

Investigating Molluscum Contagiosum Virus Protein MC008 and its Effect on Inflammatory Signalling



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By

Thomas Phelan

School of Medicine

Trinity College Dublin

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Supervisor: Prof. Gareth Brady. Co-Supervisor: Prof. Mark Little

Declaration

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Thomas Pheasant

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Abstract

Molluscum contagiosum virus (MCV) is a human-adapted poxvirus that causes a common, persistent, yet mild infection characterized by distinct, contagious, papular skin lesions. These lesions are notable for having little or no inflammation associated with them and can persist for long periods without an effective clearance response from the host. Like all poxviruses, MCV encodes potent immunosuppressive proteins which perturb innate immune pathways involved in virus sensing, the interferon response and inflammation which collectively orchestrate anti-viral immunity and clearance. Our initial investigation focused on an interaction with the E3 ligase, TRIM32, a protein shown to be involved in cGAS-STING sensing of DNA. This interaction was identified from analysis of MC008 co-purifying proteins by mass spectrometry prior to PhD project commencement. However, we were unable to discern a direct mechanism of MC008 inhibition through this protein.

Since several innate immune pathways converge at common signalling nodes, we investigated further downstream. One key node is the regulator of canonical nuclear factor kappa B (NF- κ B) activation, NF- κ B essential modulator (NEMO). In this thesis, we report that the MCV protein MC008 inhibits NF- κ B through its interaction with NEMO, disrupting its early ubiquitin-mediated activation and subsequent downstream signalling. MC008 is the third NEMO-targeting inhibitor to be described in MCV to date, with each inhibiting NEMO activation in distinct ways, highlighting a strong selective pressure to evolve multiple ways of disabling this key signalling protein.

Lay Abstract

Molluscum contagiosum (MCV) is a common human-specific pathogen that causes the formation of dome-shaped papules on the surface of the infected skin. These papules can remain present for years without an apparent immune response from the host. In comparison, infection by other poxviruses results in inflamed lesions that tend to resolve within a few weeks. This indicates that MCV possesses the ability to inhibit inflammatory signalling, likely through immunoregulatory proteins encoded in the genome. A preliminary screen of the MCV genome revealed the presence of multiple inhibitors of inflammation, one of which was MC008, and this is the focus of my PhD research.

Prior to the beginning of this PhD project, MC008 was shown to interact with a regulatory protein called TRIM32. This protein enables modification of cell signalling elements through the conjugation of ubiquitin molecules to a target protein. We subsequently confirmed the MC008 interaction with TRIM32 and the inhibition of TRIM32-driven inflammatory signalling. However, we were unable to elaborate a direct mechanism by which MC008 inhibited activation of inflammatory signalling through this protein.

Given that many signalling pathways converge at common points downstream of the sensory apparatus, we mapped the ability of MC008 to inhibit inflammatory signalling to a common node. Along with TRIM32, MC008 interacted with another key regulatory component, NEMO, a protein that is crucial to the activation of inflammatory signalling and is involved in multiple pathways. We demonstrated that MC008 inhibited NEMO association with ubiquitin, an indispensable step in the inflammatory response.

Publications & Presentations

Publications

Phelan T, Little MA, Brady G. Targeting of the cGAS-STING system by DNA viruses. *Biochemical Pharmacology*. 2020;113831.

Leacy E, Brady G, Conlon N, Scott J, Dunne J, **Phelan T**, et al. Study Protocol for DeCoMPRESS: Defining the Disease Course and Immune Profile of COVID-19 in the Immunosuppressed Patient. *HRB Open Research*. 2021;4(6).

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Dyer AH, Noonan C, McElheron M, Batten I, Reddy C, Connolly E, Pierpoint R, Murray C, Leonard A, Higgins C, Reilly P, Boran G, **Phelan T**, McCormack W, O'Neill D, Fallon A, Brady G, O'Farrelly C, Bourke N.M, Kennelly S.P. Previous SARS-CoV-2 Infection, Age, and Frailty Are Associated With 6-Month Vaccine-Induced Anti-Spike Antibody Titer in Nursing Home Residents. *Journal of the American Medical Directors Association*. 2022.

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Understanding and Exploiting how Poxviruses Evade Human Innate Immunity. Irish Research Forum. (Online) Trinity Biomedical Sciences Institute, Dublin 22nd April 2022.

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The Development of an Adaptable SARS-CoV-2 Serology Assay. SFI SPP Immunology of COVID-19 Meeting, (Online), December 2nd 2020.

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Molluscum Contagiosum Virus encodes novel inhibitors of inflammation (Flash Talk). Trinity Translational Medicine Institute, Dublin 15th of February 2019.

Poster Presentations

Molluscum Contagiosum Virus Protein MC008 Inhibits Human NFκB Activation by Preventing NEMO Ubiquitination, . Irish Society for Immunology Annual Meeting 2022, Maynooth University, Kildare, September 1st-2nd 2022.

Molluscum Contagiosum Virus Protein MC008 is a Novel Inhibitor of Human NFκB. Host-Pathogen Communication, Trinity Biomedical Sciences Institute/Online, 10th-11th November 2021.

Investigation of DNA sensing inhibition by Molluscum Contagiosum Virus protein MC008. Irish Society for Immunology Annual Meeting 2019, Royal College of Surgeons, Dublin September 19th -20th 2019.

Molluscum Contagiosum Virus encodes several novel inhibitors of inflammation including MC008 is a novel inhibitor of DNA sensing. Host-Pathogen Communication, Weizmann Institute for Science, Rehovot, Israel 11th -14th of November 2018.

