's HSD post-hoc test. Label: \*\*\*\*p  $\leq$  0.0001.

From previous data comparing IFN- $\gamma$  + TNF- $\alpha$  and VPg<sup>EMCV</sup> + TNF- $\alpha$ , it was suggested that VPg<sup>EMCV</sup> may induce more rapid cell death at the 24-hour timepoint (**Figure 3-24c**). To confirm this further, cell death induced by IFN- $\gamma$  (10 ng/mL and 100 ng/mL) + TNF- $\alpha$  was compared with VPg<sup>EMCV</sup> (0.1 ng/mL and 1 ng/mL) + TNF- $\alpha$  in HeLa cells over a 48-hour period. VPg<sup>EMCV</sup> + TNF- $\alpha$  induced more rapid and potent cell death, particularly at the 24 hour timepoint, while IFN- $\gamma$  + TNF- $\alpha$  induced slower cell death, with most cells eliminated by 48 hours (**Figure 3-48**). Although both conditions induce cell death through an iNOS-dependent pathway, these results suggest that VPg<sup>EMCV</sup> may exert additional cytotoxic effects in combination with TNF- $\alpha$ .



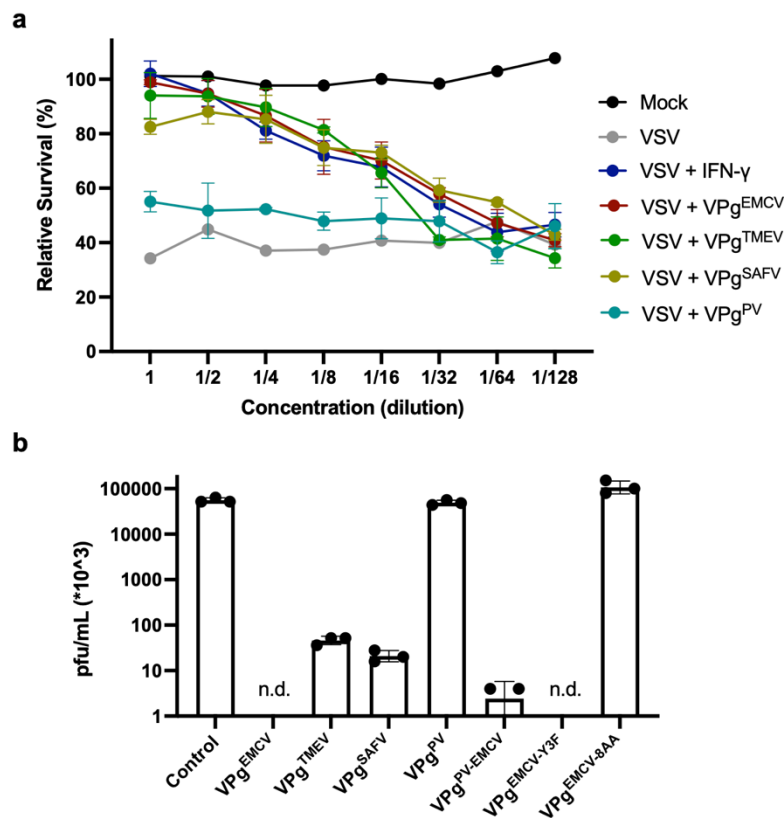
**Figure 3-48. IFN- $\gamma$  + TNF- $\alpha$  and VPg<sup>EMCV</sup> + TNF- $\alpha$  exhibit different magnitudes and kinetics of cell death.**

HeLa cells were treated with recombinant human IFN- $\gamma$  (10 or 100 ng/mL) + recombinant human TNF- $\alpha$  (10 ng/mL) or with VPg<sup>EMCV</sup> (0.1 or 1 ng/mL) + recombinant human TNF- $\alpha$  (10 ng/mL) for the indicated time points up to 48 h. Cells were fixed and stained using Amido Black 10B, then eluted for relative survival quantification. Data are presented as the mean (SD), and data are biological replicates (n = 3).

Given that VPg<sup>EMCV</sup> acts as a cytokine mimic and induces an ISG profile highly similar to IFN- $\gamma$ , the antiviral properties of *Cardiovirus* VPgs were assessed. HeLa cells were pre-treated overnight with serial dilutions of IFN- $\gamma$ , VPg<sup>EMCV</sup>, VPg<sup>TMEV</sup>, VPg<sup>SAFV</sup>, or VPg<sup>PV</sup>. The next day, cells were infected with VSV for 24 hours to evaluate antiviral activities of the VPgs. IFN- $\gamma$ , as well as the three *Cardiovirus* VPgs, exhibited

antiviral activity that prevented VSV-induced cytopathic effects (**Figure 3-49a**). As expected, VPg<sup>PV</sup> did not show comparable activity.

In a follow-up experiment, HeLa cells were pre-treated overnight with the three *Cardiovirus* VPgs, as well as VPg<sup>PV</sup>, VPg<sup>PV-EMCV</sup>, VPg<sup>EMCV-Y3F</sup>, and VPg<sup>EMCV-8AA</sup>. Cells were then infected with VSV for 24 hours, and the supernatants were collected for plaque assay on L929 cells. Pretreatment with all three *Cardiovirus* VPgs, as well as VPg<sup>PV-EMCV</sup> and VPg<sup>EMCV-Y3F</sup>, significantly reduced VSV titers, with some conditions showing VSV titers even below the limit of detection ( $< 1 \times 10^3$  PFU/mL) by plaque assay (**Figure 3-49b**). As expected, VPg<sup>PV</sup> and VPg<sup>EMCV-8AA</sup> did not reduce viral titer. Together, these experiments indicate that these *Cardiovirus* VPgs not only induce potent cell death with TNF- $\alpha$  but also exhibit antiviral activities resembling IFN- $\gamma$  activity.



**Figure 3-49. *Cardiovirus* VPgs exhibit antiviral activities similar to IFN- $\gamma$ .**

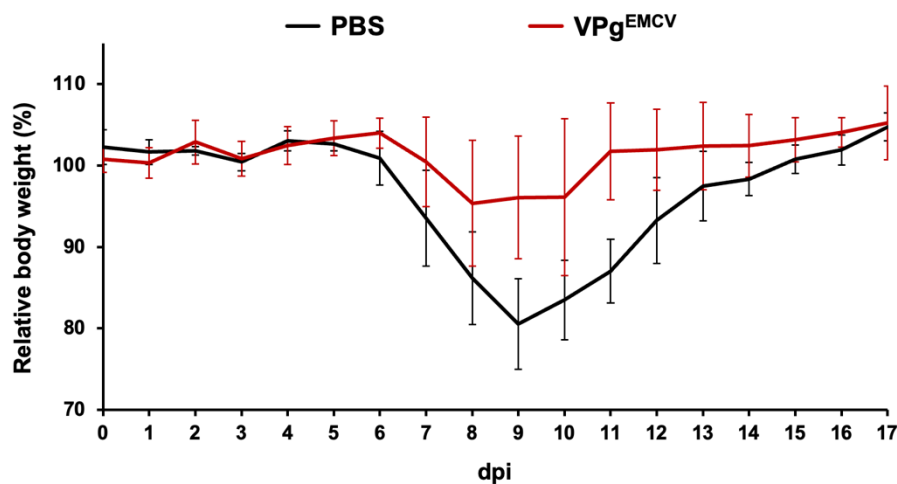
(a) HeLa cells were pre-treated overnight with 2-fold serial dilutions of recombinant human IFN- $\gamma$  (starting from 10 ng/mL) or with 2-fold serial dilutions of various VPgs as indicated (starting from 2 ng/mL). Cells were then infected with VSV (MOI 0.1) for 24 h. Cells were fixed and stained using Amido Black 10B, then eluted for relative survival quantification. Data are presented as the mean (SD). Biological replicates were  $n = 3$ , except for the Mock group ( $n = 1$ ) and VSV-only group ( $n = 1$ ).

(b) HeLa cells were pre-treated overnight with various VPgs (1 ng/mL) as indicated, then infected with VSV (MOI 0.1) for 24 h. Viral titers in the supernatants were quantified by plaque assay using L929 cells. Data are presented as the mean (SD), and individual data points are biological replicates ( $n = 3$ ). n.d.: not detected ( $< 1 \times 10^3$  pfu/mL).

