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The Molluscum Contagiosum Virus MC160 Protein Requires Both Death Effector Domains to Inhibit MAVS- and TBK1-Induced Interferon Activation

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The Molluscum Contagiosum Virus MC160 protein requires both death effector domains to inhibit MAVS- and TBK1-induced interferon activation.

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Abstract

The Molluscum Contagiosum Virus (MCV) causes a common persistent, skin infection. Two MCV immune evasion molecules, MCV MC159 and MC160 contain tandem death effector domains (DEDs). DEDs are found in several host proteins including pro-apoptotic proteins Fas-associated Death Domain (FADD) and procaspase-8 and are well characterized in innate immune signaling. MC159 blocks apoptosis induced by both Tumor Necrosis Factor (TNF) and the Fas ligand (FasL) and inhibits activation the host pro-inflammatory transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Shisler, 2001; Murao and Shisler 2005). MC160, however, does not inhibit apoptosis, but does dampen TNF-induced NF-κB activation (Shisler, 2001; Nichols and Shisler, 2006). Both MC159 and MC160 inhibit activation of type I interferons induced via the mitochondrial antiviral signaling (MAVS) pathway, an important innate signaling network that senses the presence of a viral double-stranded RNA (dsRNA) and induces an interferon (IFN) response. MC159 interacts with the protein kinases TBK-1 and IKKε. However, the molecular mechanism of MC160 is not well understood. When expressed independently, both the C-terminal domain of MC160 as well as the first DED were reported to inhibit TBK-1 induced IRF-3 activation, whereas the second DED was dispensable (Randall et al., 2013).

The goal of the current project is to identify the molecular mechanism through which MC160 inhibits activation of type I IFNs. We characterized the ability of MC160 constructs to inhibit MAVS- and TBK-1 induced activation of type I IFN. When expressed independently neither MC160 DED1 nor DED2 blocked activation the IFN-β

enhancer to the extent of full-length or the MC160 construct that expresses both DEDs. Our current analysis suggests that both DED1 and DED2 are required to fully inhibit activation of type I interferons.

Introduction

Molluscum Contagiosum Background

The Molluscum Contagiosum Virus (MCV) is a double-stranded (ds) DNA virus that belongs in the *Poxviridae* family. Since the eradication of smallpox, MCV remains the only poxvirus to exclusively infect humans. MCV infects keratinocytes and mucosal membranes, causing benign lesions that can persist for months to years in otherwise healthy individuals (Chen et al., 2013). MCV neoplasms are characterized by painless, white or flesh-colored dome-shaped papules with a shallow depression in the middle, that average from 2 to 5mm in size (Hughes et al., 2013). The infection is not typically lethal; however, in immunocompromised patients, the infection can be much more severe (Chen et al., 2013). Fatalities have occurred due to a secondary bacterial infection in those with weakened immune systems (Cotton et al., 1987). MCV primarily spreads through skin to skin contact, making children the most susceptible however, adults may contract the virus as well through sexual contact. MCV can also spread by autoinoculation or fomites. In 2010 there were 122 million cases reported, making MCV the third most common skin infection in children (Shisler, 2015).

Currently, there is no FDA approved cure or treatment for MCV lesions. Although the lesions will usually resolve on their own, some patients elect treatment (Shisler, 2015). The most common treatments of pediatric MCV include the use of cantharidin, imiquimod, curettage, cryotherapy, retinoids, and cidofovir (Coloe and Morrell, 2009). Therapies may help treat the viral infection. However, current MCV treatments are not 100 % effective and are associated with problems, such as anxiety,

painful treatments and scarring that lasts even after the infection has resolved. Cantharidin, the most common therapy, is applied topically and is absorbed by the epidermal cell membranes, thus activating neutral serine proteases eventually leading to acantholysis and intraepidermal blistering and nonspecific lysis of skin (Bertaux et al., 1988; Moed et al., 2001).

The persistence of MCV is most likely due to dampening of host innate immune responses through the production of MCV immune evasion molecules. However, the pathogenicity of MCV infections remains poorly understood. MC lesions present themselves as inflamed (I-MC) and non-inflamed (NI-MC). Histological studies suggest regression of MCV-induced lesions is observed in patients with I-MC, but not NI-MC (Vermi et al. 2011). NI-MC lesions do not provoke an inflammatory response in hosts and seem to have poor apoptotic responses, presumably because caspase-3 is inactive. In contrast, Vermi et al. reported active caspase-3 in I-MC lesions and apoptotic cell death (Vermi et al., 2001). Additionally, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activation and translocation into the nucleus followed by type I interferon (IFN) activation have been observed in cells surrounding I-MC lesions. However, neither have been observed in cells surrounding NI-MC lesions (Vermi et al., 2011). The varying responses may suggest that MCV encodes for immune evasion molecules, which may be found in NI-MC lesions. MCV immune evasion molecules inhibit several host immune responses, such as apoptosis. Because MCV cannot be cultured in mammalian cells or studied in animal models, it is especially difficult to

determine which proteins are essential for MCV to maintain its ability to impede host innate immune responses.

Molluscum Contagiosum Virus Immune Evasion Strategy

Like all poxviruses, MCV replicates within the cytoplasm of host cells. To ensure successful replication, MCV encodes a myriad of immune evasion molecules, which dampen host immune responses. For example, the MC54 protein is a critical binding protein of Interleukin-18 (IL-18) that is secreted when cells are infected and inhibits the pro-inflammatory activity of IL-18 (Xiang and Moss, 2003). MC148 is a viral chemokine which inhibits chemotaxis by binding to CCR8 and CxC12α (Luttichau et al., 2000; Jin et al., 2011). MC007 is a protein that is expressed in the mitochondria and stimulates cellular proliferation by inhibiting retinoblastoma protein (pRb) (Mohr et al., 2008). The MC66 protein inhibits apoptosis induced by ultraviolet light and hydrogen peroxide (Shisler et al., 1998). MC132 inhibits NF-κB activation by targeting p65 for degradation (Brady et al., 2015). Similarly, MC005 interacts with the IKK complex and inhibits NF-κB activation, by NF-κB essential modulator (NEMO) (Brady et al., 2017). MC163 prevents mitochondrial membrane permeabilization (MMP), an important apoptotic event controlled by tumor necrosis factor-alpha (TNF-α) or carbonyl cyanide 3-chlorophenylhydrazone (CCCP) (Beaury et al., 2017). MC159 inhibits activation of the extrinsic apoptotic pathway and inhibits the activation of NF-κB (Shisler and Moss, 2001; Murao and Shisler, 2005). MC160 inhibits TNF-α induced NF-κB activation (Nichols and Shisler 2006).

MCV Protein	Function	Mechanism
MC007	Inhibits pRb	Binds pRb
MC54	Inhibits IL-18	Binds IL-18
MC148	Inhibits chemotaxis	Binds CCR8 and CxC12α
MC66	Inhibits apoptosis	Blocks H202 and UV-induced apoptosis
MC132	Inhibits NF-κB activation	Induces degradation of p65
MC005	Inhibits NF-κB activation	NEMO-regulated IκB kinase activation
MC159	Inhibits apoptosis, IFN-β activation, and activation of NF-κB	Apoptosis: Binds procaspase-8, FADD, TRAF2 NF-κB: interacts with the IKKγ IFN-β: Binds TBK1 and IKKε
MC160	Inhibits NF-κB activation Inhibits IFN-β activation	Binds procaspase-8, FADD, and Hsp90
MC163	Inhibits apoptosis	Localizes to mitochondria to prevent MMP

Table 1. Known functions of the MCV Immune Evasion Molecules

Molluscum Contagiosum Virus MC159 and MC160 Proteins

MC159 and MC160 are viral homologs that belong to a group of proteins known as viral FADD-like IL-1β–converting enzyme (FLICE)-like inhibiting proteins (v-FLIPs) (Randall and Shisler, 2013). A distinctive feature of v-FLIPS is the presence of tandem death effector domains (DEDs) (Figure 2). Proteins containing DEDs affect numerous signaling pathways, including apoptotic cell death and activation of pro-inflammatory transcription factors IRF3 and NF-κB (Garvey et al., 2002; Shisler, 2014). The expression of MC159 inhibits Fas- and TNF-α-induced apoptosis and interacts with fas-associated protein with death domain (FADD) and procaspase-8, ultimately inhibiting apoptosis (Bertin et al., 1997; Shisler and Moss, 2001). MC159 expression also suppresses TNF-α-induced activation of NF-κB by directly interacting with the IKKγ regulatory subunit and TNF receptor-associated factor 2 (TRAF 2) (Randell et al., 2012). NF-κB is a transcription factor that is known to regulate host immune responses during an infection and is activated by TNF-α. When TNF-α binds to TNFR-1 the signalsome, containing TNFR1-associated death domain protein (TRADD), TRAF2, and reception-interacting protein (RIP), is formed (Chen et al., 2002). The signalsome then goes on to recruit and activate the IKK complex composed of IKKβ, IKKα, and a regulatory subunit, IKKγ. As long as NF-κB is inactive, it will remain in the cytoplasm sequestered by IκBα. Upon activation of the IKK complex by pathogens (PAMPs) or pro-inflammatory cytokines (TNF-α), the activated IKK complex phosphorylates IκBα protein (Hayden et al., 2004). Phosphorylation of IκBα leads to its ubiquitination and degradation, thereby freeing NF-κB allowing for translocation into the nucleus,

subsequently activating target genes, such as type I IFNs (Hayden et al., 2004).