Abbreviations

Abbreviation	Description
4E-BP1	Eukaryotic initiation factor 4E-binding protein 1
ANOVA	Analysis of variance.
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CdCl ₂	Cadmium chloride
cGAMP	Cyclic guanosine monophosphate–adenosine monophosphate
cGAS	Cyclic GMP–AMP synthase
cIAP1	cellular inhibitor of apoptosis protein 1
CMV	Cytomegalovirus
dH ₂ O	Distilled water
DMEM	Dulbecco's Modified Eagle Medium
DOX	Doxycycline
dsDNA	Double-stranded DNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERGIC	ER Golgi intermediate compartment
EV	Empty vector
EVs	Enveloped virions
G3BP1	GTPase-activating protein SH3 domain–binding protein 1
GAG	Glycosaminoglycans
Gas6	Growth arrest-specific 6
HA	Hemagglutinin
HaCaT	High sensitivity of human epidermal keratinocytes
HEK293T	Human embryonic kidney 293T
HeLa	Henrietta Lacks
HIV	Human immunodeficiency virus
IFNs	Interferons
IKK	IκB kinase
IL-6	Interleukin 6
IRF	Interferon regulatory factor
ISRE	Interferon-stimulated response element
MAVS	Mitochondrial antiviral-signalling protein
MCV	Molluscum contagiosum virus
MPX	Monkeypox virus
mRNA	Messenger RNA
MTORC	Mammalian Target of Rapamycin complexes
MV	Mature virions
MyD88	Myeloid differentiation primary response 88
NAP1	NF-Kappa-B-Activating Kinase-Associated Protein 1
NEMO	NF-kappa-B essential modulator

NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHL	NCL-1, HT2A, LIN-41
NIK	NF- κ B-inducing kinase
NPH I	Nucleotide phosphohydrolase I
PAK1	p21 (RAC1) activated kinase 1
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PEI	Polyethylenimine
PIAS γ	Protein inhibitor of activated STAT γ
PI3K	Phosphatidylinositol-3-kinase
Poly(dA:dT)	Poly(deoxyadenylic-deoxythymidylic)
Poxins	Poxvirus immune nucleases
PRR	Pattern recognition receptors
Rac1	Ras-related C3 botulinum toxin substrate 1
RAP94	RNA polymerase associated factor
Raptor	Regulatory associated protein of mTOR
Rictor	Rapamycin-insensitive companion of mTOR
RIG-I	Retinoic acid-inducible gene I
RING	Really Interesting New Gene
RNA	Ribonucleic acid
SDS-PAGE	Sodium dodecyl-sulphate-polyacrylamide gel electrophoresis
SINTBAD	Similar to NAP1 TBK1 adaptor
STIM1	Stromal interaction molecule 1
STING	Stimulator of interferon genes
TAB2/3	TGF-beta activated kinase 1 binding protein 2/3
TAK1	TGF- β -activated kinase 1
TANK	TRAF family member-associated NF-kappa-B activator
TBK1	Tank-binding kinase 1
TET	Tetracycline
TI-IFNs	Type I interferons
TIR	Toll/interleukin-1 (IL-1) receptor
TLRs	Toll-like receptors
TMB	3,3',5,5'-Tetramethylbenzidine
TRAF	TNF receptor associated factor
TRIF	TIR-domain-containing adapter-inducing interferon- β
TRIM32/56	Tripartite motif 32/56
TRIS	Trisaminomethane
UBAN	Ubiquitin binding in ABIN and NEMO
VACV	Vaccinia virus
VARV	Variola virus
VETF	Vaccinia early transcription factor
vFLIPS	Viral FLICE-like proteins

Chapter 1 Introduction

1.1 Host Innate Immune Signalling

1.1.1 Overview

This chapter contains extracts originally published in Biochemical Pharmacology. Phelan T, Little MA, Brady G. Targeting of the cGAS-STING system by DNA viruses. 2020;174:113831

The existence of pattern recognition receptors (PRRs) was predicted by Charles Janeway Jr., before innate mechanisms of pathogen-associated molecular pattern (PAMP) recognition had been described (1). The first example of a PRR was the ‘Toll’ protein found to induce expression of host defence mechanisms in the fruit fly *drosophila melanogaster* (2). The first human homolog of this protein was identified in humans by Janeway and Ruslan Medzhitov in 1997 and was subsequently termed Toll-like receptor (TLR) (3). Since then, multiple TLRs and PRR families have been identified, with each playing a crucial role in host immune defence.

The primary, innate, front-line mechanism for detecting viral infection is the vast array of PRRs displayed in the cytosol and endosomal compartments of cells. These PRRs include TLRs 3, 7, 8, and 9, the RIG-I-like receptors MDA5 and RIG-I, and cytosolic DNA sensors such as the AIM2 inflammasome and cyclic GMP-AMP synthase (cGAS). PRRs can detect a variety of viral ligands including single-stranded (ss) and double-stranded (ds) DNA and RNA, the uncapped 5′ triphosphate of viral RNA and unmethylated CpG viral DNA. These signatures of viral infection are known as PAMPs due to biochemical distinctions or cellular localisation, enabling the innate immune system to effectively distinguish between self and non-self (4).

Activation of PRR pathways during viral infection, while distinct at the point of sensing, overlap considerably in their use of downstream signalling components. They culminate

in the activation of common transcription factors sets which drive interferon and pro inflammatory cytokine production with diverse effects on a wide variety of cell types. Type I interferons (IFNs) are induced by the combined action of IRF-family and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) transcription factors (5). Type I IFNs, such as IFN- α and IFN- β , are predominantly induced during the antiviral response and exert their influence by binding to interferon- α/β receptors which activate Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signalling pathway. This results in the upregulation of hundreds of interferon-stimulated genes (ISGs) that restrict viral replication, promote growth arrest, and induce apoptosis (6, 7). Type I IFNs exert a cell-intrinsic antimicrobial state on stimulated cells or trigger antiviral behaviour in innate and adaptive immune cells. For example, type I IFNs induce monocytes to produce IL-18, which in turn activates natural killer (NK) cells resulting in the production of IFN- γ , a type II IFN. This is a key cytokine in the antiviral response required for macrophage activation and links innate and adaptive immune responses (8). NF- κ B activation is also induced in response to viral infection, leading to the production of a wide variety of proinflammatory cytokines and chemokines including IL-1 β , TNF- α , and IL-6. Proinflammatory cytokines have important central roles in orchestrating inflammation through induction of proinflammatory genes, recruitment, and activation of various immune cells along with the upregulation of diverse cell surface molecules (9, 10). Therefore, both NF- κ B- and IRF-activating pathways collaborate in complex ways, with both required for an optimal response to invading viral pathogens (11).