Finally, the potential of VPg<sup>EMCV</sup> to act as an antiviral agent during influenza A virus infection was tested *in vivo* using the Influenza A virus (IAV, H1N1 PR8 strain). C57BL/6 mice were prepared ( $n = 5$  per group) and intranasally administered 30  $\mu$ L of PBS (control) or VPg<sup>EMCV</sup> (10  $\mu$ g/mL) one day before and one day after infection with

500 PFU/body of IAV. This infection dose was selected to induce significant weight loss, while avoiding lethality, which was known from previous experiments. Mouse body weight was monitored daily for 17 days (**Figure 3-50**).

In the PBS group, weight loss began around day 6-7 post-infection, peaked around day 9, and reached an average of approximately 80% of the starting body weight, followed by gradual recovery until day 17. In contrast, mice administered with VPg<sup>EMCV</sup> exhibited only mild weight loss, including at the peak timepoint (8-10 days), suggesting an antiviral effect of VPg<sup>EMCV</sup> *in vivo*. Although these *in vivo* analyses in this study remain preliminary, this experiment still supports the potential of VPg<sup>EMCV</sup> to exert antiviral activity by intranasal administration. Overall, these results show that VPg<sup>EMCV</sup> is effective in inducing TNF- $\alpha$ -dependent cell death *in vitro* in several cell lines. In addition, VPg<sup>EMCV</sup> and two other *Cardiovirus* VPgs also showed antiviral activity *in vitro*. Furthermore, VPg<sup>EMCV</sup> exhibited antiviral activity not only *in vitro* but also *in vivo*.



**Figure 3-50. Intranasal administration of VPg<sup>EMCV</sup> exhibits antiviral activity against influenza A virus *in vivo*.**

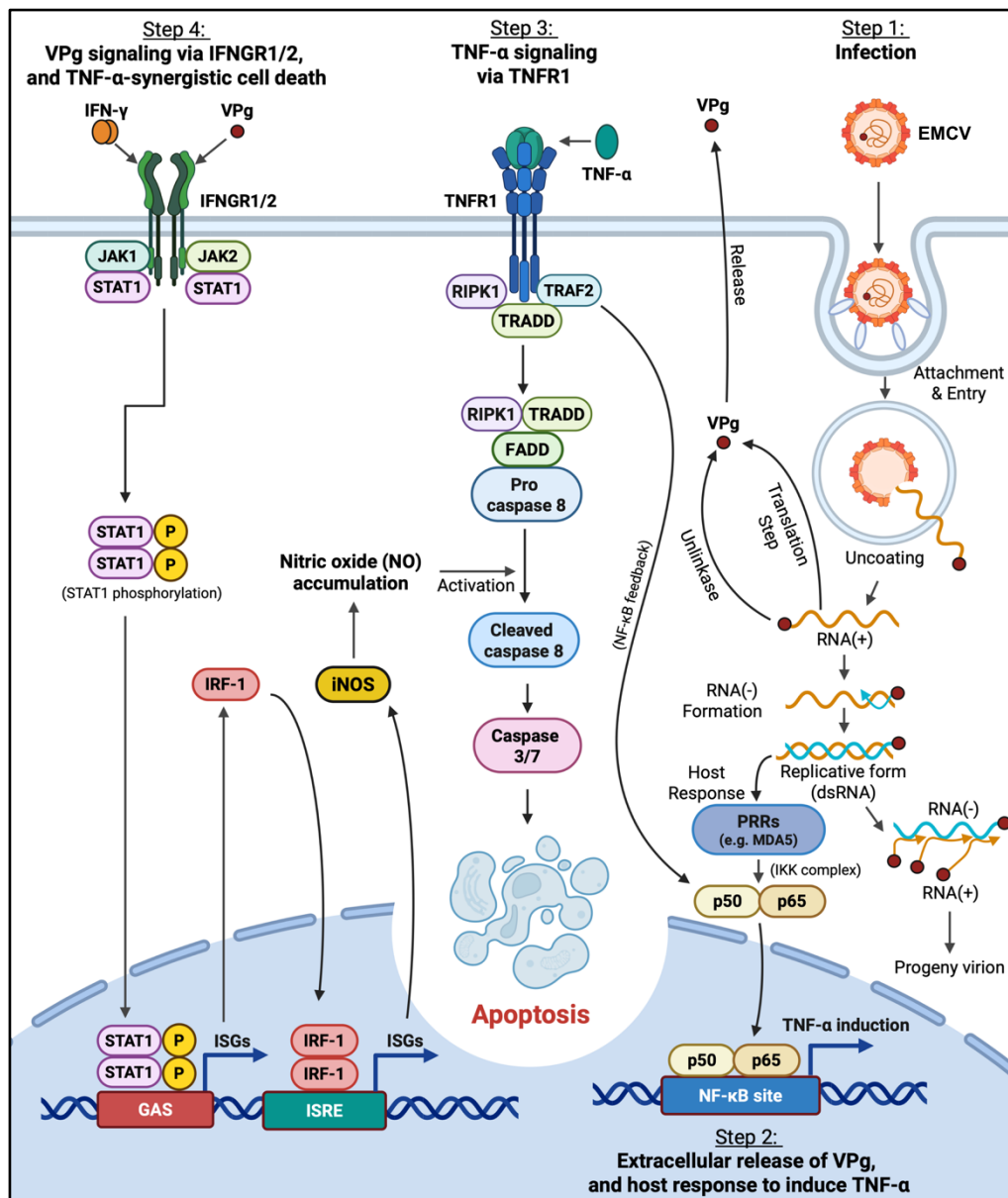
C57BL/6 mice (n = 5 per group) were intranasally administered with 30  $\mu$ L of PBS (control) or VPg<sup>EMCV</sup> (10  $\mu$ g/mL) both 1 day pre- and post-influenza A virus (IAV) infection. Mice were infected intranasally with IAV (500 PFU/body) on day 0 post-infection. Body weights were measured daily for 17 days and plotted. Data are presented as the mean (SD), and data represent each mouse (n = 5).

## **Chapter 4**

### **Discussion**

## 4-1 Overview of pathways and mechanisms identified in this study

In this study, the most central finding is that VPg<sup>EMCV</sup> not only functions as an essential protein during viral RNA synthesis, but also possesses a previously unrecognized, “noncanonical” host-modulating function. Specifically, VPg<sup>EMCV</sup> functions as an IFNGR agonist and induces downstream signaling that is quite indistinguishable from canonical IFN- $\gamma$  signaling. Based on this discovery, a schematic describing the sequence of events from EMCV infection to TNF- $\alpha$  and VPg-dependent apoptosis was constructed, and this is summarized in **Figure 4-1**.



**Figure 4-1. Overall schematic of EMCV infection, extracellular VPg release, and VPg/TNF- $\alpha$  synergy.**

(This figure was created using BioRender.)

As shown in this schematic, the overall process can be divided into 4 major phases. Step 1 is the infection phase, in which the EMCV virion attaches to its host entry receptor and releases its genomic RNA into the cytoplasm. VPg is cleaved from the viral RNA by VPg unlinkase (protein TDP2) and is also generated during polyprotein processing, as described previously in the Introduction (Section 1-2-2). Although the detailed EMCV life cycle and the canonical function of VPg have already been discussed earlier and will not be explained here, it is evident that free VPg is ultimately generated within infected cells and is released extracellularly.

Based on well-established current knowledge, extracellular VPg may be produced both through VPg unlinkase-mediated cleavage and as a consequence of polyprotein processing. Importantly, as shown in **Figure 3-13**, the kinetics of VPg release sufficient to exert activity closely mirror the increase in EMCV viral titers, occurring approximately 6 hours post-infection in HeLa cells. In addition, as discussed in the Introduction (Section 1-2-2), *Picornaviridae*, including *Cardiovirus* and *Enterovirus*, are lytic viruses that primarily exit host cells through cell lysis. Consistent with this, detection of cytoplasmic proteins such as GAPDH and viral proteins such as 3D<sup>Pol</sup> in post-infected culture supernatants (**Figure 3-19a**) strongly suggests that VPg is released predominantly through passive cytosolic leakage associated with virus-induced cytopathic effects.

Step 2 shows the host response phase. During viral replication, formation of the replicative form (RF) dsRNA allows recognition by PRRs as PAMPs. The host response to EMCV RNA has been well characterized, with MDA5 identified as the primary sensor (see Section 1-2-3). In brief, downstream innate immune signaling activates the NF- $\kappa$ B pathway, leading to induction of inflammatory cytokines, including TNF- $\alpha$ . Notably, although EMCV is known to antagonize type I IFN signaling through certain viral proteins (see Section 1-2-3 for details), this study clearly shows that sufficient TNF- $\alpha$  is produced during infection to induce potent, non-cell-autonomous cell death (**Figure 3-20**).