Much is known about the homology, binding partners and function of MC159. In contrast, little is known about the binding partners and function of the MC160 protein. MC160 expression prevents TNF-α-induced NF-κB activation by interacting with procaspase-8 and heat shock protein 90 (Hsp90), thereby inhibiting the IKK complex. (Nichols and Shisler, 2006; Nichols and Shisler, 2009). Even with the ability MC160 has to bind to FADD and procaspase-8 through DEDs, the MC160 protein does not inhibit apoptosis (Shisler and Moss, 2001). Recently, both MC159 and MC160 proteins were reported to inhibit activation of type I IFNs (Randall et al., 2013; Balachandran et al., 2007).

Activation of Type I Interferons

When the immune system is compromised, the first line of defense is triggered by the innate immune system. Pattern-recognition receptors (PRRs) recognize foreign viral RNA or DNA, also called pathogen-associated molecular patterns (PAMPs) and subsequently activates immune signaling cascades, such as the RIG-I/IRF3 pathway (Bender et al., 2015). The innate immune response is responsible for activating type I IFNs, which are crucial to hosts during viral infections. The innate immune system utilizes type I IFNs, such as IFN-β and IFN-α, which have three important functions. IFNs activate antimicrobial states in infected cells and neighboring cells to stop the infection from spreading. IFNs promote antigen presentation and natural killer cell functions. Finally, IFNs activate adaptive immunity, thus activating highly specific B cell and T cell responses as well as immunological memory (Ivashkiv et al., 2014).

During a poxvirus infection, dsRNA accumulates in the cytoplasm and is detected by PRRs retinoic acid inducible gene 1 (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), triggering an innate immune response. RIG-I and MDA5 form a complex with a mitochondria-localized adaptor protein, mitochondrial antiviral signaling (MAVS) protein (Jacobs et al., 2014). The complex activates a MAVS "signalosome" consisting of TRAF3, TRAF6, TRAF family member associated NF-κB (TANK) activator and TRADD (Jacobs and Coyne, 2013). The complex ultimately leads to activation of type I IFNs and pro-inflammatory cytokines by activation of TANK binding kinase 1 (TBK-1) and IKKε, which in turn phosphorylates and activates the transcription factor interferon regulatory factor 3 (IRF3) (Figure 1). Virus-induced phosphorylated IRF3 is one of several host transcription factors that induces IFN-β production. IFN-β, in turn, signals through the Janus Kinase/Signal Transducers and Activators of Transcription (JAK/STAT) pathway, thereby stimulating the production of IRF7. The positive feedback regulation of IRF7 on IFN-α and other IFN-stimulated genes (ISGs) is critical to fully activate type I IFNs (Honda and Taniguchi, 2006). The MAVS signaling pathway also activates NF-κB, which translocates into the nucleus to help drive the expression of type I IFNs (Bender et al., 2015). NF-κB and IRF3 work synergistically to induce the production of type I IFNs along with other inflammatory cytokines in response to a viral infection.

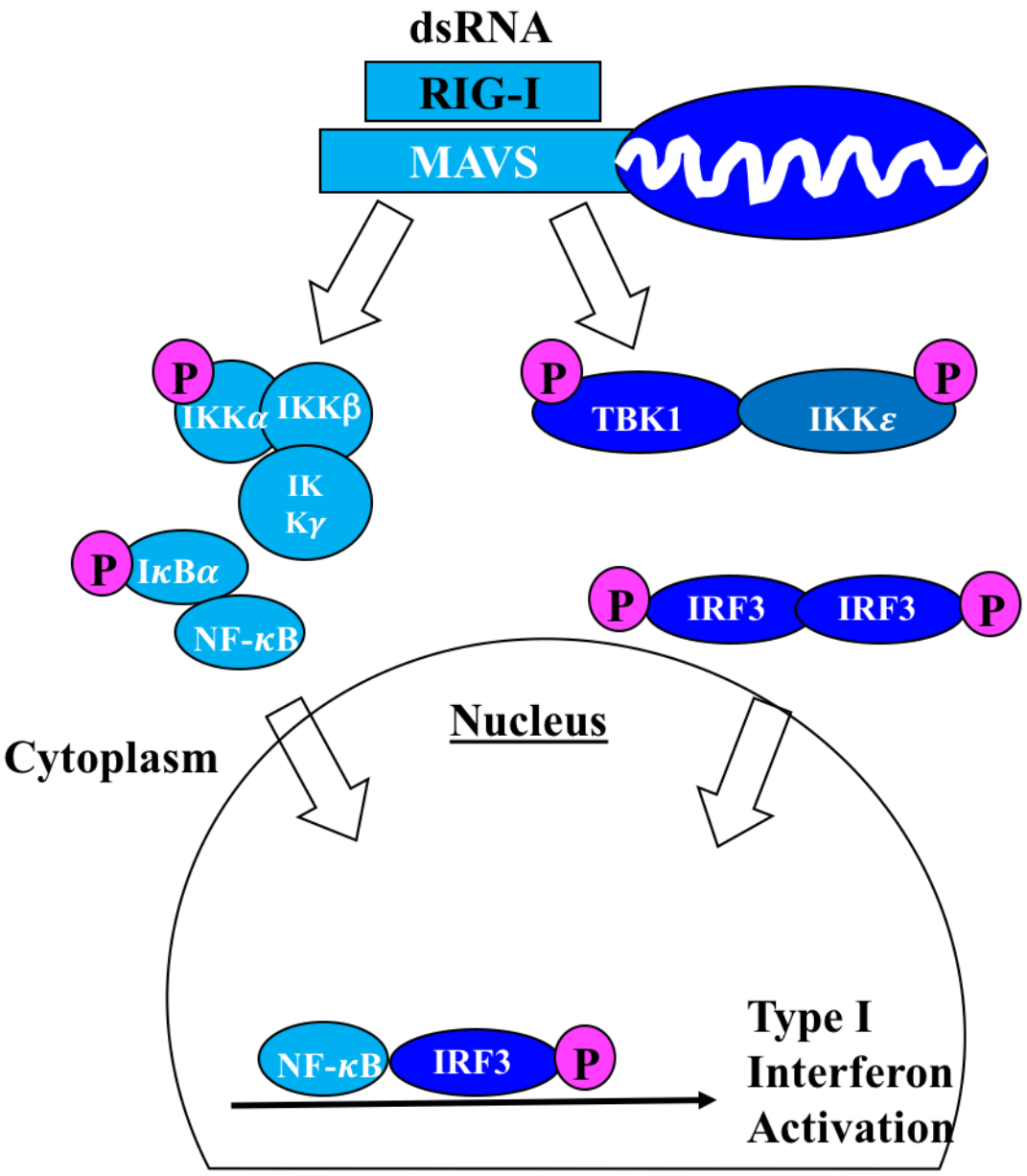

Figure 1: dsRNA is a by-product of a Poxvirus infection, and acts as a PAMP. Upon infection, PRRs detect dsRNA, thereby initiating a signaling cascade by MAVS to fight off the viral infection.

MC159 and MC160 dampen MAVS-mediated activation of type I IFNs

MC159 and MC160 antagonize MAVS signaling. MC159 disrupts activation of type I IFNs by binding to TBK-1 and IKKε. Although MC160 inhibits IFN-β activation, the molecular mechanism and binding partner of the MC160 protein remains unknown. To better understand the molecular mechanism by which MC160 expression inhibit IFN signaling, a panel of MC160 truncation mutants were generated (Nichols and Shisler 2009) (Figure 2). Previously, Randall et al. reported MC160 expression inhibited TBK-1-induced IRF3 activation (Randall et al., 2013). When transiently transfected into cells, every MC160 construct had the ability to inhibit IRF3-regulated luciferase activity, with the exception of MC160D2 (Randall et al., 2013). Because the IFN-β-enhancer element contains binding sites for several transcription factors including, NF-κB, IRF3 and IRF7, we wanted to determine if the same regions of MC160 were required to inhibit activation of type I IFNs. To this end, the MC160 truncation mutants were characterized by their ability to inhibit MAVS and TBK-1-induced IFN-β luciferase activity. MC160 constructs were expressed in 293T cells along with either pMAVS or pTBK-1. Results were analyzed through various luciferase assays observing relative fold change in MAVS- or TBK-1-mediated IFN-β luciferase activity. Protein expression was confirmed via immunoblots. Notably, the constructs containing both DEDs together, MC160 and MC160N, showed the most inhibition of MAVS- and TBK-1-induced IFN-β luciferase activity, suggesting that both DEDs may be required to inhibit this pathway.