1.1.2 Ubiquitination

Post-translational modifications, such as ubiquitination, vastly increase the complexity and functional diversity of the proteome, enabling swift and dynamic cellular responses (12). Ubiquitination is a reversible process that occurs through a sequential enzymatic cascade (**Figure 1.1.1**). Ubiquitination is initiated through an ATP-dependent reaction that forms a thioester-bond between glycine 76 on ubiquitin molecule and the cysteine residue on a E1 ubiquitin activating enzyme (13). The ubiquitin molecule is then transferred to the cysteine residue of an E2 ubiquitin conjugating enzyme. An E3 ubiquitin ligase then facilitates the transfer of the ubiquitin molecule from the E2 to the internal lysine residue of the target substrate (13, 14). These substrates can be mono- or poly-ubiquitinated and this process is reversible through the action of de-ubiquitinating enzymes. In humans, only 2 E1, approximately 30 E2 and over 600 E3 enzymes have been identified to date (14).

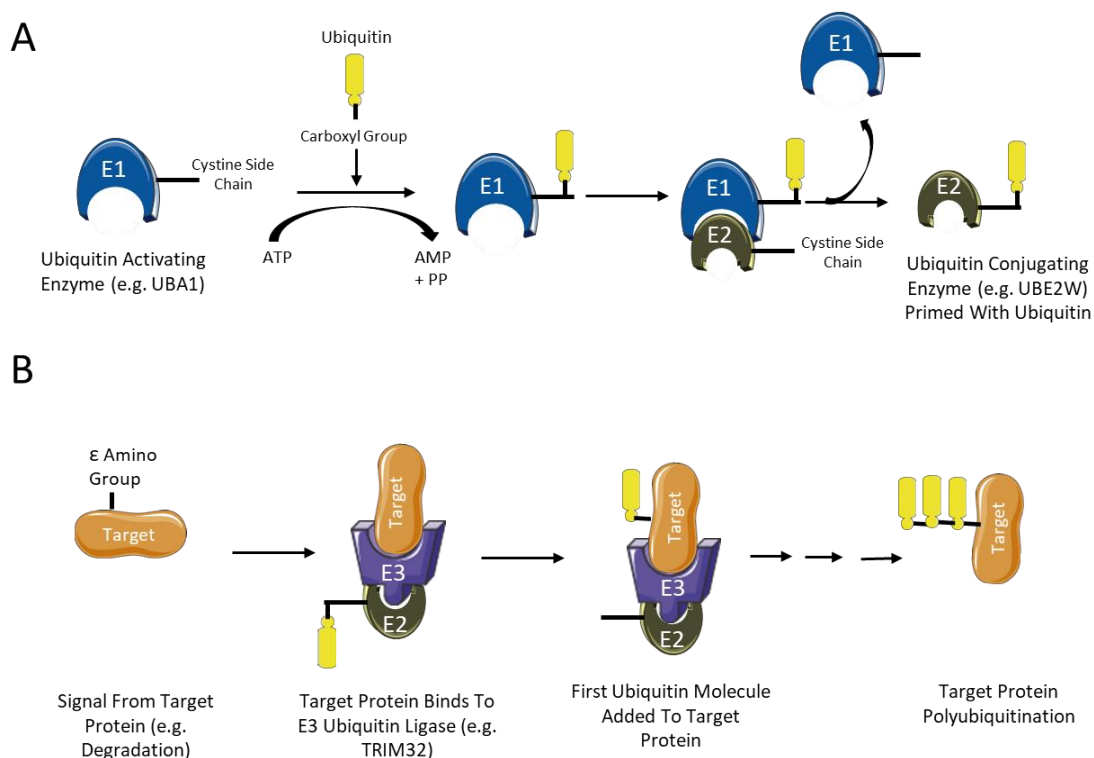


Figure 1.1.1 The ubiquitination process. (A) The E1 ubiquitin activating enzyme is conjugated to a ubiquitin molecule through an ATP dependent process involving the cystine side chain of the E1 and the carboxy terminal of ubiquitin, resulting in the formation of a thioester bond. The activated ubiquitin molecule is now transferred to an E2 ubiquitin conjugating enzyme, again forming a thioester bond. (B) The E2 interacts with the E3 ligase, which detects a specific target protein. The E3 transfers the ubiquitin molecule to the ϵ -amino group of lysine on the target protein forming an isopeptide bond with the carboxyl terminus of the ubiquitin molecule. Repetition of this process leads to poly-ubiquitination of the target protein. The forms of the individual proteins were adapted from Servier Medical Art (<https://smart.servier.com/>), licensed under a Creative Common Attribution 3.0 Generic License.

Ubiquitination events during PRR signalling present as crucial modifications that can promote or inhibit signalling, depending on the type of ubiquitin involved. Following the initial transfer of a ubiquitin molecule to the lysine residue of a target substrate, additional ubiquitin molecules can bind to any of the following 7 lysine residues of ubiquitin (K6, K11, K27, K29, K33, K48, K63) or the n-terminal methionine (Met-1), through repetitions of the process to build up chains of variable length and configuration (14). These ubiquitin configurations have various functions including signal transduction, protein kinase activation, and protein degradation. The best characterised of these configurations in innate immunity are K63- and K48-linked which play crucial roles in NF- κ B activation and proteasomal degradation of target proteins, respectively (15).

1.1.3 Toll-Like Receptors

Humans express 10 known TLRs located on the cell membrane or in an endosome, with each one detecting distinct molecular patterns (**Table 1**). TLRs are transmembrane proteins containing 20–27 extracellular leucine-rich repeats (LRR) that form a distinctive horseshoe-shaped structure, and have an intracellular toll–interleukin 1 (IL-1) receptor

(TIR) domain (16). Ligand binding induces dimerization of two TLR ectodomains, bringing the TIR domains together, thereby enabling them to interact with TIR domain-containing cytoplasmic adaptor molecules (16). Five of these adaptors have been identified in mammals; myeloid differentiation primary-response protein 88 (MyD88), TIR domain-containing adaptor protein (MAL), TIR domain-containing adaptor protein inducing IFN- β (TRIF), TRIF-related adaptor molecule (TRAM), and sterile α - and armadillo-motif-containing protein (SARM) (16). MyD88 is used by all TLRs, except for TLR3, and leads to proinflammatory cytokine production. TRIF is utilized by TLR3 and TLR4 and is associated type I IFN production. Different combinations of these adaptors influence downstream signals. MAL facilitates the recruitment of MyD88 and TRAM facilitates recruitment of TRIF (17). SARM is a negative regulator of TRIF-dependent TLR signalling (17, 18).