In Step 3, TNF- $\alpha$  produced during the host response phase is sensed by non-infected cells, primarily through binding to TNFR1. Under physiological conditions, TNF- $\alpha$  signaling through TNFR1 promotes inflammatory responses via NF- $\kappa$ B activation or the MAP kinase cascade, rather than directly inducing apoptosis, as described in Section 1-3.

Step 4 occurs almost simultaneously with Step 3. Extracellularly released VPg<sup>EMCV</sup> binds directly to IFNGR1/2 and functions as a cytokine mimic of canonical IFN- $\gamma$ . Although viral replication, cell lysis, and extracellular release of VPg should also occur during infection with other *Picornaviridae* such as *Enterovirus*, this study revealed that VPg<sup>EMCV</sup> and some other *Cardiovirus* VPgs uniquely possesses this host-responsive activity, capable of triggering IFNGR downstream signaling.

Upon binding to IFNGR1/2, VPg<sup>EMCV</sup> activates the JAK-STAT1 signaling pathway in a manner very similar to IFN- $\gamma$ , leading to induction of IRF-1 and upregulation of iNOS. iNOS-mediated NO accumulation plays a crucial role in sensitizing cells to TNF- $\alpha$ -dependent signaling through TNFR1. This synergy of TNF- $\alpha$  signaling (Step 3) and VPg-induced NO production (Step 4) activates Caspase 8 by its cleavage, and ultimately results in potent apoptotic cell death.

## **4-2 VPg and IFNGR binding and implications for structural studies**

### 4-2-1 Summary of current findings

The findings regarding amino acid residues of VPg<sup>EMCV</sup> that are required for its functional activity and physical interaction with IFNGR are summarized in **Figure 4-2**. First, it was demonstrated that the C-terminal region of *Cardiovirus* VPg contains 8 highly conserved amino acids when comparing EMCV, SAFV, and TMEV. In contrast, VPg<sup>PV</sup> showed markedly reduced activity; however, this activity was restored when the 8 C-terminal amino acids were replaced with those from EMCV. In addition, it was confirmed that the third tyrosine residue, which is essential for the canonical function of VPg during viral RNA synthesis, is not required for its activity as an IFNGR agonist.

Further analysis using alanine scanning revealed that the 15th glutamine and 16th leucine residues of VPg<sup>EMCV</sup> are critical for maintaining its IFNGR agonist activity. Notably, the corresponding residue at position 16 in VPg<sup>SAFV</sup> and VPg<sup>TMEV</sup> is valine, a closely related hydrophobic amino acid, and these VPgs retained full activity, suggesting interchangeability of closely-related amino acids at this position. Importantly, single mutations at either position 15 (Q15A) or position 16 (L16A) did not completely abolish activity, as comparable STAT1 phosphorylation was still observed at higher concentrations (100 ng/mL). In contrast, the double mutant (Q15A/L16A) completely lost functional activity. These observations suggest that while each residue

individually contributes to IFNGR agonist activity, at least one of the two residues must be present to maintain partial function, and that both residues together are likely important for stable/efficient interaction with the IFNGR complex.

Consistent with this, the co-immunoprecipitation experiments in this study showed stronger association of VPg<sup>EMCV</sup> with IFNGR1 than with IFNGR2. This raises the possibility that residues Q15 and L16 participate in direct binding to IFNGR1; however, this hypothesis will require additional evidence to be definitively confirmed.

An unresolved aspect of VPg<sup>EMCV</sup> function is the requirement for the N-terminal region. Truncation of VPg<sup>EMCV</sup> from 20 to 17 amino acids, removing the N-terminal GPY residues while preserving the C-terminal region, resulted in a significant reduction in activity, particularly in TNF- $\alpha$ -dependent cell death sensitization. Further truncation to 13 amino acids, removing the GPYNETA sequence, completely abolished activity. Moreover, the isolated C-terminal 8 amino acid sequence (TLQLLDIQ) exhibited no detectable activity, despite evidence that the QL motif within this region are the critical amino acids for proper function.

Taken together, these results indicate that the N-terminal region of VPg<sup>EMCV</sup> is also required to maintain IFNGR agonist activity and cell death-sensitizing function. However, several observations suggest that the specific N-terminal amino acid sequence may be less important than the overall length of the peptide. VPg<sup>SAFV</sup> and VPg<sup>TMEV</sup> possess divergent N-terminal sequences yet retain their activity, and grafting the conserved C-terminal 8 amino acids of EMCV to VPg<sup>PV</sup> restored its function. In addition, VPg<sup>PV-EMCV</sup>, which contains 22 amino acids, exhibited activity comparable to VPg<sup>EMCV</sup>, indicating that an exact length of 20 amino acids is not strictly required. These findings altogether suggest that the peptide length, rather than the precise N-terminal sequence, is important for stable interaction with IFNGR and downstream signaling.

| Name (Label)                  | Sequence of VPg                           | Amino Acids | Apoptosis w/ TNF- $\alpha$ | p-STAT1 Induction | IFNGR1/2 Binding |
|-------------------------------|-------------------------------------------|-------------|----------------------------|-------------------|------------------|
| VPg <sup>EMCV</sup>           | GPYNETARVKPK <b>TLQLLDIQ</b>              | 20          | ++++                       | ++++              | ++++             |
| VPg <sup>SAFV</sup>           | AA <b>Y</b> AGRARAQ <b>KQALQVLDIQ</b>     | 20          | ++++                       | ++++              | Not tested       |
| VPg <sup>TMEV</sup>           | AA <b>Y</b> SGGKKGE <b>KQTLQVLDVQ</b>     | 20          | ++++                       | ++++              | Not tested       |
| VPg <sup>PV</sup>             | G <b>A</b> YTLGPNKKPNV <b>PTTIR</b> TAKVQ | 22          | +                          | -                 | Not tested       |
| VPg <sup>PV-EMCV</sup>        | G <b>A</b> YTLGPNKKPNV <b>PTLQLLDIQ</b>   | 22          | ++++                       | ++++              | Not tested       |
| VPg <sup>EMCV-Y3F</sup>       | GP <b>F</b> NETARVKPK <b>TLQLLDIQ</b>     | 20          | ++++                       | ++++              | Not tested       |
| VPg <sup>EMCV-17AA</sup>      | NETARVKPK <b>TLQLLDIQ</b>                 | 17          | +++                        | +++               | Not tested       |
| VPg <sup>EMCV-13AA</sup>      | RVKPK <b>TLQLLDIQ</b>                     | 13          | -                          | -                 | Not tested       |
| VPg <sup>EMCV-8AA</sup>       | <b>TLQLLDIQ</b>                           | 8           | -                          | -                 | Not tested       |
| VPg <sup>EMCV-Q15A</sup>      | GPYNETARVKPK <b>TLALLDIQ</b>              | 20          | ++                         | ++                | -                |
| VPg <sup>EMCV-L16A</sup>      | GPYNETARVKPK <b>TLQALDIQ</b>              | 20          | ++                         | ++                | No input         |
| VPg <sup>EMCV-Q15A/L16A</sup> | GPYNETARVKPK <b>TLAALDIQ</b>              | 20          | +                          | -                 | No input         |

**Figure 4-2. Comparison of all synthetic VPgs used in this study.**

The table compares amino acid sequences, peptide length, and relative activities compared with VPg<sup>EMCV</sup>, including apoptosis with TNF- $\alpha$ , STAT1 phosphorylation, and IFNGR1/2 binding. Labels: ++++ (comparable/equal to VPg<sup>EMCV</sup>), +++ (slightly weak), ++ (weak), + (very weak/almost no activity), - (no activity). The third tyrosine residue required for canonical VPg activity is indicated in blue. The eight conserved C-terminal amino acids are highlighted in yellow, and the critical QL residues are indicated in red.

#### 4-2-2 Comparison of human and murine IFN- $\gamma$ and IFNGR, detailed comparison of VPg and IFN- $\gamma$

There are clear differences between IFN- $\gamma$  and VPg<sup>EMCV</sup>. One prominent observed difference is species cross-reactivity. Human and murine IFN- $\gamma$  share approximately 41% amino acid identity, and are generally unable to cross-react with the receptor of the other species [127, 128]. This lack of cross-reactivity in IFN- $\gamma$  is different with other cytokines, for example TNF- $\alpha$ , which has approximately 79% amino acid homology between human and mouse, and is known to cross-react between species [129, 130]. In contrast to IFN- $\gamma$ , VPg<sup>EMCV</sup> was found in this study to induce signaling and biological effects in both human and murine cell lines. VPg<sup>EMCV</sup> also exhibited antiviral activity in mice. These findings suggest that VPg<sup>EMCV</sup> is capable of cross-species activity, at least between human and mouse.