Figure 2: MCV MC160 wild type and truncation mutant proteins (Nichols & Shisler, 2009). The full length MC160 protein contains two tandem death effector domains (DEDs) at residues 5-79 for DED1 and 97-175 for DED2, as well as a C-terminal region at residues 225-371. An HA-tag was engineered into the N-terminal region of each construct for protein detection.

Methods and Materials

DNA Preparations

DH5α Escherichia Coli (E.Coli) bacteria carrying recombinant DNA were isolated using the PureYield Plasmid Miniprep system (Promega). Bacteria were cultured in 3mLs of Luria Broth (LB) supplemented with 100μg/mL of ampicillin. After incubating the bacteria in an incubator shaker at 37°C at 225 rpm for 17-20 hours, the bacteria were placed into a 1.5mL microcentrifuge tube, and centrifuged at 14,000 rpm for 1 minute. The supernatant was discarded, and the pellet was resuspended in 600μL of 1x Tris-EDTA buffer (TE). The plasmid DNA was harvested by following the Promega miniprep protocol by adding 100μL of cell lysis buffer, followed by 350μL of neutralization solution buffer and centrifuged for 3 minutes at 14,000 rpm. The supernatant was pipetted into a mini-column and centrifuged for 30 seconds at 14,000 rpm to collect the plasmid DNA. After discarding the supernatant, 30μL of TE was added to the mini-column, and subsequently centrifuged for 1 minutes at 14,000 rpm. The plasmid DNA was quantified with a BioDrop Duo UV/VIS Spectrophotometer (Denville Scientific). The plasmids used in the study include IFN-β firefly luciferase from provided by J. Hiscott, McGill University, Montreal, Canada, Flag-MAVS and IRF3provided by Fanxiu Zhu from Florida State University, Tallahassee, FL, Renilla TK and NF-κB firefly luciferase (Promega), pCI, Flag-tagged TBK-1 protein was a gift from Siddharth Balachandran (Fox Chase Cancer Center, Philadelphia), and HA-MC160/pCI, HA-MC160N/pCI, HA-MC160D1/pCI, HA-MC160D2/pCI, MC160C/pCI (Nichols and Shisler, 2009).

Cell Culture

Human Embryonic Kidney 293T (HEK 293T) cells, provided by ATCC, were cultured in Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin and streptomycin. The cells were grown in a 250mL tissue culture flask, treated with vacuum gas plasma for consistent cell attachment and growth, with vent cap, sterilized (VWR) and kept in a humidified incubator at 37°C with 5% CO_2. Once 293T cells reached about 80% confluency, the media was aspirated and the cells were washed with 3mLs of 1x phosphate buffered saline (PBS). The cells were passaged with 2mLs of trypsin and 8mLs of DMEM with 10% FBS and diluted at a ratio of 1:5 or 1:10.

Transfections and Luciferase Assays

HEK 293T were seeded at 2.5×10^5 cells per well on a tissue culture 12 well plate (VWR) and kept in a humidified incubator at 37°C. At 80% confluency, the cells were transfected using pIFN-β luciferase reporter gene, which is an enhancer that drives firefly luciferase activity as well as the control reporter pRenilla-TK to drive sea pansy luciferase activity. Plasmids obtained from minipreps were utilized for transfections. 100ng of Renilla TK, 200ng of IFN-β luciferase reporter, and varying concentrations ranging from 50ng-500ng of pFlag-MAVS or pTBK-1 and MC160 constructs (MC160, MC160N, MC160D1, MC160D2, MC160C) for a total of 1μg of DNA per well. For transfections, the Mirus Trans-IT 2020 was used at a ratio of 3μL/1μg of total DNA, along with 100uL of Opti-MEM (Gibco) per well. All transfections were repeated 3 times each in triplicate.

The cells were harvested at 16-24 hours post transfection using a Dual-Luciferase® Reporter Assay System (Promega) with 150μL per well of 1x passive lysis buffer on a rocker for 30 minutes at room temperature. After rocking, 10μL of each lysate was added to an opaque 96 well plate (Costar). Firefly luciferase activity was measured by adding 50μL of Luciferase Assay Reagent II. Luminescence was quantified using a SpectraMax M5 reader and analyzed with SoftwareMax Pro. Following the firefly quantification, 50μL of 1x Stop & Glo® Reagent was added to the same plate and read to quantify the Renilla values. The data was analyzed, averaged and normalized to obtain the values of cells expressing pCI only to show relative fold change in IFN-β luciferase activity. A student t-test was performed to analyze statistical significance of each result ($p < 0.05$).

Immunoblots

Lysates from luciferase assays were used for the immunoblotting. After harvesting 293T cells with 1x passive lysis buffer, 130μL of each lysate was added to a 1.5mL centrifuge tube, the samples consisted of pCI only, pFlag-MAVS or pFlag-TBK 1, MC160, MC160N, MC160D1, MC160D2, and MC160C. The samples were centrifuged for 1 minute at 14,000 rpm at 4°C. 100μL of the supernatant was collected for each sample and added into a new 1.5mL centrifuge tube without disturbing the pellet. 20μL of 6x of sample buffer was added to each sample followed by 5% of 2-Mercaptoethanol. The samples were boiled at 100°C for 5 minutes. After boiling, the samples were placed on ice for immediate use, or stored at -80°C for later use.

Lysates were run on a 10% SDS-PAGE prepared in the laboratory. In the first

well, 3μL of the protein ladder (NEB) was loaded. In wells 2-10, 15μL of each sample was added to the corresponding well and the gel was run in 1X SDS-PAGE (0.25mM Tris, 1.92mM glycine, 0.001% SDS, and deionized water) at 60 mA for 45 minutes. The proteins were transferred to nitrocellulose paper at 90 volts for 1 hour with transfer buffer (200mL methanol, 3.06g Tris, 14.39g glycine). After the transfer, the membrane was either blocked for 1 hour at room temperature with gentle agitation or overnight at 4°C in 5% milk (Bob's Red Mill Non-Fat Dry Milk) dissolved in 1X Tris buffered saline with Tween®20 (TBST).

After blocking, the membrane was probed with primary antibody (anti-HA or anti-Flag, Sigma) diluted 1:1000 in 0.5% milk TBST for 1 hour at room temperature with gentle agitation. The membrane was washed 3 times for 10 minutes each with 0.5% milk TBST and subsequently probed with secondary antibody (goat-anti-mouse-HRP) diluted 1:5000 in 0.5% milk TBST for 1 hour at room temperature with gentle agitation. The membrane was then washed 3 more times for 10 minutes each with 0.5% milk TBST. The membrane was removed from the 0.5% milk TBST solution and patted dry. Bands corresponding to flag-tagged or HA-proteins were visualized by chemiluminescence using the SuperSignal™ West Femto (ThemoScientific) reagent, following the manufacturer's protocol. The blots were imaged using a FluorChem E system and saved for further analysis.

Results

MC160 Inhibits MAVS-mediated IFN-β activation

Because MCV cannot replicate in tissue culture, the MC160 wild-type and mutant MC160 expression vectors were transfected into HEK 293T cells. Both MC159 and MC160 proteins inhibit MAVS-mediated activation of the IFN-β enhancer (Randall et al., 2013). The mechanism by which MC159 impedes innate immune responses is well studied. However, the molecular mechanism by which MC160 expression antagonizes MAVS signaling remains undetermined. Randall et al. reported the second DED of MC160 was dispensable for inhibiting IRF3 activation (Randall et al., 2013). Because the IFN-β-enhancer element contains binding sites for multiple transcription factors including NF-κB, IRF3 and IRF7 (J. Hiscott, McGill University, Montreal, Canada), the goal of this study was to determine the region of the MC160 protein necessary to inhibit MAVS-mediated IFN-β activation.

Previously, mutagenesis was performed on the MC160 protein to divide the full length protein into individual regions composed of an N-terminal region (residues 1-220), a C-terminal region (residues 224-371) and the two individual DEDs (residues 5-79 for DED1 and 97-175 for DED2) (Figure 2). Each region was engineered with an HA-tag on the N-terminal regions to confirm protein expression via immunoblot (Nichols and Shisler, 2009). In the current study, 293T cells were transiently transfected with varying concentrations of pHA-MC160 mutants, as well as pMAVS, pIFN-β-luc and Renilla enhancer in each well. 16-24 hours post transfection, the cells were lysed and analyzed

using a dual-reporter assay where the firefly luciferase gene was under the transcriptional control of the IFN-β enhancer (J. Hiscott, McGill University, Montreal, Canada).