TLR	Ligand	Location	References
TLR-1:TLR-2 heterodimer	Lipomannans (mycobacteria)	Cell Surface	(19)
TLR-2:TLR-6 heterodimer	Diacyl and triacyl lipopeptides (bacteria) Lipoteichoic acids (Gram-positive bacteria) Cell-wall β -glucans (fungi)		(20)
TLR-3	Double-stranded RNA (viruses) poly I:C	Endosome	(21)
TLR-4	LPS (Gram-negative bacteria)	Cell Surface	(22)
TLR-5	Flagellin (bacteria)	Cell Surface	(23)
TLR-7	Single-stranded RNA (viruses)	Endosome	(24)
TLR-8	Single-stranded RNA (viruses)	Endosome	(25)
TLR-9	DNA with unmethylated CpG (bacteria and DNA viruses)	Endosome	(26)
TLR-10	Unknown	Cell Surface	(27)

Table 1. Toll-like receptors, their associated ligands and cellular location.

TLR signalling leads to activation of NF- κ B (**Figure 1.1.2**), activator protein 1 (AP-1) and IRF transcription factors, depending on the receptor stimulated (28, 29). Following MyD88 recruitment to the TIR domain of a TLR, the amino terminus contains a death domain which interacts with the death domain on two serine/threonine protein kinases- IL-1 receptor-associated kinase 4 (IRAK4) and IRAK1 (30). The IRAK proteins and MyD88 form a signalling scaffold, also referred to as the Myddosome, and recruit enzymes such as TNF receptor-associated factor 6 (TRAF6). TRAF6, in combination with the E2 enzyme complex Ubc13/Uev1A and the E1 enzyme UBA1 (31, 32), can produce poly-ubiquitin chains which serve as a scaffold for other signalling molecules (30). These include the poly-ubiquitin-binding adaptor proteins TGF-beta activated kinase 1 binding protein 1 (TAB1), (TAB2), and the serine/threonine kinase transforming growth factor- β (TGF- β)-activated kinase 1 (TAK1)(33). The IRAK complex

phosphorylates TAK1 which, in turn can, phosphorylate the I κ B kinase (IKK) complex which consists of NF-kappa-B essential modulator (NEMO), IKK α and IKK β (30). Simultaneously, TAK1 also phosphorylates mitogen-activated protein kinases (MAPKs), leading to AP-1 activation (30).

NEMO is a 419-amino acid protein composed of multiple domains including two coiled-coil regions (CC1 and CC2), along with a leucine zipper (LZ) and a zinc finger (ZF), arranged into an elongated structure (34). The beginning of the CC1 domain interacts with the C-terminus of IKK proteins (34, 35). This domain has also been shown to auto-inhibit NEMO in its resting state by preventing ubiquitin binding to the ubiquitin binding in ABIN and NEMO (UBAN) domain (36). Conformational changes in the IKK complex due to NEMO activation lead to trans-autophosphorylation of the IKK β T-loop serine's which facilitate enhanced kinase activity (37, 38). The CC2 domain and leucine zipper form the UBAN domain of NEMO. This region also regulates the conformational change that allows phosphorylation of IKK β by TAK1 on serine-177 and -181 in the activation loop, leading full IKK activation (39, 40). The C-terminal zinc finger has also been shown to be another ubiquitin binding domain (41). Activated IKK β phosphorylates I κ B, an inhibitory protein that is constitutively bound to cytoplasmic NF- κ B dimers (typically p50/p65 in canonical NF- κ B activation) (42). Phosphorylated I κ B is subsequently tagged with ubiquitin for proteasomal degradation. This releases NF- κ B, enabling it to translocate into the nucleus and regulate gene expression.

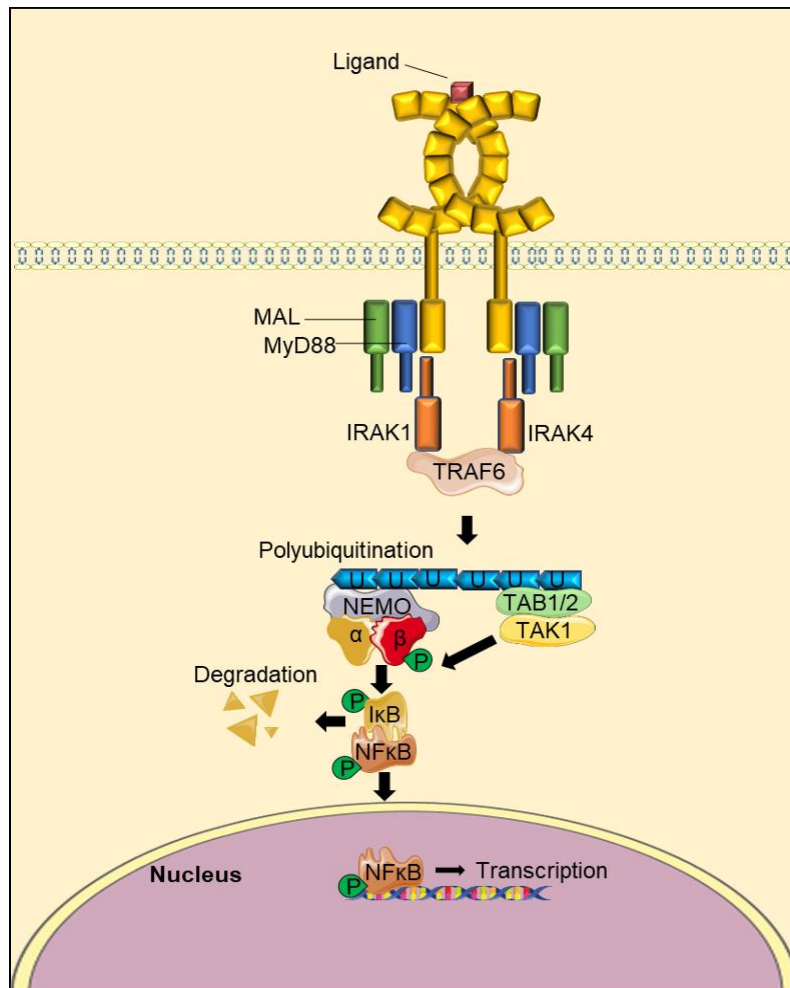


Figure 1.1.2 MyD88 dependent TLR signalling pathway. Ligand binding to a TLR induces dimerization of the ectodomains, bringing the cytoplasmic TIR domains into close proximity to each other. Some TLRs use both MyD88 and MAL adaptor proteins to propagate downstream signalling. MyD88 recruits IRAK1 and IRAK4, along with TRAF6. The IRAK proteins phosphorylate TRAF6 leading to induction of E3 ligase activity and generation of a poly-ubiquitin scaffold. This scaffold recruits and activates a complex containing the proteins TAB1/2 and TAK1. TAK1 phosphorylates IKK β which subsequently phosphorylates the cytoplasmic inhibitor, I κ B, leading to its degradation and the release of NF- κ B. NF- κ B (p50/p65) can then translocate into the nucleus where it drives transcription of NF- κ B responsive genes. The forms of the individual proteins were adapted from Servier Medical Art (<https://smart.servier.com/>), licensed under a Creative Common Attribution 3.0 Generic License.