To further examine differences between different species, amino acid sequences of IFN- $\gamma$  from human, mouse, pig, and chicken are compared (**Figure 4-3**). Conserved amino acids across species are highlighted in yellow, while residues conserved specifically among mammals are shown in red (all IFN- $\gamma$  sequences of different species referenced from [131]). Although certain residues are conserved, IFN- $\gamma$  sequences differ significantly among species. Importantly, VPg<sup>EMCV</sup>

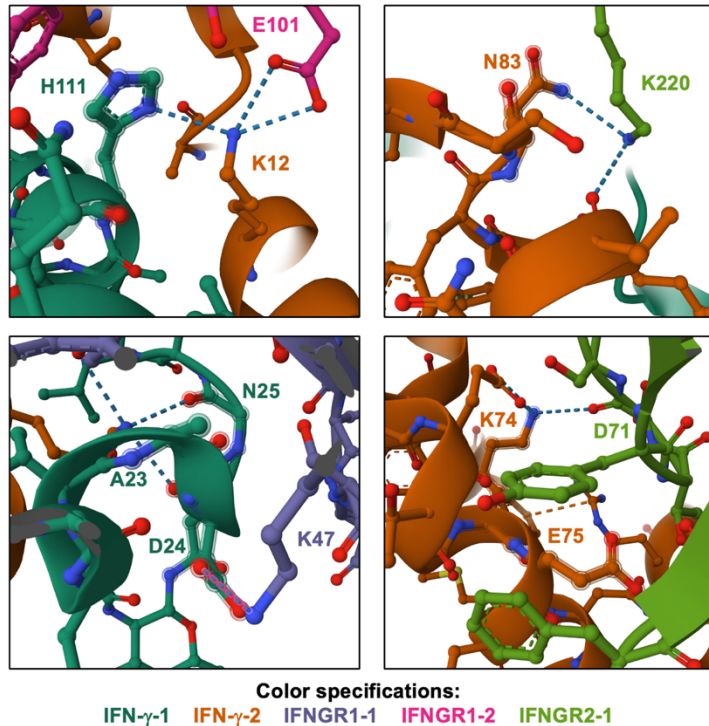
(GPYNETARVVKPKTLQLLDIQ) does not share any sequence homology with IFN- $\gamma$  from any of these species, supporting the conclusion that VPg<sup>EMCV</sup> does not share detectable sequence homology with IFN- $\gamma$ , and therefore does not represent a sequence-based mimic.

|                    |                  |            |
|--------------------|------------------|------------|
|                    | 33               |            |
| MKYTSYILAFQLCIVL   | GSLG-CYCQDPYVKEA | ENLK       |
| MNATHCILALQLFLMAV  | SG--CYCHGTVIESL  | ESLN       |
| MSYTTYFLAFQLCVTL   | CFSG-SYCQAPFKKEI | TILK       |
| MTCQTYNLFVLSVIMIYY | GHTASSLNLVQLQDDI | DKLK       |
|                    |                  |            |
|                    | 92               |            |
| KNWKEES            | DRKIMQS          | QIVSFYFKLF |
| RNWQKDG            | DMKILQS          | QIISFYLR   |
| KNWKEES            | DKIIQS           | QIVSFYFKFE |
| KNWTERNE           | KRIILS           | QIVSMYLE   |
|                    |                  |            |
|                    | 143              |            |
| EKLITNYS           | VTDLNV           | QRKA       |
| MSIAKFE            | VNNPQV           | QRQAFN     |
| EKLIPVD            | NLQIQ            | KAISEL     |
| MDLAKL             | PMNDL            | RIRKA      |

**Figure 4-3. Comparison of human, mouse, pig and chick IFN- $\gamma$  amino acid sequences.**

Amino acid sequence homology among IFN- $\gamma$  from human, mouse, pig, and chick is shown. Sequences highlighted in yellow indicate conserved amino acids (identical or closely related) across all four species, while red letters indicate residues conserved among mammals only.

Regarding the IFN- $\gamma$ -IFNGR complex, as described in the Introduction (Section 1-4-2), IFN- $\gamma$  functions as a homodimer and binds with IFNGR to form a hexameric 2:2:2 complex comprised of IFN- $\gamma$ , IFNGR1, and IFNGR2 [87]. Previous studies have identified histidine 111 (H111) of human IFN- $\gamma$  as critical for its biological activity, and the H111D mutant exhibiting significantly reduced antiviral activity and receptor recognition [132]. A major paper published many years later [87] further showed that a triple mutant (K74A/E75Y/N83R) loses binding to IFNGR2, while retaining binding to IFNGR1. In contrast, although the H111D mutation disrupts IFNGR1 binding at one interface, complete loss of binding to both IFNGR1 and IFNGR2 requires multiple mutations in IFN- $\gamma$  (A23E, D24E, N25K, K74A, E75Y, N83R, H111D) [87]. These findings highlight the complexity of IFN- $\gamma$ -IFNGR interactions, in which two IFN- $\gamma$  engage both IFNGR1 and IFNGR2 through multiple binding sites. A schematic of the critical IFN- $\gamma$  residues involved in IFNGR1/2 binding is shown in **Figure 4-4**.



**Figure 4-4. Critical binding sites of human IFN- $\gamma$  within the IFNGR complex.**

Key IFN- $\gamma$  residues involved in IFNGR binding are shown: residue H111 for IFNGR1 binding (top left); residues A23, D24, and N25 for IFNGR1 binding (bottom left); residue N83 for IFNGR2 binding (top right); and residues K74 and E75 for IFNGR2 binding (bottom right).

(Structural data were retrieved from the RCSB Protein Data Bank, PDB ID: 6E3L, deposited by Jude K. M., Mendoza J. L., and Garcia K. C., released February 2019.)

Comparison of human and murine IFN- $\gamma$  sequences (reference [131]) reveals that most amino acid residues critical for IFNGR1/2 binding in human IFN- $\gamma$  are not conserved in murine IFN- $\gamma$ , with the exception of D25, which is substituted with glutamic acid (E) in murine IFN- $\gamma$ , which is a closely related residue (**Figure 4-5**). These sequence differences possibly explain the lack of cross-reactivity between human and murine IFN- $\gamma$ . However, it has been reported that human IFN- $\gamma$  can still exert very weak activities on murine IFNGR, and vice versa, but this is at concentrations much higher than IFN- $\gamma$  of the same species [127].

Binding with hLFNGR1      Binding with hLFNGR2      Binding with hLFNGR1

20      30      70      90      105      115

HSDVADNGTLF ... VETIKEDMNVKFFNSNKKKRD ... VQRKAIHELIIQ      Human

GIDVEE-KSLF ... ISVIESHLITNFFSNSKAKD ... VQRQAFNELIR      Mouse

24-26      74-75      83      111

**Figure 4-5. Comparison of human and murine IFN- $\gamma$  sequences in human IFNGR1/2 binding regions.**

Known human IFN- $\gamma$  residues involved in binding to IFNGR1 or IFNGR2 are shown in orange. The murine IFN- $\gamma$  residue E25 is shown in blue as a closely-related amino acid of D25 in human IFN- $\gamma$ .

Taken together, these observations show that IFN- $\gamma$ -IFNGR signaling depends on a highly complex, multivalent interaction rather than a single critical binding residue. Single-point mutations in IFN- $\gamma$  often reduce STAT1 phosphorylation and downstream gene induction, but rarely abrogate downstream signaling completely [87]. This complexity most likely explains the extremely weak cross-species activity observed for IFN- $\gamma$  under non-physiological concentrations.

In this context, it is surprising that VPg<sup>EMCV</sup>, a peptide comprised of merely 20 amino acids, can bind to the IFNGR complex and activate downstream signaling. The double mutation of Q15/L16 completely abrogated STAT1 phosphorylation and biological activity, while single mutants (Q15A or L16A) still retained weak activity at higher concentrations, despite significantly reduced or even undetectable receptor binding (in the case of Q15A). This behavior could be perhaps similar to that of IFN- $\gamma$  single point mutants, which may retain partial activity despite impaired physical interactions with IFNGR1/2 [87, 127].