A MAVS expression plasmid was overexpressed in 293T cells using 400ng of pMAVS, 300ng of MC160 constructs and 300ng of the reporter genes (200ng IFNβ and 100ng Renilla TK). Expression of either wild type MC160 or MC160N resulted in approximately a 54-fold and 15-fold decrease in IFN-β activity, respectively, when compared to cells expressing the empty vector pCI and MAVS. Although MC160C expression did not inhibit to the same degree as MC160 or MC160N, relative to pCI co-transfected with MAVS, expression of MC160C showed a decrease of IFN-β luciferase activity roughly 2.3 fold. Interestingly, MC160DED1 and MC160DED2 did not inhibit IFN-β enhancer controlled luciferase activity when MAVS was overexpressed compared to MC160 and MC160N. A mixing experiment was conducted to evaluate whether the co-expression of MC160D1 and MC160D2 could inhibit IFN activity as well as MC160N expression, but when expressed together as separate proteins, there was no inhibition in IFN-β enhancer controlled luciferase activity (Figure 3). The presence of MC160 constructs and the flag-tagged MAVS protein in the lysates was confirmed via immunoblot utilizing anti-FLAG and anti-HA (Figure 4). MAVS expression was confirmed in every sample, as well as expression of each MC160 construct. The MC160D1 protein expressed a much lighter band compared to all other mutants.

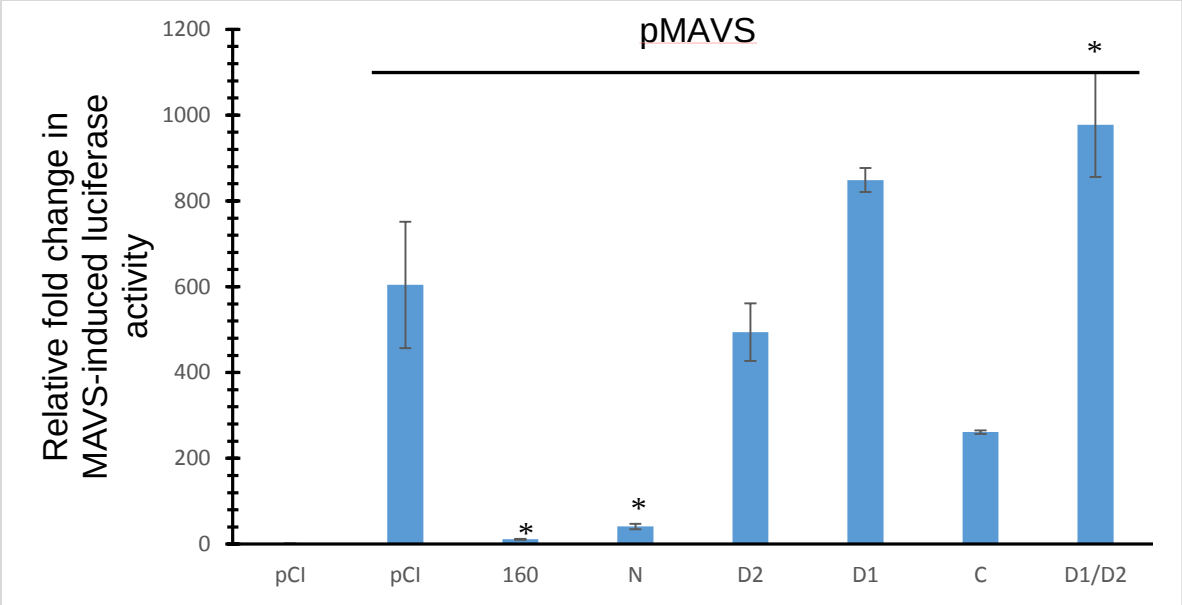

Figure 3: The effect of MC160 truncation mutants on IFNβ luciferase activity in the presence of higher concentrations of MAVS. Subconfluent 293T cells were transfected with pIFNβ-luc (200ng); pRenilla-TK (100ng); pMAVS (400ng); and MC160 constructs (300ng) (D1/D2 mixing experiment had 150ng of D1 and 150ng of D2). 16-24 hours post transfection the cells were lysed and firefly and sea pansy luciferase activities were recorded. Luciferase data was determined based on the ratio of firefly to sea pansy luciferase values. All values were normalized to cells expressing the reporters and the empty vector. Values are shown as mean ± standard deviations (SD). All experiments were performed in triplicate, n=3. *Statistical significance was determined by a $p < 0.05$.

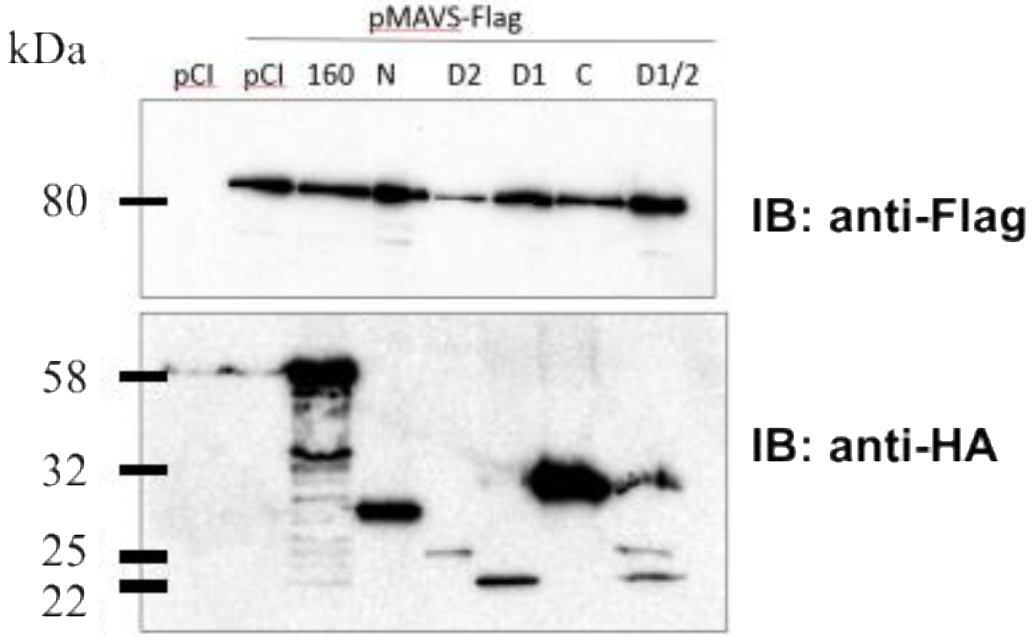

Figure 4: Expression of Flag-MAVS and HA-tagged MC160 proteins. Membranes were probed with anti-HA (1:1000) and anti-Flag (1:1000) antibodies. Bands corresponding to Flag-MAVS or the indicated MC160 mutant protein were visualized by chemiluminescence.

A similar MAVS experiment was conducted with a lower concentration of MAVS and higher concentrations of MC160 constructs. 293T cells were transfected with 200ng of IFN-β enhancer luciferase gene, 100ng of Renilla TK, 200ng of MAVS and 500ng of MC160 constructs. Again the MC160N protein, which contains both DEDs, inhibited IFN-β enhancer controlled luciferase activity compared to pCI stimulated with MAVS. MC160C expression also inhibited MAVS-induced activation of on the IFN-β enhancer controlled luciferase activity when compared to pCI co-transfected with MAVS. However, IFN-β luciferase activation was about 2.9-fold higher in lysates expressing MC160C than in MC160N. Even when transfected at higher concentrations, MC160D1 did not inhibit activation of IFN-β luciferase activity (Figure 5). In this experiment, when we transfected with higher amounts of MC160D2 relative to lower amounts of MAVS, we observed slight inhibition of IFN-β luciferase activity, but not to the levels seen with MC160N expressing cells. Expression of MC160 mutants were verified via immunoblot (Figure 6). Interestingly, in this experiment the MC160D2 protein was detected at levels similar to the MC160N protein. Therefore, it seemed unlikely that expression levels of MC160D2 contributed to less inhibition of MAVS-induced IFN-β luciferase activity relative to the inhibition observed in MC160N expressing cells. Taken together, the data suggests both MC160 DEDs are required to inhibit MAVS-induced IFN-β activation.