In the non-canonical NF- κ B pathway (**Figure 1.1.3**), a subset of TNF receptor family members, such as lymphotoxin β 1 and CD40L ligand, act on NF- κ B-inducing kinase (NIK) to induce p100 or p105 processing (43, 44). P100 is an I κ B-like molecule predominantly associated with RELB. NIK, in its inactive state, is continuously tagged with ubiquitin for degradation by TRAF proteins but these are degraded following ligand binding, enabling NIK accumulation in the cytoplasm (45). NIK accumulation leads to phosphorylation of IKK α which subsequently phosphorylates serines 866 and 870 in the carboxy terminus of p100 (46). This triggers the poly-ubiquitination of p100 by the E3 ligase β -transducin repeats-containing protein (β -TrCP) which processes it into mature p52. The p52-RELB dimer can then translocate into the nucleus leading to activation of NF- κ B (44). Conversely, p105 processing is constitutive but acts in the same way to produce mature p50. However, it does not require a stimulus leading to high steady levels of both p105 and p50 (44, 47).

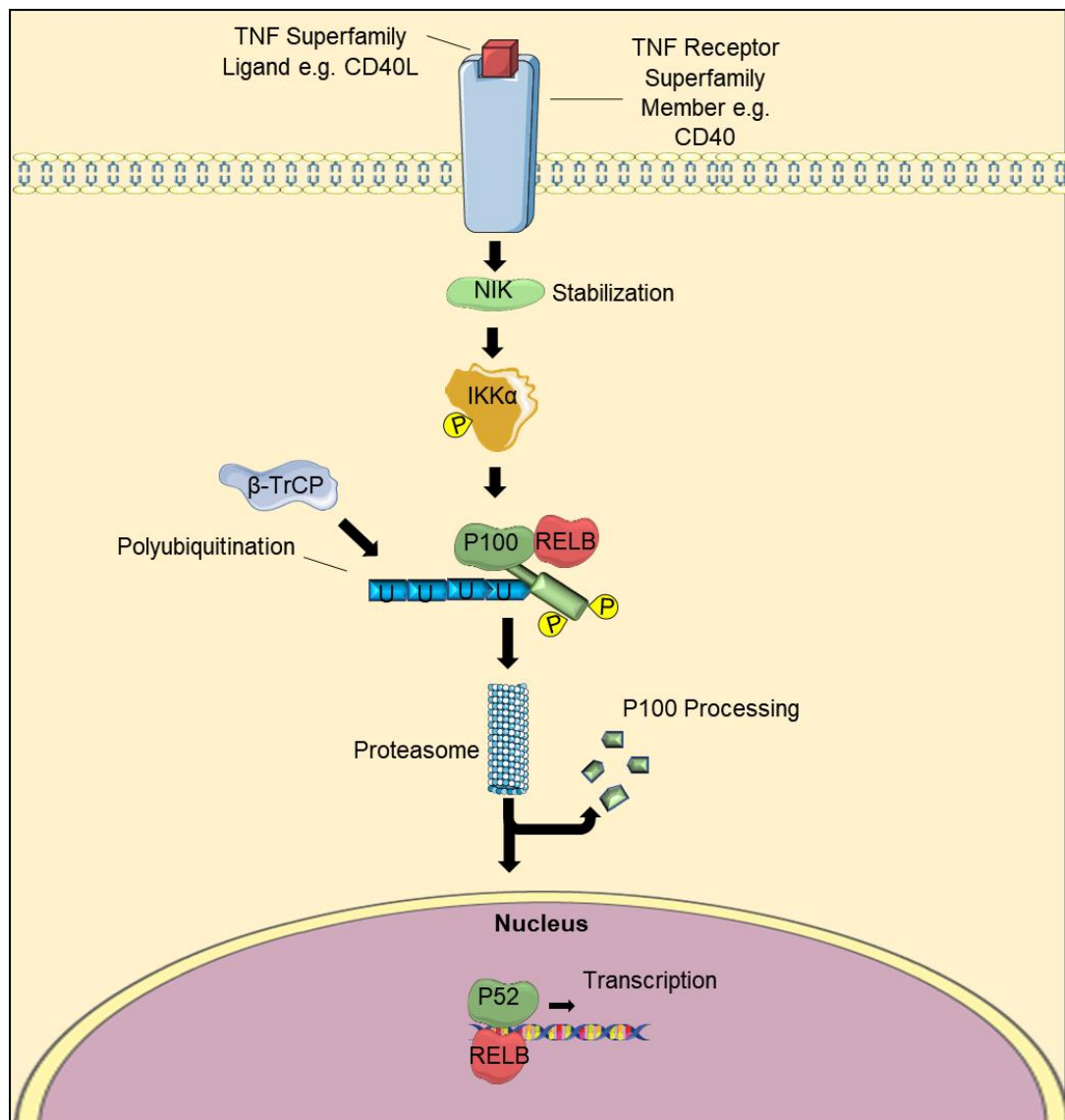


Figure 1.1.3 Non-canonical NF-κB pathway signalling. Ligand binding to a TNF superfamily member induces stabilisation of NIK which can the phosphorylate IKKα. Phosphorylated IKKα subsequently phosphorylates the C-terminal of p100 leading to ubiquitin-dependent processing into mature p52 by the proteasome. The p52-RELB dimer can then translocate into the nucleus and drive transcription of NF-κB responsive genes. The forms of the individual proteins were adapted from Servier Medical Art (<https://smart.servier.com/>), licensed under a Creative Common Attribution 3.0 Generic License.