Another unresolved question is whether VPg<sup>EMCV</sup> functions as a monomer or forms dimers/multimers, alike IFN- $\gamma$  homodimers. Although multimerization cannot be excluded, it appears unlikely from current data that dimerization or oligomerization is a stable or required state for VPg<sup>EMCV</sup>, given its small size and successful filtration using the 3 kDa cut-off filter. Conventional techniques such as Native-PAGE may be poorly suited for assessing dimerization/oligomerization of such small peptides, particularly as free VPg<sup>EMCV</sup> appears as a smear in Western blots. While approaches such as nuclear magnetic resonance (NMR) spectroscopy or dynamic light scattering (DLS) could be theoretically applied, technical challenges will surely remain due to the peptide's size.

Based on current data, the requirement for the length of VPg<sup>EMCV</sup> for activity (>17 amino acids) is likely due to the necessity of VPg<sup>EMCV</sup> to stably dock onto IFNGR1/2 to trigger downstream signaling. An alternative hypothesis which was considered was that VPg<sup>EMCV</sup> might enter cells and interact with intracellular regions of IFNGR1/2. However, this appears somewhat unlikely, as large antibody-bound VPg<sup>EMCV</sup> still retained biological activity (**Figure 3-18a**). In addition, VPg<sup>EMCV</sup> lacks currently-known cell-penetrating peptide (CPP) motifs [133, 134], particularly within the N-terminal region required for activity. While additional studies will be necessary to map the interaction interface and docking to IFNGR1/2, current evidence strongly

supports a model in which VPg<sup>EMCV</sup> bind to the extracellular domain of IFNGR, alike IFN- $\gamma$ . However, this binding is most likely structurally distinct to canonical IFN- $\gamma$ .

#### *4-2-3 Future approaches to strengthen evidence for physical binding and structural analysis*

Although the co-immunoprecipitation studies provide solid evidence that VPg<sup>EMCV</sup> binds IFNGR, additional experiments could further strengthen this conclusion. While other experiments were not feasible within the time and resource constraints of the study, several potential experimental strategies could be envisioned to extend these findings further.

One straightforward approach would be to use techniques such as surface plasmon resonance (SPR) to directly assess binding between VPg<sup>EMCV</sup> and IFNGR. SPR should also allow determination of the binding affinity (K<sub>d</sub>) and kinetics, and would also enable a comparison between the binding between *Cardiovirus* VPg and IFN- $\gamma$ . These data would be quite valuable given the cross-species activity of VPg<sup>EMCV</sup>, which has been confirmed in at least for both human and murine IFNGR.

Other than binding assays, one ultimate goal of future structural studies would be to construct a detailed model of VPg<sup>EMCV</sup> interaction with IFNGR1/2. Techniques such as X-ray crystallography or cryo-electron microscopy (cryo-EM) could theoretically provide high-resolution structure information. However, these methods may have technical challenges due to the extremely small size of VPg<sup>EMCV</sup> compared with IFN- $\gamma$  and IFNGR1/2. As an initial step, *in silico* approaches, such as structure prediction and docking simulations using tools like AlphaFold Multimer, may be a more practical starting point. Key parameters that should be incorporated in future computational modeling will be briefly discussed.

First and probably most important, is the requirement of residues Q15 and L16 of VPg<sup>EMCV</sup>. The complete loss of activity observed with the Q15A/L16A double mutant (**Figure 3-17, Figure 3-29**) strongly suggests that these residues play a direct role in engagement with IFNGR1/2. Second, the importance of N-terminal peptide length must be considered: full-length VPg<sup>EMCV</sup> (20 amino acids) exhibits efficient activity, while truncated VPg<sup>EMCV</sup> below 13 amino acids completely abrogates both activity and binding (**Figure 3-16, Figure 3-31c**), indicating that a sufficient peptide length is possibly required for a stable interaction with IFNGR1/2.

Third, data from other VPgs should be integrated. VPgs from other *Cardiovirus*, as well as the VPg<sup>PV-EMCV</sup> hybrid, show comparable activity to VPg<sup>EMCV</sup> and are likely to engage IFNGR in a similar manner (**Figure 3-15b, Figure 3-31b**). Poliovirus VPg (VPg<sup>PV</sup>), on the other hand, lacks this activity (**Figure 3-14c, Figure 3-31b**) and should exhibit much weaker or absent binding. Fourth, co-immunoprecipitation data suggest that VPg<sup>EMCV</sup> interacts more strongly with IFNGR1 than with IFNGR2, although detectable binding to IFNGR2 was also observed (**Figure 3-34**). This raises the possibility that VPg<sup>EMCV</sup> primarily binds with IFNGR1, while either 1) indirectly recruiting IFNGR2 or 2) interacting with both subunits differently, but stronger to IFNGR1. It is quite likely that the latter may be the actual case, as single overexpression of IFNGR2 still showed partial pulldown bands of VPg<sup>EMCV</sup> (**Figure 3-34**).

Fifth, the state of VPg<sup>EMCV</sup> (monomer, dimer, or oligomer) remains unsolved. While dimerization or multimerization cannot be excluded, current evidence suggests that VPg<sup>EMCV</sup> is unlikely to exist as a stable dimer under physiological conditions. If VPg<sup>EMCV</sup> functions as a monomer, a possible binding model would be the 1:2:2 complex (VPg<sup>EMCV</sup>:IFNGR1:IFNGR2), which is distinct from the canonical 2:2:2 IFN- $\gamma$ -IFNGR1/2 complex. Nevertheless, because VPg<sup>EMCV</sup> oligomerization has not been experimentally addressed in any way, alternative stoichiometries should also be considered in future modeling. This may be quite important, given the possibility that VPg<sup>EMCV</sup> induces a distinct IFNGR conformational state compared with IFN- $\gamma$ , resulting in biased/noncanonical receptor signaling.

Finally, given the lack of sequence homology between VPg<sup>EMCV</sup> and IFN- $\gamma$ , together with the observed cross-species activity of VPg<sup>EMCV</sup>, it is highly likely that VPg<sup>EMCV</sup> engages a binding site(s) distinct from canonical IFN- $\gamma$ . An important parameter for future modeling will be the identification of IFNGR1/2 regions that are conserved between species, as these conserved residues may be the potential VPg<sup>EMCV</sup> interaction sites.

After identifying possible residues from *in silico* modeling, *in vitro* experiments could be designed for actual validation. These include generation of point-mutated IFNGR1 and IFNGR2 expression plasmids based on predicted interaction sites, followed by co-immunoprecipitation or other binding assays. In addition, antibodies capable of detecting VPg<sup>EMCV</sup> mutants (particularly L16A and Q15A/L16A) would allow more precise comparison of binding between WT and the mutant VPg<sup>EMCV</sup> variants.

These experiments altogether would significantly strengthen the understanding of VPg<sup>EMCV</sup>-IFNGR1/2 interactions. However, these ideas are suited for future studies which may build upon the findings of this study, given the scope and technical demands of these experiments.

#### **4-3 Potential IFNGR-independent effects of VPg<sup>EMCV</sup>**

Another peculiar phenomenon observed in this study is the differences between IFN- $\gamma$  and VPg<sup>EMCV</sup> regarding gene downregulation. VPg<sup>EMCV</sup> uniquely downregulated a large subset of genes that are possible completely unrelated to canonical IFN signaling. Moreover, although not all downregulated genes were validated by RT-qPCR, at least the top 5 genes remained significantly downregulated even in IFNGR1/2 DKO cells. However, the magnitude of downregulation was reduced in DKO cells compared with WT cells, indicating that this response is not fully independent of IFNGR signaling, but rather partially IFNGR-dependent.

First, it is very unlikely that this phenomenon results from residual IFNGR expression, such as due to an incomplete knockout. STAT1 phosphorylation was completely abrogated in IFNGR1/2 DKO cells following both IFN- $\gamma$  and VPg<sup>EMCV</sup> treatment (**Figure 3-30c**), and ISG upregulation by VPg<sup>EMCV</sup> or IFN- $\gamma$  was completely lost in these cells by RT-qPCR analysis (**Figure 3-41b**). These results strongly support that this reason of partial downregulation is not due to an incomplete knockout of IFNGR1/2.