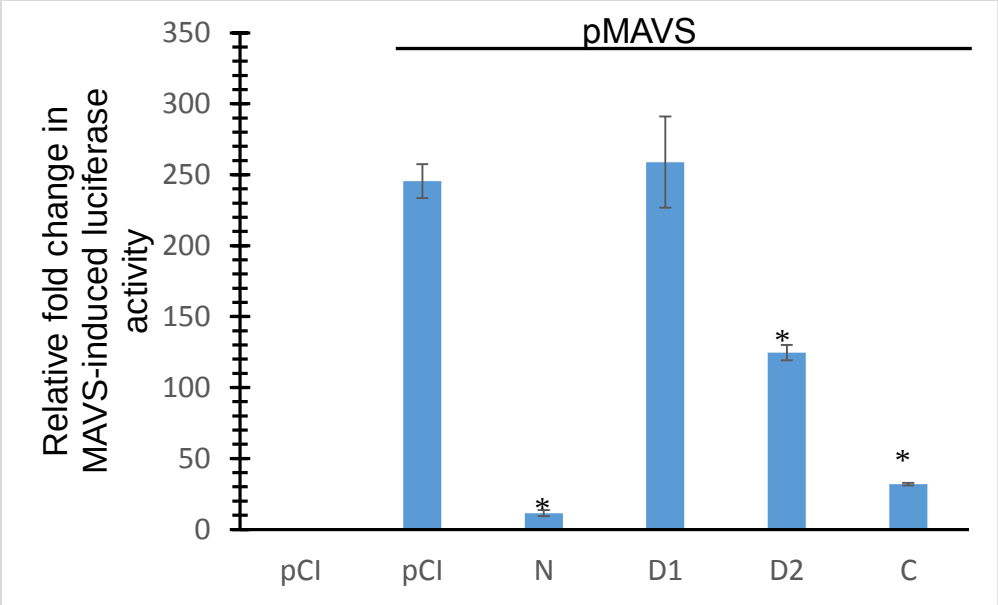

Figure 5: The effect of MC160 truncation mutants on IFN-β luciferase activity in the presence of lower concentrations of MAVS. Sunconfluent 293T cells were transiently transfected with pIFN-β-luc (200ng); pRenilla-TK (100ng); pMAVS (200ng); and MC160 constructs (500ng). The results were collected using a dual-luciferase reporter assay. IFN-β enhancer gene controls firefly luciferase activity, while Renilla drives the sea pansy luciferase activity. Luciferase data was determined based on the ratio of firefly to sea pansy luciferase values. All values were normalized to cells expressing the reporters and the empty vector. Values are shown as mean ± standard deviations (SD). All experiments were performed in triplicate, n=3. *Statistical significance was determined by a $p < 0.05$.

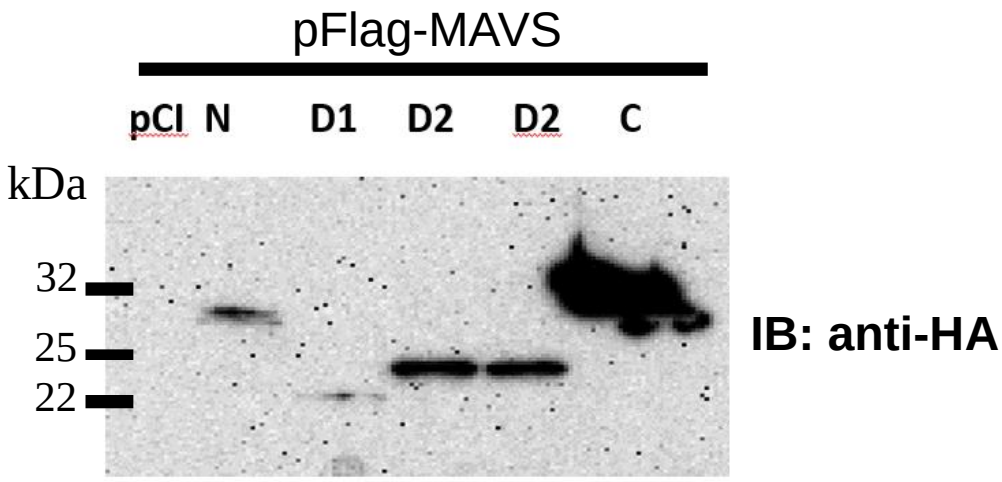

Figure 6: Immunoblots were used to verify the presence of MC160 constructs and MAVS in the lysates. MAVS was detected by using a FLAG antibody (1:1000), while MC160 mutants were detected by an HA antibody (1:1000). (**D2 samples were loaded twice).

MC160 Inhibits TBK-1-mediated IFN-β Activation

During infection, MAVS recruits TBK-1 to complex with IKKε at the mitochondria. The TBK-1/ IKKε phosphorylates transcription factors IRF3 and IRF7, which eventually leads to IFN-β production (Sharma et al., 2003). To this end, it was of interest to investigate which region(s) of the MC160 protein is/are crucial to maintain proficiency in dampening TBK-1-mediated type I IFN activation in hosts. In the first TBK-1 experiment, 293T cells were transiently transfected with 400ng of pTBK-1, 300ng of MC160 constructs, 200ng of pIFN-β-luc and 100ng of pRenilla-TK. MC160 constructs containing both DEDs, MC160 and MC160N, inhibited activation of the IFN-β enhancer regulated luciferase by approximately 378.78-fold and 11.64-fold difference, respectively compared to pCI transfected cells overexpressing pTBK-1. However, MC160D1, MC160D2 and MC160C failed to inhibit TBK-1 induced activation of IFN-β. (Figure 7). This result was surprising given that Randall et al. previously reported both MC160D1 and MC160C blocked TBK-1 induced activation of IRF3 (Randall et al., 2013). The expression levels for the corresponding immunoblot are consistent. Flad-TBK-1 was detected in all the samples. Again, the wild-type MC160 protein and MC160N are expressed very well. MC160C expression is also very abundant however D2 was very faint and D1 was undetectable in this sample (Figure 8).

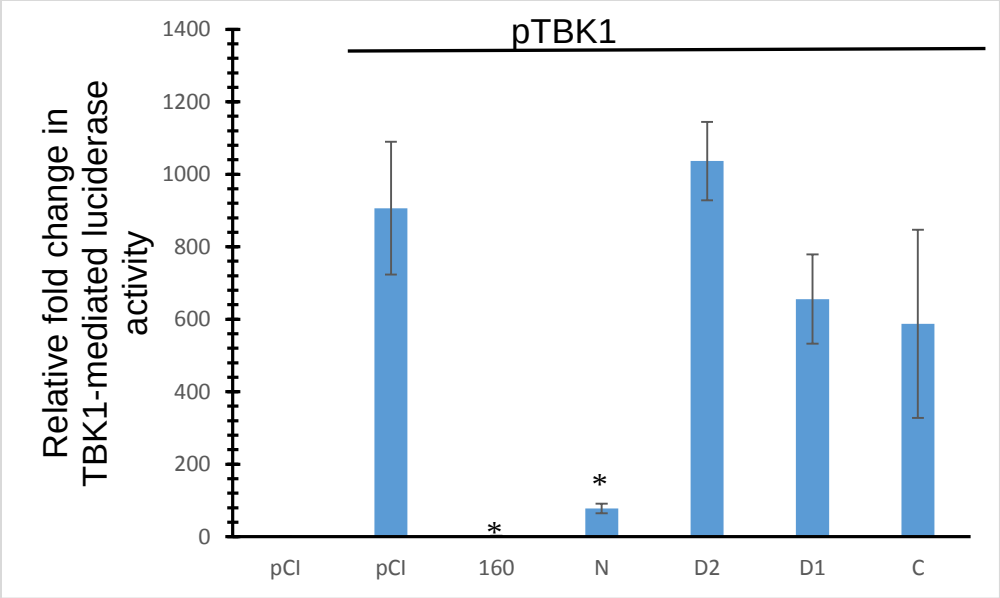

Figure 7: The effect of MC160 truncation mutants on TBK-1-mediated IFN-β enhancer controlled luciferase activation. TBK-1 was expressed at a greater concentration than the MC160 constructs. 293T cells were transfected with pIFN-β-luc (200ng); pRenilla-TK (100ng); pTBK-1 (400ng); and MC160 mutants (300ng). 16-24 hours post transfection the cells were lysed and firefly and sea pansy luciferase activities were recorded. Luciferase data was determined based on the ratio of firefly to sea pansy luciferase values. All values were normalized to cells expressing the reporters and the empty vector. Values are shown as mean ± standard deviations (SD). All experiments were performed in triplicate, n=3. *Statistical significance was determined by a $p < 0.05$.