TLR3 and TLR4 can activate IRFs through recruitment of the adapter molecule TIR-domain-containing adapter-inducing IFNβ (TRIF) which activates another member of the

TRAF family, TRAF3, catalysing the formation of a poly-ubiquitin scaffold (48) (**Figure 1.1.4**). This scaffold recruits IKK ϵ and TBK1 which phosphorylate IRF3 on its C-terminal, alleviating autoinhibition and enabling dimerization (49, 50). IRF3 can then translocate into the nucleus and bind to interferon stimulated response elements (ISREs) in the promoter leading to IFN induction (51). IFNs create a positive feedback loop in nearby cells through activation of JAK-STAT signalling, leading to the induction of interferon stimulated genes (ISGs) (52).

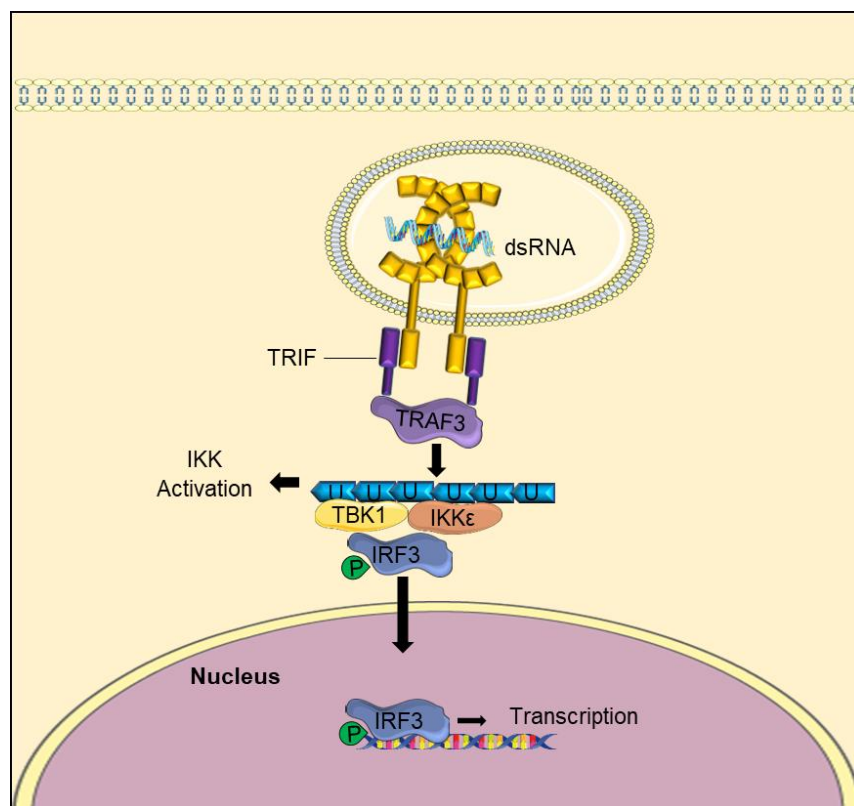


Figure 1.1.4 TRIF dependent TLR signalling pathway. Sensing of dsRNA by endosomal TLRs require the use of the TRIF adaptor protein to recruit TRAF3. TRAF3 forms a poly-ubiquitin scaffold that recruits the kinases TBK1 and IKK ϵ . These kinases subsequently phosphorylate IRF3 leading to nuclear translocation and transcription of IRF3 responsive genes. The forms of the individual proteins were adapted from Servier Medical Art (<https://smart.servier.com/>), licensed under a Creative Common Attribution 3.0 Generic License.

1.1.4 RIG-I Pathway

The RIG-I pathway detects uncapped 5'-triphosphate dsRNA within cells through its RNA-helicase-like domain and utilizes many of the same downstream signalling elements as the TLRs (53, 54) (**Figure 1.1.5**). The RIG-I PRR contains a caspase recruitment domain (CARD) which can dimerize with CARD domains on other proteins. Following ligand binding, RIG-I undergoes a conformational change exposing the N-terminal CARD domain (55). This domain interacts with the CARD domain of the adapter mitochondrial antiviral signalling protein (MAVS), which is attached to the outer mitochondrial membrane. MAVS CARD domains form prion-like aggregates, resulting in aggregates of other MAVS CARD domains (56). The MAVS proline-rich region then binds to TRAF proteins and signal transduction from this point proceeds in an analogous manner to TLR signalling, leading to NF- κ B and IRF activation (57).