Several additional observations scattered throughout the study further suggest the presence of VPg<sup>EMCV</sup> activities that are not entirely dependent on the IFNGR1/2–JAK/STAT1–iNOS pathway. For example, inhibition of iNOS using L-NAME or 1400W (**Figure 3-24c**), or inhibition of JAK signaling using Tofacitinib (**Figure 3-26a**), did not completely suppress cell death induced by VPg<sup>EMCV</sup> + TNF- $\alpha$ . This is actually different with IFN- $\gamma$  + TNF- $\alpha$  treatment, where cell death was almost completely abrogated by using the same inhibitors (**Figure 3-24c**). Importantly, Tofacitinib fully abolished STAT1 phosphorylation, iNOS induction (**Figure 3-26b**), and NO production (**Figure 3-27b**), indicating that the residual cell death observed with VPg<sup>EMCV</sup> is unlikely to be explained by efficiency of the inhibitors, such as incomplete pathway inhibition.

Consistent with this observation, knockout of IFNGR1 or IFNGR2 did not fully eliminate (but significantly reduced) cell death induced by VPg<sup>EMCV</sup> + TNF- $\alpha$  (**Figure**

**3-30b**). Together, these findings suggest that VPg<sup>EMCV</sup> exhibits additional effects beyond canonical IFNGR signaling, which may contribute to the observed differences between VPg<sup>EMCV</sup> and IFN- $\gamma$ -mediated cell death, including the rapid cell death induced by VPg<sup>EMCV</sup> (**Figure 3-24c**, **Figure 3-48**).

Gene ontology analysis of VPg<sup>EMCV</sup>-downregulated genes revealed significant enrichment of pathways related to DNA damage response and DNA repair (**Figure 3-39a**). Some notable examples include *DDIT4* (DNA damage-inducible transcript 4), a hypoxia-responsive gene that negatively regulates mTOR signaling [135, 136], and *TGIF1*, which interacts with transcriptional regulators such as c-Jun and HDAC1 and is associated in the cell cycle and apoptosis [137, 138]. Other downregulated genes include *DUSP1*, a negative regulator of MAP kinase signaling [139], and some well-known proto-oncogenes such as *MYC* [140].

Another notable gene is *IFRD1* (interferon-related developmental regulator 1), which is induced during glutamine starvation, and has been reported to inhibit autophagy [141], as well as interacting with HDAC1 [142]. Despite the name “interferon-related,” *IFRD1* expression was not significantly affected by IFN- $\gamma$  or IFN- $\beta$  treatment. Downregulation of these genes further support the idea that VPg<sup>EMCV</sup>-induced downregulation involves mechanisms distinct from canonical IFN signaling. In total, more than 200 genes were selectively downregulated by VPg<sup>EMCV</sup>.

An initial hypothesis considered was that VPg<sup>EMCV</sup> might induce a “noncanonical” downstream IFNGR signaling that selectively induces gene downregulation. However, past literature suggests that these modified IFNGR agonists modulate only the magnitude of ISG induction, rather than inducing gene downregulation. For example, IFN- $\gamma$ -responsive genes such as *ICAM1*, *PARP14*, *GBP1*, and *IDO1* have been shown to be “tunable” depending on the ligand-receptor interactions, while other ISGs (e.g., *HLA-A* and *CHAC1*) remain relatively stable [87]. This pattern differs from this study, where VPg<sup>EMCV</sup> selectively downregulated numerous genes unrelated to IFN signaling while inducing ISGs in a manner nearly identical to IFN- $\gamma$ . These past reports argue against altered IFNGR downstream signaling as the trigger of gene downregulation.

Instead, the current idea is that VPg<sup>EMCV</sup> triggers gene downregulation through an IFNGR-independent mechanism, while canonical IFNGR signaling synergistically modulates the magnitude of this response. This idea is consistent with the observation that gene downregulation occurs in IFNGR1/2 DKO cells, albeit at reduced levels.

Binding of certain cytokines to extracellular components other than its canonical receptor has been reported previously. For example, IFN- $\gamma$  from multiple species can bind heparan sulfate (HS) in the extracellular matrix (ECM) via a conserved C-terminal KRKR motif (see **Figure 4-3** for the location of motif) independently of IFNGR binding, and this interaction has been shown to modulate biological activity and reduce systemic toxicity *in vivo* [128, 143]. Although VPg<sup>EMCV</sup> does not contain a KRKR motif, this past literature suggests that such short motifs can exert biological effects through noncanonical extracellular interactions.

One possible extension of this hypothesis is that VPg<sup>EMCV</sup> directly interacts with an unidentified extracellular or cell-surface component, which initiates gene downregulation independently of IFNGR, while IFNGR-dependent downstream signaling enhances/accelerates apoptosis-related processes. Supporting this idea, downregulation of certain genes (e.g., *H4C3* and *DUSP1*) occurred earlier (**Figure 3-40a**, **Figure 3-40c**) than ISG induction (**Figure 3-38a**, **Figure 3-38c**), suggesting that gene downregulation may be an early preceding event, rather than a result from apoptosis. Therefore, the current model in mind of VPg<sup>EMCV</sup>-dependent gene downregulation (although requiring further validation) is that VPg<sup>EMCV</sup> initiates transcriptional repression through an IFNGR-independent mechanism, while IFNGR-dependent signaling, particularly iNOS/NO induction, amplifies this response, potentially through stress/DNA damage-associated feedback loops. RNA-seq analysis, rather than RT-qPCR, comparing VPg<sup>EMCV</sup>-treated WT and IFNGR1/2 DKO cells across multiple time points could further dissect the time-dependent relationship between gene downregulation and apoptosis, although such analyses were beyond the scope of the present study.

Finally, identification of the IFNGR-independent binding partner(s) of VPg<sup>EMCV</sup> still remains as an important future question. As one important observation, VPg<sup>EMCV</sup>-Q15A/L16A retained partial cytotoxicity at higher concentrations (100 ng/mL) (**Figure 3-17d**) despite the complete loss of STAT1 phosphorylation at the same concentration (**Figure 3-29**). This could indicate that the Q15/L16 motif is critical for IFNGR signaling but could be dispensable for possible other VPg<sup>EMCV</sup> functions. This further supports the existence of at least one additional IFNGR-independent mechanism which is contributing to downregulation of unique genes.

## 4-4 Biological significance of *Cardiovirus* VPg

### 4-4-1 Biological relevance of the newly-identified function of VPg in the context of EMCV infection

The biological relevance of the IFNGR agonist function of VPg<sup>EMCV</sup> has several important implications in the context of EMCV infection. From the data presented in this study, VPg<sup>EMCV</sup> signaling, through induction of iNOS/NO in synergy with TNF- $\alpha$ , may contribute to the suppression of viral replication both *in vitro* and *in vivo*. In addition, VPg<sup>EMCV</sup> alone exhibited antiviral activity, consistent with its ability to function as an IFNGR agonist. Although iNOS inhibition *in vivo* significantly increased viral load and disease severity, it is important to consider the actual relative contribution of this newly identified VPg<sup>EMCV</sup> function within the broader context of host-pathogen interactions.

Because virus infection experiments in this study were restricted to EMCV, the discussion here focuses primarily on only EMCV infection. As explained in the Introduction (Section 1-2-3), EMCV encodes multiple viral proteins that actively modulate host responses to facilitate viral replication. Within this complex host-EMCV relationship, this study identifies a previously unrecognized factor during EMCV infection, which is the synergistic interaction between EMCV-derived VPg<sup>EMCV</sup> signaling and host-derived TNF- $\alpha$ .

Interestingly, a certain study published in 2005 [144] reported that iNOS expression and total nitrite levels are upregulated during EMCV infection. Notably, this past study also showed that heat-inactivated EMCV (54 °C for 20 min) retained the ability to induce iNOS, suggesting a mechanism independent of viral RNA sensing. The authors of this paper further showed that this event occurred independently of type I IFN signaling and PKR, from experiments using IFNAR-deficient macrophages. However, the authors ultimately proposed that empty EMCV viral capsids were responsible for iNOS induction. This conclusion remained quite speculative and there are no follow-up studies, to the best of my knowledge. It is possible that the observed iNOS induction in this earlier study may instead have been the activity of extracellularly released VPg<sup>EMCV</sup>.

As discussed in Section 1-2-3, several EMCV proteins actively suppress type I IFN responses, which is consistent with the 2005 observation [144] that IFN- $\beta$

signaling was dispensable for iNOS induction. In contrast, there are few studies which have examined IFN- $\gamma$  production during EMCV infection, but IFN- $\gamma$  has been reported to be induced during EV71 infection [145]. In this case, however, viral proteins such as 2A and 3D were shown to attenuate IFN- $\gamma$  signaling by suppressing IRF-1 induction [145]. These past findings suggest that *Picornaviridae* could perhaps employ broad strategies to suppress both type I and type II IFN signaling through diverse mechanisms.