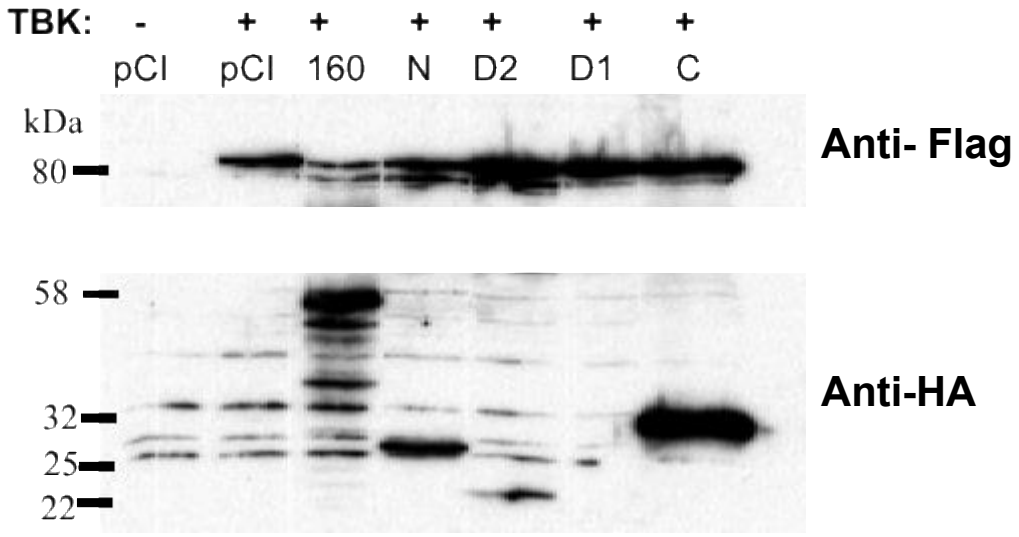

Figure 8: Expression of Flag-TBK-1 and HA-tagged MC160 proteins, probed with anti-HA (1:1000) and anti-Flag (1:1000) antibodies in 0.5% milk TBST for 1 hour. Bands corresponding to Flag-TBK-1 or the indicated MC160 mutant protein were visualized by chemiluminescence.

A second TBK-1 study was performed to assess the effects of less TBK-1 and more MC160 mutants on IFN-β enhancer controlled luciferase activity. 293T cells were transiently transfected with 200ng of pIFN-β-luc, 100ng of pRenilla-TK, 200ng of pTBK-1 and 500ng of MC160 constructs. Expression of MC160 inhibited IFN-β enhancer controlled luciferase activity about 86.3-fold better than pCI co-transfected with TBK-1. Similarly, MC160N expression on IFN-β enhancer controlled luciferase activity was approximately 15-fold lower when compared to pCI with TBK-1. MC160C inhibited activation of IFN-β, but not as strong as either MC160 or MC160N. Neither MC160D1nor MC160D2 inhibited IFN-β activation when compared to cells transfected with the empty vector and TBK-1 plasmids (Figure 9). These finding were unanticipated as Randall et al. reported that all MC160 mutants, except MC160D2, inhibited TBK-1-induced IRF3 activation (Randall et al., 2013). A corresponding immunoblot to verify expression of the MC160 constructs in the lysates was conducted (Figure 10). Flag-TBK-1 is present in all samples. However, the sample containing the MC160 protein seemed to show a lower amount of TBK-1 compared to all other samples. MC160C protein levels are very high and are similar to wild type MC160. MC160N protein expressed a band comparable to the band associated with MC160D2, indicating similar protein levels. While MC160D1 showed a very faint band compared to all the other constructs.

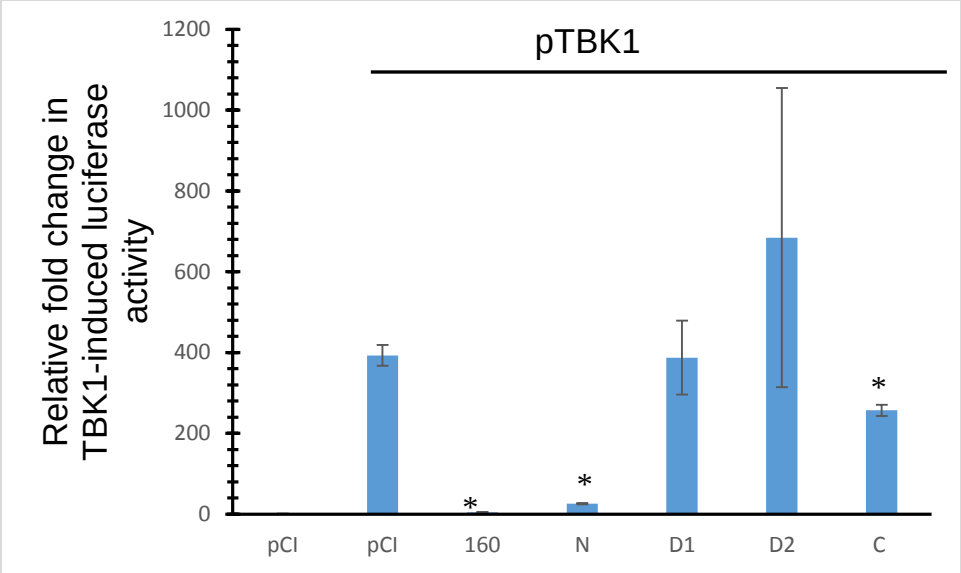

Figure 9: The effect of MC160 truncation mutants on IFN-β luciferase activity in the presence of lower concentrations of TBK-1. MC160 constructs and TBK-1 were overexpressed in 293T cells (500ng and 200ng, respectively) along with pIFN-β-luc (200ng); and p-Renilla-TK (100ng). Data was obtained based on the ratio of firefly to sea pansy luciferase values. The values were normalized to cells expressing the reporters and the empty vector. Values are shown as mean ± standard deviations (SD). All experiments were performed in triplicate, n=3. *Statistical significance was determined by a $p < 0.05$.

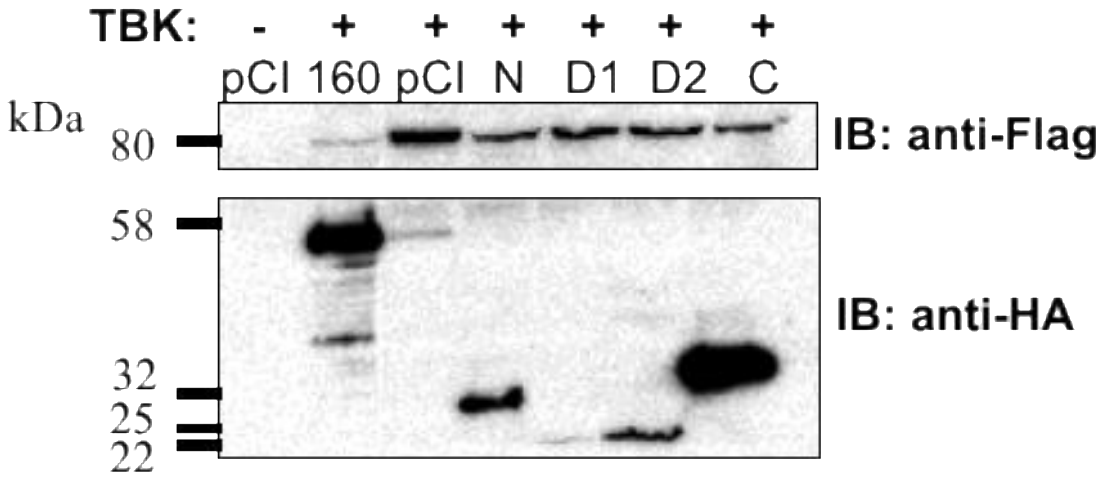

Figure 10: Expression of Flag-TBK-1 and HA-tagged MC160 proteins. Lysates were run on an SDS-PAGE and proteins were subsequently transferred to a nitrocellulose membrane. The membranes were probed with anti-HA (1:1000) and anti-Flag (1:1000) antibodies in 0.5% milk TBST for 1 hour. Bands corresponding to Flag-TBK-1 or the indicated MC160 mutant protein were visualized by chemiluminescence.

MC160 Titrations expressed with MAVS or TBK-1

The overexpression of MC160 or MC160N, both of which have the tandem DEDs together on one protein, inhibited MAVS- and TBK1 induced IFN-β enhancer controlled luciferase activity (Figure 3 through Figure 7). While MC160C expression inhibited IFN-β enhancer controlled luciferase activity in most cases, it was never comparable to the inhibition seen when MC160 or MC160N were expressed. In contrast, the luciferase data revealed no statistically significant inhibition in IFN-β enhancer controlled luciferase activity when MC160D1 or MC160D2 were overexpressed with either MAVS or TBK-1. Although the degree of IFN-β luciferase activity varied among MC160D1, MC160D2, and MC160C, none displayed levels comparable to MC160 or MC160N, which were both statistically significant compared to the empty vector co-transfected with either TBK-1 or MAVS. However, one concern was that based on immunoblots, neither MC160D1 nor MC160D2 seemed to express consistently to the same level as MC160 full-length or MC160N, potentially due to protein insolubility or inability of the protein to fold correctly. However, further studies are needed to determine that reasoning. Therefore, one possible explanation as to why neither D1 nor D2 inhibited could be due to lower protein levels in the cells. To this end, a titration of the MC160 mutant expression plasmids was conducted with either MAVS or TBK-1.