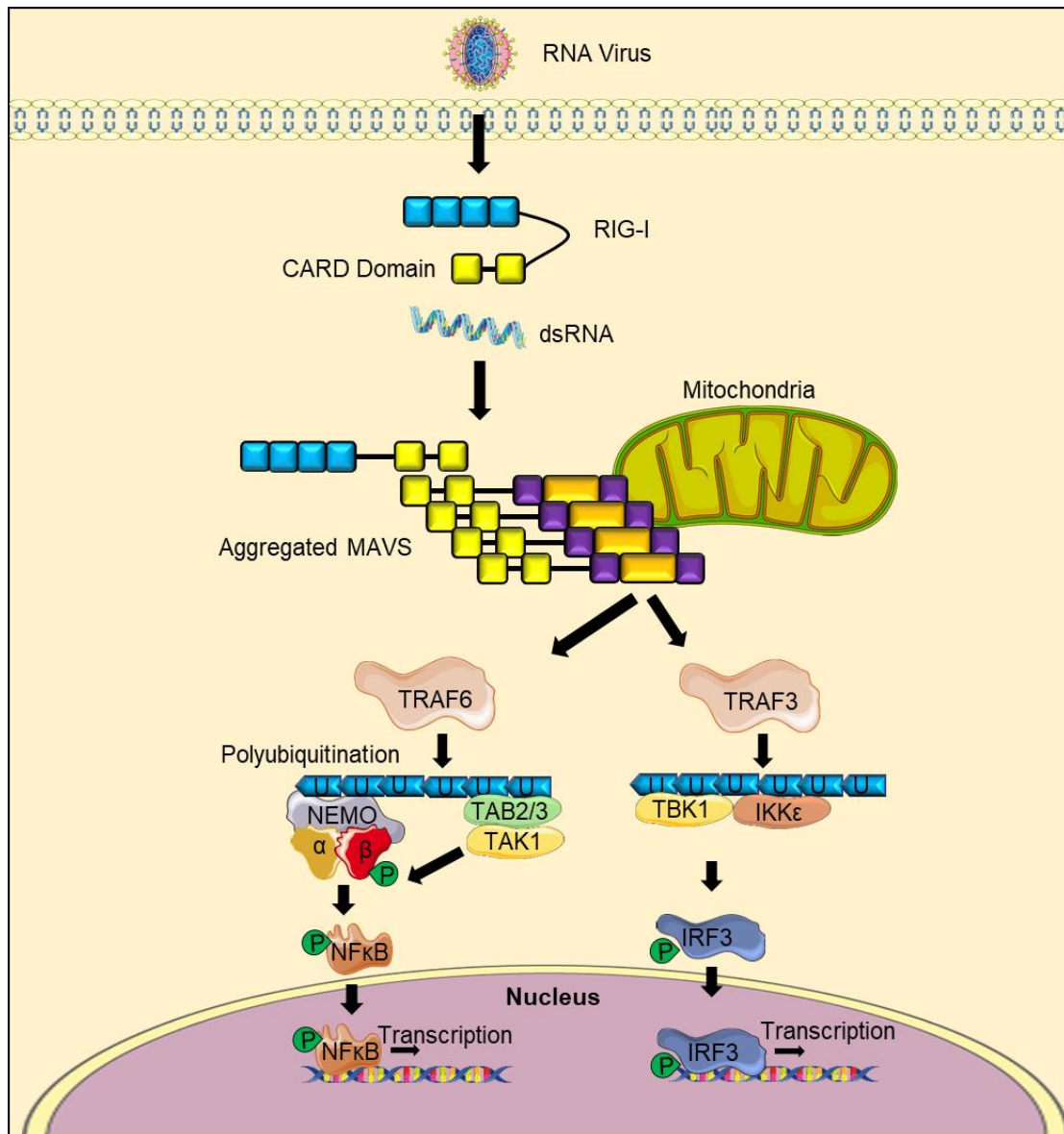


Figure 1.1.5 The RIG-I signalling pathway. Following binding of viral RNA, RIG-I undergoes a conformational change enabling a CARD-CARD domain interaction with MAVS. MAVS forms aggregates with other MAVS CARD domains and subsequently interacts with TRAF proteins to drive NF-κB and IRF signalling. The forms of the individual proteins were adapted from Servier Medical Art (<https://smart.servier.com/>), licensed under a Creative Common Attribution 3.0 Generic License.

1.1.5 TNF Induction of NF- κ B

TNF signalling is another crucial pathway involved in NF- κ B activation (**Figure 1.1.6**) and apoptosis. In most cells TNF- α signalling occurs by binding to the TNF-R1. This results in receptor trimerization and the formation of the TNFR1-associated signalling complex. The adaptor proteins receptor interacting protein 1 (RIP1) and TNF receptor associated death domain (TRADD) are recruited via their respective death domains (58). This enables the TRADD to interact with the TRAF2 adaptor protein which can then activate cellular inhibitors of apoptosis 1 and 2 (c-IAP1, c-IAP2). RIP1 is modified by lysine 63-linked (K63) ubiquitin chains directed by C-IAP1/2 which serve as a docking site for the TAB/TAK complex. c-IAP1/2 promote self-ubiquitination leading to the recruitment of the linear ubiquitin chain assembly complex (LUBAC) (59). LUBAC consists of SHANK-associated RH-domain interactor (SHARPIN), heme-oxidized IRP2 ubiquitin ligase 1 (HOIL-1L), and HOIL-1L-interacting protein (HOIP) which together form the E3 ligase complex (60). This complex generates M1-linked ubiquitin chains which act in combination with K63-linked ubiquitin chains to activate the IKK complex, thus leading to NF- κ B activation (61).