In addition to IFN suppression, EMCV proteins such as L and 2A have been reported to inhibit host apoptosis [47-49], while other pathways such as inflammasome activation and autophagy may be accelerated during infection ([50, 51], also mentioned in Section 1-2-3). Despite these antiapoptotic strategies of EMCV, this study clearly demonstrates that robust apoptosis occurs during EMCV infection in both HeLa and THP-1 cells (Section 3-1). Importantly, viral titers increased to a similar extent upon inhibition of VPg<sup>EMCV</sup>-associated signaling components at different levels, including IFNGR1/2, iNOS, and Caspase 8 (**Figure 3-43**). These results strongly indicate that iNOS/NO-dependent, Caspase-8-mediated apoptosis plays a critical role in limiting EMCV replication. Taken together with the past reports regarding viral suppression of IFN-related pathways, VPg<sup>EMCV</sup> acting as an IFNGR agonist, together with induction of host TNF- $\alpha$ , may be one of the few remaining crucial antiviral mechanisms during EMCV infection, previously masked by the complexity of host-pathogen interactions.

Finally, one observation that is unresolved in this study is the presence of unknown cytotoxic activity in the void fractions that should not contain VPg<sup>EMCV</sup>. Specifically, fraction 7-8 from size-exclusion chromatography (**Figure 3-8a**, **Figure 3-8b**) and the 10-50 kDa fraction from size-based filtration (**Figure 3-10d**) exhibited some cytotoxicity at their original concentrations. Although these fractions contained TNF- $\alpha$  (**Figure 3-9c**), they should lack VPg<sup>EMCV</sup> (**Figure 3-19b**), raising the possibility that additional factor(s) could perhaps contribute to cell death. This activity was observed in both acid-treated and non-treated samples, which excludes acid inactivation artifacts. Neutralization of TNF- $\alpha$  using etanercept (**Figure 3-6b**, **Figure 3-6d**) or knockout of TNFR1 (**Figure 3-6c**, **Figure 3-6e**) completely abolished cytotoxicity and Caspase 8 cleavage, indicating that this unidentified component(s) likely also acts in synergy with TNF- $\alpha$  signaling.

The identity of this factor still remains completely unknown, and could represent host-derived proteins (e.g., cytokines or large chemokines) or viral proteins within the 10-50 kDa range, which includes a large fraction of EMCV-encoded proteins. Given the complexity of EMCV-host interactions, it is unlikely that VPg<sup>EMCV</sup> and TNF- $\alpha$  alone explain all the host outcomes during EMCV infection. However, identification of this additional cytotoxic component was beyond the scope of the present study, and is an independent but another possible important topic for future investigation.

#### *4-4-2 Conceptual implications of the noncanonical host-modulating function of *Cardiovirus* VPg in host-pathogen interactions*

When identifying a peculiar host-responsive factor during viral infection, it is important to consider not only its biological function or what the factor does during infection, but also why such a function exists in the context of host-pathogen interactions. While the biological significance of the newly identified function of VPg<sup>EMCV</sup> is discussed in detail, the remaining question is why this function exists specifically in *Cardiovirus* but not in *Enterovirus*, and why the virus has evolved to acquire such a host-responsive mechanism. At first glance, this function does not appear to benefit the virus, given that both *in vitro* and *in vivo* data indicate that VPg<sup>EMCV</sup> exerts antiviral effects and reduces viral load in the host.

At least 2 possibilities can be considered regarding how this IFN- $\gamma$ -like activity of *Cardiovirus* VPg may influence host-pathogen interactions, and these may help explain why *Cardiovirus* has evolved to gain such a function. First, although this remains speculative, reduction of viral load through VPg<sup>EMCV</sup> and TNF- $\alpha$ -dependent cell death could ultimately benefit the virus over a longer timescale. In the mouse infection experiments presented in this study, inhibition of iNOS resulted in increased viral load, more severe symptoms, and earlier death. Previous reports have shown that intravenous administration of either TNF- $\alpha$  or IFN- $\gamma$  during EMCV infection exhibits therapeutic effects [146-148], and when interpreted together with the data of this study, it can be strongly suggested that acceleration of apoptosis by VPg<sup>EMCV</sup> + TNF- $\alpha$  signaling should suppress viral replication *in vivo*.

However, from the perspective of viral transmission, excessive viral replication leads to rapid host death thereby reducing opportunities for viral spread to new hosts. In this context, this IFN- $\gamma$ -like activity of *Cardiovirus* VPg may work to partially

attenuate viral load. This could prolong host survival and could potentially enhance viral transmission to new hosts. This interpretation is also related with the fact that although EMCV strongly suppresses IFN responses through other viral proteins [45, 46], host-induced TNF- $\alpha$  is induced as a critical inflammatory cytokine capable of synergizing with VPg<sup>EMCV</sup>. Nevertheless, this model implies a very balanced and sensitive system in which viral proteins such as L and 2A inhibit apoptosis in certain contexts [47-49], while VPg<sup>EMCV</sup> and TNF- $\alpha$  accelerates apoptosis in others. These opposing activities may operate differently, or differ in significance comparing different cell types, tissues, or phases of infection, which reflects the intricate nature of host-virus co-evolution. Although still speculative, this remains as the most possible explanation for why *Cardiovirus* may have evolved to gain such a function.

A second possible consequence of VPg<sup>EMCV</sup> activity is the promotion of tissue damage through non-cell-autonomous apoptosis, which should contribute to viral pathogenesis. One example derives from a past study of TMEV [149], another *Cardiovirus* that should possess VPg with IFN- $\gamma$ -like activity similar to that of VPg<sup>EMCV</sup>. This past report shows that TMEV infection in mice induces encephalitis and hippocampal cell death, despite viral replication being detected primarily in non-hippocampal regions of the brain. The authors of this paper reported apoptosis, assessed by TdT-mediated dUTP Nick End Labeling (TUNEL) staining, and accumulation of reactive oxygen species (ROS) within the hippocampus. This observation is consistent with the model proposed in the present study, in which extrinsic apoptosis is induced in a non-cell-autonomous manner through the synergy of TNF- $\alpha$  signaling and extracellularly released VPg. Taken together, these findings raise the possibility that VPg<sup>EMCV</sup>, together with TNF- $\alpha$ , may contribute to tissue damage by inducing apoptosis in uninfected cells. However, whether this tissue damage in the central nervous system (CNS) benefits *Cardiovirus* still remains unclear.

Induction of apoptosis in the CNS may not directly enhance viral replication, yet from the host perspective, VPg<sup>EMCV</sup> and TNF- $\alpha$ -dependent apoptosis may act like a “double-edged sword.” While this synergy reduces viral load and promotes survival of the host, it may simultaneously accelerate tissue damage in non-infected cells. In the EMCV mouse model presented in this study, intraperitoneal iNOS inhibition increased viral load and disease severity. However, it is known that EMCV predominantly targets organs such as the heart and brain, and it remains uncertain whether pharmacological

inhibition of iNOS by 1400W i.p. can effectively reach the CNS due to the blood–brain barrier. This may also be directly related to the previous reports describing therapeutic effects of intravenous IFN- $\gamma$  or TNF- $\alpha$  administration [146-148], which may not directly induce neurotoxicity as this should not reach the CNS. In contrast, accumulation of VPg in the CNS during infection, as observed in TMEV, may have pathological consequences. Although speculative, localized inhibition of iNOS within the brain could have different outcomes than i.p. administration of 1400W.

The reason why *Enterovirus* lack this IFN- $\gamma$ -like function of VPg (or exhibit very weak activity) compared with *Cardiovirus* also remains unknown. One possible idea is that *Enterovirus* VPg could somehow represent an ancestral form, like a “prototype,” while *Cardiovirus* VPg have acquired the specific C-terminal amino acids, particularly residues such as Q15 and L16, to obtain IFN- $\gamma$ -like activity. However, whether this evolutionary aspect ultimately benefits viral transmission or disease pathogenesis cannot yet be determined. Nevertheless, this study highlights a functional divergence within the *Picornaviridae* family, in which VPg exhibits distinct host-modulating functions between *Cardiovirus* and *Enterovirus*, despite both viruses having a conserved genome structure and similar replication cycle. These findings indicate that very small differences in viral proteins may significantly influence host-pathogen interactions and viral evolution.