MC160 mutants were transiently transfected into 293T cells at the indicated concentrations with MAVS or TBK-1 at 200ng. Because MC160N produced such a strong inhibitory effect on IFN-β luciferase activity, a low concentration of 50ng and a high of 100ng of MC160N were tested. The luciferase data showed approximately a 3.8-

fold and 5.88-fold difference between MC160N expressed at 50ng or 100ng, respectively, when compared to pCI with MAVS (Figure 11). Similarly, MC160N expressed at 50ng or 100ng with 200ng of TBK-1 was roughly 3.66- and 5.57-fold less compared to the empty vector pCI co-transfected with TBK-1 (Figure 13). Upon analyzing the D1 and D2 titrations, with a low of 100ng and a high of 500ng, neither DED inhibited MAVS or TBK-1 induced IFN-β enhancer controlled luciferase activity (Figure 11, Figure 13). The lysates for each titration were run on an SDS-PAGE, and blotted with anti-FLAG and anti-HA to validate the luciferase results for titrations with MAVS and TBK-1, respectively (Figure 12, Figure 14). For the titration with MAVS, only MC160N expressed at 50ng and 100 ng inhibited IFN-β enhancer controlled luciferase activity, while neither DED at 100ng or 500ng inhibited IFN-β luciferase activity. Confirming MC160 mutant protein expression via immunoblot showed that the D1 and D2 protein expression at 500ng transfection had more similar protein levels to the MC160N protein at 50ng. These results suggest that the decreased protein levels in D1 and D2 are not correlated to the inability to inhibit MAVS-induced IFN-β enhancer controlled luciferase activity (Figure 12). Likewise, the MC160 titration with TBK-1 showed that MC160N expressed at 50ng and 100ng inhibited IFN-β luciferase activation, while all other mutants at various concentration did not. Surprisingly, D1 expressed at 500ng increased IFN-β enhancer controlled luciferase activity by approximately 1.67-fold compared to the pCI co-transfected with TBK-1 (Figure 13). The enhanced expression of luciferase activity could be due to binding to procaspase-8 through MC160 DEDs. Procaspase-8 when converted to caspase-8 results in disassembly of the MAVS-complexes in order to

limit duration and intensity of signaling (Rajput et al., 2011). Whether, MC160D1 when expressed independently binds and interferes with this caspase-8 function is under investigation. The immunoblot measuring the titration of MC160 protein expression with TBK-1 showed good expression of MC160N at 50ng and 100ng. However, D1 levels were hardly detectable, even at 500ng. Interestingly, the protein levels for D2 at 100ng and 500ng were more similar to MC160N protein levels at 50 ng (Figure 14). Based on the collected data, there does not seem to be a correlation between protein expression and inhibition of IFN-β firefly luciferase activity.

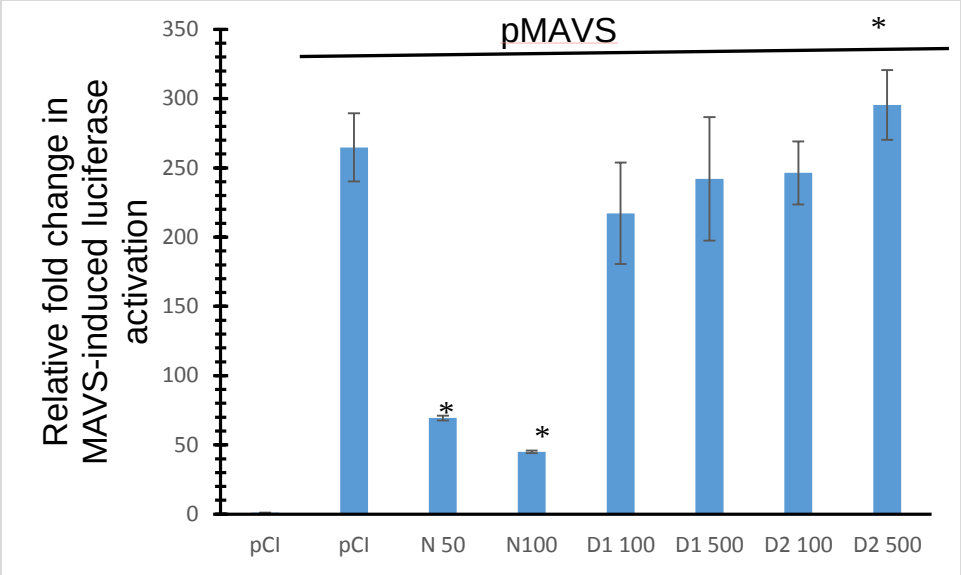

Figure 11: The effect of MC160 mutant titrations on IFNβ luciferase activity in the presence of MAVS. Subconfluent 293T cells were transfected with pIFNβ-luc (200ng); pRenilla-TK (100ng); pMAVS (200ng); and MC160 constructs at varying concentrations. MC160N inhibits IFNβ activity at concentrations as low as 50ng. Neither DED showed inhibition of IFN-β activity, despite increasing amounts of MC160 mutant. Values are shown as mean ± standard deviations (SD) (n=3). *Statistical significance was determined by a $p < 0.05$.

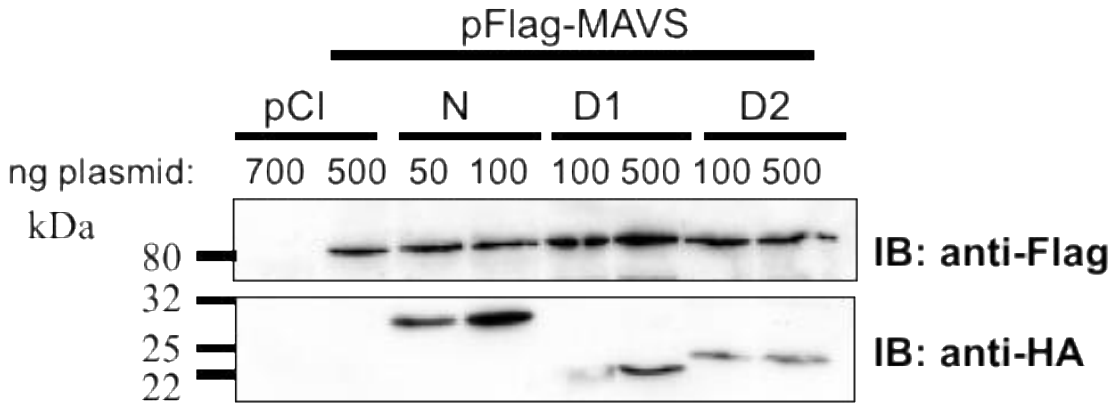

Figure 12: Expression of Flag-MAVS and HA-tagged MC160 proteins titrations. Membranes were probed with anti-HA (1:1000) and anti-Flag (1:1000) antibodies. Bands corresponding to Flag-MAVS or the indicated MC160 mutant protein were visualized by chemiluminescence.

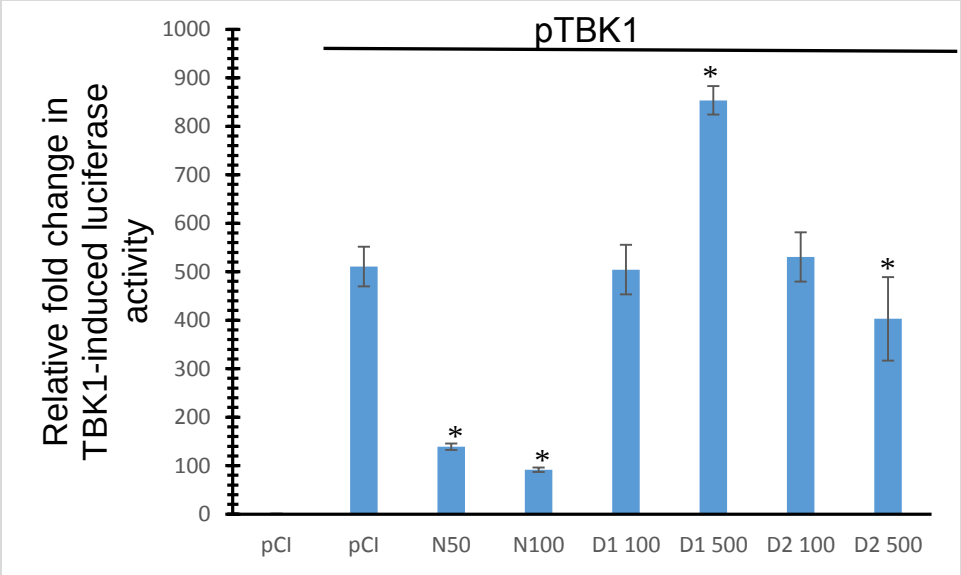

Figure 13: The effect of MC160 truncation mutants on IFNβ luciferase activity in the presence of TBK-1. 293T cells were transfected with pIFNβ-luc (200ng); pRenilla-TK (100ng); pTBK-1 (200ng); and MC160 constructs at varying concentrations. MC160N inhibits IFNβ activity at concentrations as low as 50ng. Neither DED showed inhibition of IFN-β activity, despite increasing amounts of MC160 mutants. Values are shown as mean ± standard deviations (SD) (n=3). *Statistical significance was determined by a $p < 0.05$.

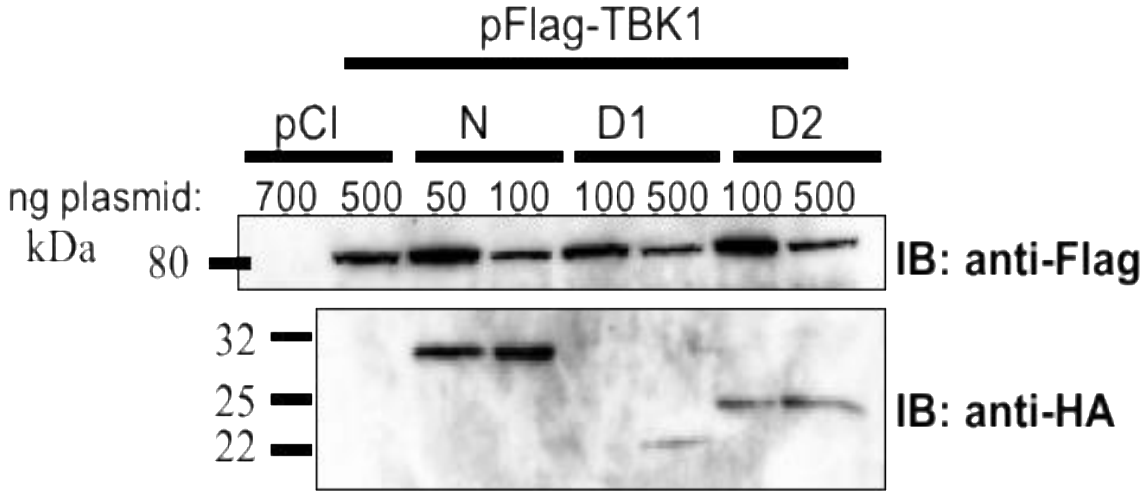

Figure 14: MC160 titration with TBK-1 expression immunoblot. Confirmation of MC160 mutant expressions are shown here. The membrane was probed with primary antibodies, anti-FLAG (1:1000) and anti-HA (1:1000), followed by a secondary antibody, goat-anti-mouse-HRP (1:5000) each for 1 hour at room temperature. Protein expression was visualized by chemiluminescence.

Discussion

MC159 and MC160 proteins were previously identified as viral proteins that inhibit MAVS-mediated IFN activation (Randall et al. 2013). The purpose of this thesis was to identify the region of the MC160 protein required to inhibit activation of type I IFNs. While Randall et al. presented evidence that all MC160 constructs with the exception of MC160D2 inhibit TBK-1 induced activation of IRF3 (Randall et al., 2013), our data suggests inhibition of TBK-1 or MAVS-induced IFN-β luciferase activity is exclusive to MC160 and MC160N, with MC160C showing partial inhibition of IFN-β under certain conditions. Thus, the data presented here suggest that MC160 requires both DEDs to inhibit TBK-1 or MAVS-induced IFN-β activation.

One possible explanation as to why the MC160N protein inhibits activation of IFN-β and the D1 and D2 mutants do not could be attributed to expression levels. When MC160N, D1, and D2 expression vectors were transfected at equal concentrations of plasmid DNA, bands corresponding to MC160N in immunoblots was often detected at higher concentrations relative to D1 and D2 (Figures 4, 6, 8 and 10). However, this explanation seems unlikely for several reasons. 1) When the MC160N expression vector was transfected at as little as 50ng of plasmid DNA, MC160N still strongly inhibited relative to 500 ng of MC160D1 and MC160D2. 2) Based on the immunoblots for each titration experiment, protein levels of MC160N at 50ng was more comparable to MC160D1 at 500ng when MAVS was overexpressed (Figure 12). 3) MC160D2 at 500 ng does not inhibit activation of type I IFNs, but does prevent activation of TNF-induced NF-κB activation (Nichols and Shisler 2009). 4) MC160D1 inhibits TBK-1 induced IRF3

activation (Randall et al., 2013) when transfected at equal concentrations to that used in in the experiments presented herein. Therefore, the most likely explanation of the results is that *MC160 requires both DEDs to inhibit activation of type I IFNs*. Further, when MC160D1 and MC160D2 were co-transfected in cells to determine if individual DEDs mixed together could inhibit TBK-1 or MAVS-induced IFN-β luciferase activity to levels similar to MC160N, MC160D1 and MC160D2 failed to inhibit under these conditions (Figure 3). Thus, both DEDs are required to be on the same protein to inhibit TBK-1 or MAVS-induced IFN-β enhancer controlled luciferase activity.

While MC160C partially inhibited MAVS and TBK-1-induced IFN-β luciferase activation, it was not to the same degree as the regions containing both DEDs. MC160C expression was detected at higher concentrations than wild-type MC160 or MC160N. Thus, reduced inhibition of activation of IFN-β by MC160C cannot be attributed to differences in expression levels. Interestingly, MC160C binds to Hsp90 (Nichols and Shisler, 2009). During virus infection, Hsp90 forms a bridge between IRF3 and TBK-1, thereby stabilizing the interaction and enabling a better signal through the pathway (Yang et al., 2006). Perhaps, MC160C when expressed at higher amounts than TBK1, competes with TBK1 for binding to MAVS signaling complexes, but when TBK1 is at higher levels than MC160C, TBK1 outcompetes MC160C for binding to MAVS complexes.

In conclusion, the MCV MC160 protein inhibits MAVS-mediated signaling through interactions mediated by the MC160 DEDs. The future experiments of this project would be to identify the cellular binding partner of MC160. We surmise that the MC160 protein inhibits the MAVS signaling pathway at the TBK-1 level, therefore

running co-immunoprecipitations with Flag-TBK-1 would be advantageous. Additionally, running a yeast two-hybrid system using MC160 and TBK-1 would be useful in determining the binding partner of MC160 protein in the MAVS pathway. Another future direction would be to investigate whether MC160 expression can block activation of TBK-1 induced by other pathways. We have preliminary data showing LPS induced phosphorylation of TBK-1 in RAW 264.7 cells (Figure 15). RAW 264.7 cells could be transfected with the MC160 constructs and treated with LPS, which signals through TLR4, eventually leading to the translocation of NF-κB and IRF3 into the nucleus, thus inducing IFN production. Similarly, instead of LPS, treatment with Poly I:C, a synthetic mimic of dsRNA produced during viral infections, would signal through TLR3, thus inducing IFN production. MC160 inhibits phosphorylation of TBK-1 induced by the overexpression of TBK-1 (Randall et al., 2013), therefore TBK-1 phosphorylation would not be excepted in cells expressing MC160.

Because MCV cannot replicate in either tissue culture or animal models, overexpressing viral proteins by transfecting 293T cells was appropriate to study the effects of MC160 expression on MAVS-mediated activation of type I IFNs. To validate that MC160 functions similarly during a live virus infection, recombinant vaccinia viruses could be used as a surrogate virus to express and study MC160 in the context of a virus infection.

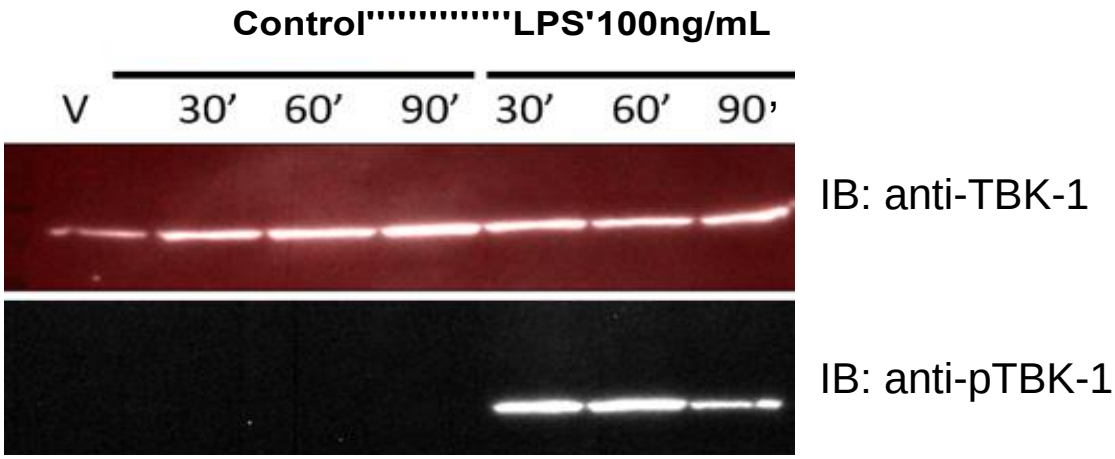

Figure 15: LPS induced TBK-1 phopsorylation of RAW 264.7 cells. The vehicle consists of 2% dimethyl sulfoxide (DMSO) diluted in PBS. LPS is detected by TLR4 and signals down the pathway to activate type I IFNs. Treating MC160 constructs with LPS would verify the ability of the MC160 protein to inhibit TBK-1 phosphorylation in this pathway.

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