#### 4-4-3 Limitations and associated challenges

There are several limitations to this study, particularly regarding the *in vivo* experiments. Although the *in vivo* experiments clearly demonstrate the significance of iNOS during EMCV infection, these mouse experiments remain relatively primitive and requires further confirmation for reproducibility. As discussed earlier, more detailed analyses, such as examining apoptotic markers in tissues/organs with or without iNOS inhibition, would provide a clearer understanding of tissue-specific effects, especially in organs such as the brain or heart, which are known targets of EMCV infection.

An attempt was made to measure DNA fragmentation levels in the serum at day 2 post-infection; however, no significant increase was observed compared with nitrite levels or EMCV RNA levels. Measuring DNA fragmentation at later time points (e.g., 3-4 days post-infection, when clinical symptoms were more severe), or directly

assessing apoptosis in specific organs, may have been better for evaluating tissue-localized cell death.

Further infection experiments using recombinant viruses with mutant VPg, as well as genetically modified mouse models, would be valuable to more precisely define the biological contribution of VPg<sup>EMCV</sup>. However, both of these experiments should present multiple challenges. First, regarding recombinant virus generation, beyond practical constraints such as biosafety permits and facility limitations, engineering VPg-mutant viruses itself will be very difficult. Theoretically and logically, replacement of EMCV VPg with poliovirus VPg should abolish IFN- $\gamma$ -like activity and increase viral load, while replacing poliovirus VPg with EMCV VPg should reduce viral load by gaining this IFN- $\gamma$ -like function. However, experimental attempts conducted through a collaboration with a laboratory specializing in recombinant virus generation showed that such mutations severely impaired viral replication, making these mutant viruses nonviable.

This outcome is not exactly surprising, given the essential role of VPg in viral RNA synthesis. It is also likely that VPg proteins from different *Picornaviridae* members are not interchangeable, despite conservation of the viral life cycle. Rather than swapping VPgs entirely, attempts to generate VPg point-mutant viruses also resulted in impaired replication. Although this could be the result of various issues, at present, no mutant viruses that exhibited increased viral titers could be generated. Therefore, it remains very difficult at this point to distinguish whether reduced viral titers result from disruption of the canonical function of VPg during replication, or from loss of the IFN- $\gamma$ -like activity. These results, although not indicating impossibility, clearly demonstrate the technical difficulty of generating and utilizing recombinant VPg-mutant viruses without careful experimentation and validation.

A second approach involves the use of genetically modified mouse models. For example, *Nos2* knockout mice (*Nos2*<sup>-/-</sup>) could validate the effects observed using iNOS inhibition by 1400W, and to assess whether 1400W has any off-target effects. Importantly, *Nos2*<sup>-/-</sup> mice are known to be viable, as reported previously [150].

Further insight could also be gained by comparing iNOS inhibition with IFNGR knockout mouse models, such as *Ifngr1*<sup>-/-</sup> or *Ifngr2*<sup>-/-</sup> mice, or by using knockouts of its downstream Caspase 8 (*Casp8*<sup>-/-</sup>). These experiments could definitively validate that increased viral load and disease severity from iNOS inhibition is the actual result of inhibiting IFNGR1/2-mediated, iNOS/Caspase 8-dependent apoptosis triggered by

VPg<sup>EMCV</sup>. However, each of these knockout mice models presents additional complexities. Although *Ifngr1*<sup>-/-</sup> mice are known to be viable [151, 152], these mice exhibit altered immune states, and the contribution of endogenous IFN-γ induction/signaling during EMCV infection cannot be excluded without proper experimentation. This means that comparison with *Ifng*<sup>-/-</sup> mice with WT mice may be required, or by utilizing double knockout mice models such as *Ifngr1*<sup>-/-</sup>*Ifng*<sup>-/-</sup>. Although these mice should be viable [128], knockout mice models could perhaps introduce unexpected phenotypes.

Regarding Caspase 8-related mice models, *Casp8*<sup>-/-</sup> mice are known to be embryonically lethal due to accelerated/uncontrolled necroptosis [153]. Therefore, knockout mouse models such as *Casp8*<sup>-/-</sup>*Ripk3*<sup>-/-</sup> or *Casp8*<sup>-/-</sup>*Mkl1*<sup>-/-</sup> would be required, along with using appropriate controls (*Ripk3*<sup>-/-</sup> or *Mkl1*<sup>-/-</sup> mice), to remove contributions from necroptosis [153, 154]. Additionally, previous studies using *Tnf*<sup>-/-</sup> mice infected with EMCV showed rapid death [146], which is consistent with the findings of this study. However, detailed analysis of organ/tissue-specific pathology and apoptotic markers in these mice, particularly in regions such as the hippocampus, could further clarify the contribution of TNF-α–dependent extrinsic apoptosis in EMCV pathogenesis.

Finally, although neutralization of VPg<sup>EMCV</sup> using the anti-VPg<sup>EMCV</sup> antibody generated in this study could have been a more direct approach, this was clearly ineffective *in vitro* (**Figure 3-18a**). Under all these circumstances, pharmacological inhibition of iNOS using 1400W was the most practical approach within the constraints of this study. Compared with broader inhibitors such as JAK inhibitors (e.g., Tofacitinib), which should suppress multiple cytokine-related signaling pathways *in vivo*, selective iNOS inhibition by 1400W was considered to minimize off-target effects, while still allowing assessment of the functional relevance of the VPg<sup>EMCV</sup>–IFNGR1/2–iNOS-Caspase 8 pathway during EMCV infection.

#### **4-5 Potential applications of *Cardiovirus* VPg and concluding remarks**

This study revealed that VPg<sup>EMCV</sup> and some other *Cardiovirus* VPgs can function in at least two distinct ways: first, as an inducer of potent cell death in synergy with TNF-α, and second, as an antiviral agent. These two aspects will be briefly discussed, together with potential future directions.

This study clearly demonstrated that VPg<sup>EMCV</sup> functions in both human and murine cells, inducing cell death synergistically with TNF- $\alpha$  across multiple cell types (**Figure 3-47**). Based on this property, one potential future application could be to target malignant tumors, given that VPg<sup>EMCV</sup> appears to induce cell death more rapidly than IFN- $\gamma$  and exhibits cross-species activity. However, this concept remains at a very early stage, and extensive future studies, especially *in vivo*, would be required to evaluate efficacy and toxicity of VPg<sup>EMCV</sup>. As one initial experiment, well-established mouse tumor models, such as subcutaneous implantation of B16 melanoma cells, could be used to compare tumor growth with or without administration of VPg<sup>EMCV</sup> or other *Cardiovirus* VPgs. It would also be important to assess whether exogenous TNF- $\alpha$  is required to induce tumor-specific cell death *in vivo*, or whether endogenous TNF- $\alpha$  levels are sufficient in the tumor microenvironment.

Another potential application is the use of VPg<sup>EMCV</sup> or other *Cardiovirus* VPgs as antiviral agents, for example as priming stimuli that could substitute for IFN- $\gamma$ . In this study, overnight treatment with VPg<sup>EMCV</sup>, as well as some other *Cardiovirus* VPgs exhibited clear antiviral activity against VSV *in vitro*, significantly reducing viral titers (**Figure 3-49a, Figure 3-49b**). In addition, preliminary *in vivo* experiments suggested antiviral efficacy, as intranasal administration of VPg<sup>EMCV</sup> one day before and after IAV infection attenuated weight loss in mice compared with the untreated control (**Figure 3-50**). Nevertheless, these *in vivo* observations remain preliminary and will require further validation, including reproducibility testing. It would also be important to examine alternative routes of administration (e.g., intraperitoneal or intravenous delivery) and to carefully evaluate toxicity/safety. In this context, determining parameters such as dose-dependent efficacy and LD<sub>50</sub> values, and comparing these with recombinant IFN- $\gamma$ , would be essential to assess the feasibility of using VPg<sup>EMCV</sup> as a therapeutic agent.

In conclusion, although many questions remain unanswered, several of which are very complex and extend beyond the scope of this work, this study clearly identified a previously unrecognized noncanonical function of VPg<sup>EMCV</sup> as an IFNGR agonist that induces downstream signaling highly similar to that of IFN- $\gamma$ . While the molecular target(s) regarding the IFNGR-independent effects of VPg<sup>EMCV</sup> also require future investigation, these unique properties may be utilized once better understood. In addition to its cross-species activity, the relative ease of synthesis of VPg<sup>EMCV</sup> and other *Cardiovirus* VPgs, consisting of merely 20 amino acids, may be advantageous

in terms of production speed and cost. Overall, this discovery should open multiple avenues for future research, including structural studies to elucidate VPg–IFNGR interactions, virological studies addressing host-pathogen interactions, and investigations into potential therapeutic applications.

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