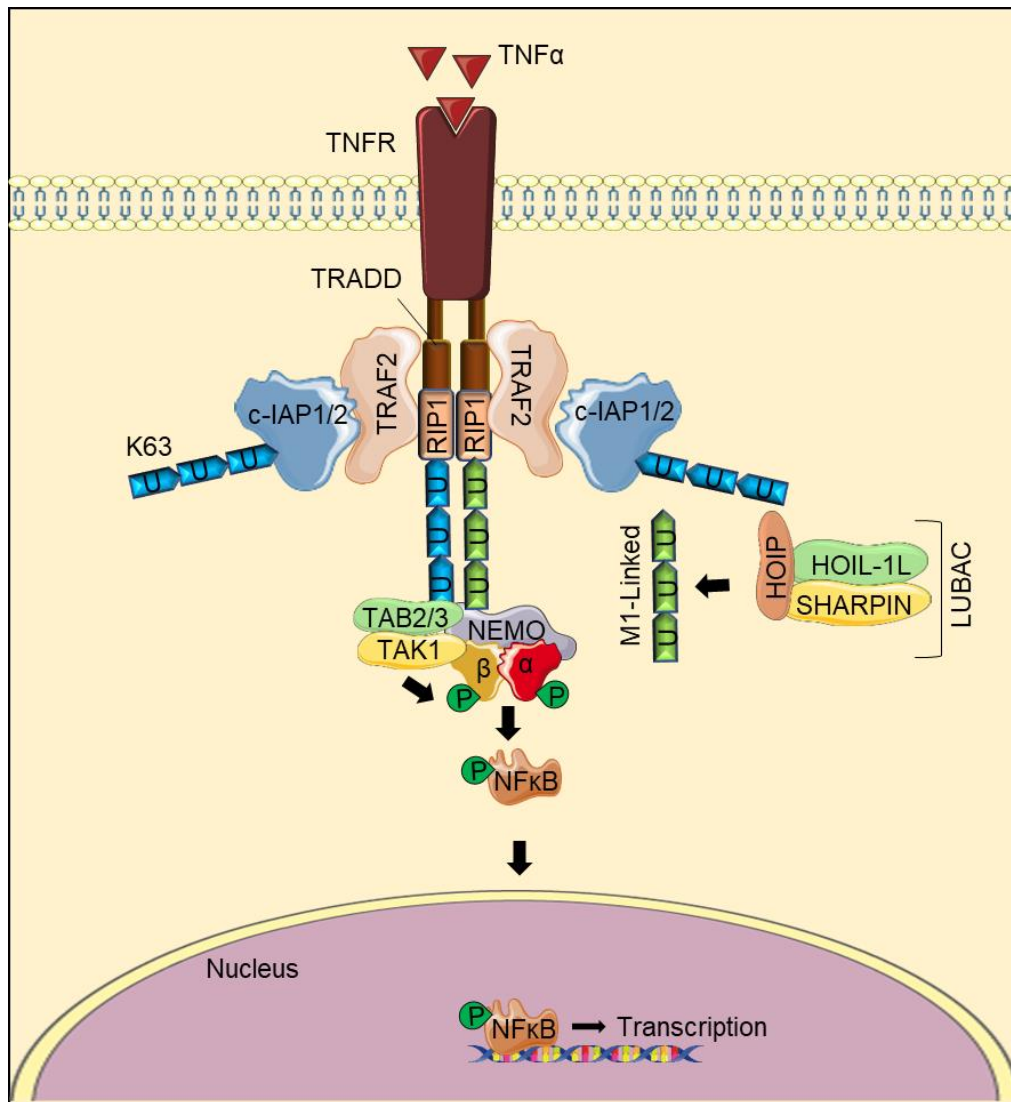


Figure 1.1.6 TNF α induced NF- κ B activation. Following TNF binding to TNFR, RIP1 and TRADD are recruited through their death domains. TRADD interacts with TRAF2 which subsequently activates c-IAP1/2. C-IAP1/2 can then poly-ubiquitinate RIP1, providing a scaffold that the TAB-TAK and IKK complexes can bind. Self-ubiquitination of c-IAP1/2 leads to the recruitment of the LUBAC complex which generates M1-linked ubiquitin chains. These ubiquitin chains also bind to NEMO, therefore complementing IKK complex activation and subsequent NF- κ B activation. The forms of the individual proteins were adapted from Servier Medical Art (<https://smart.servier.com/>), licensed under a Creative Common Attribution 3.0 Generic License.

1.1.6 cGAS-STING Pathway

Molecular phylogenetic evidence suggests that the cGAS-STING pathway evolved around 600 million years ago in a common ancestor of humans and cnidarians, though its antiviral function appears to be a more recent development (62). cGAS was first identified in 2013 by Sun et al. and is now recognised as a crucial element in the host DNA immune response pathway (63). cGAS acts in conjunction with the downstream effector, stimulator of interferon genes (STING), which was discovered in 2008 (64). Together, they respond to dsDNA to induce the production of interferons and proinflammatory cytokines via activation of IRF and NF- κ B family transcription factors (9).

A conformational change is triggered upon direct binding of cGAS to dsDNA, inducing catalytic activity of this enzyme which converts adenosine triphosphate (ATP) and guanosine triphosphate (GTP) into a second cyclic messenger called 2'3' cyclic GMP-AMP (cGAMP). This binds to STING with a high affinity compared to cGAMP molecules with other combinations of phosphodiester linkages (65, 66). The extent of cGAS activation is influenced by several factors. The length of the DNA bound to cGAS has a significant impact on the enzymatic activity with short dsDNA (< 20 bp) inducing activation less efficiently (67). Large cGAS complex formation is enhanced by GTPase-activating protein SH3 domain-binding protein 1 (G3BP1). G3BP1 enhances cGAS oligomerisation, promoting its DNA binding ability, and is crucial for mediating DNA sensing independent of DNA length (68). The E3-ligase, tripartite motif 56 (TRIM56), has been shown to induce mono-ubiquitination of cGAS at K335 in the amino-terminal regulatory domain. TRIM56 mono-ubiquitination of cGAS has been shown to increase DNA binding and promotes cGAS dimerization (69). While TRIM56 was previously believed to also poly-ubiquitinate STING in response to dsDNA detection by cGAS (70),

more recent research disputed this finding using two-step co-immunoprecipitation. This method involves two rounds of antibody purification prior to immunoblotting and is designed to minimise coprecipitating contaminants. Sciuto et al (71) provides a detailed explanation and protocol for this type of immunoprecipitation. Using a two-step co-immunoprecipitation method, it was demonstrated that TRIM56 did not directly interact with STING and is likely ubiquitinating another element co-purifying with this adapter. These researchers observed a similar effect for tripartite motif 32 (TRIM32), previously thought to drive direct activation of STING by poly-ubiquitination (72, 73). TRAF6 has also been shown to poly-ubiquitinate cGAS and positively regulate its activation and downstream induction of IFNs (74). However, the mechanistic requirement of this poly-ubiquitination remains to be fully elucidated.

STING resides at the endoplasmic reticulum (ER) in an inactive state through interactions with the Ca^{2+} sensor, stromal interaction molecule 1 (STIM1). This interaction is disrupted in response to a conformational change in STING induced by cGAMP binding, resulting in STING trafficking through the ER Golgi intermediate compartment (ERGIC) (75, 76). Recently, Cryo-electron microscopy revealed the mechanism by which cGAMP activates STING. Binding of cGAMP to the ligand binding domain of STING leads to a 180° conformational change relative to the transmembrane domain, enabling STING oligomerisation and translocation (77). This translocation of STING enables the C-terminal tail to insert into a groove in TBK1 located between the scaffold and dimerization domain and the kinase domain of adjacent TBK1 subunits. STING oligomerisation is essential for activation of TBK1 as it enables trans-autophosphorylation of adjacent TBK1 molecules, a process that would not be otherwise feasible due to geometric molecular constraints (78, 79). Phosphorylation of the C-terminal tail of STING by TBK1 then provides a docking site for IRF3 where it becomes

phosphorylated by TBK1 (80). Activated IRF3 dimers then translocate into the nucleus upon activation where they bind target gene promoters and activate transcription of genes including type I interferons (**Figure 1.1.7**) (81).

While robust IRF activation by the cGAS-STING signalling pathway is well-characterised, NF- κ B is also activated by the system and plays an important role in regulating gene expression in the DNA sensing response (64). However, the molecular events leading up the activation of NF- κ B in this pathway remain poorly understood. A number of proteins are believed to be involved in linking the two activation events including the E3 ligases TRIM32, TRIM56, TRAF6, the kinases TBK1, IKK α and IKK β , and the ubiquitin binding protein NF-kappa-B essential modulator (NEMO) (9, 82, 83). The involvement of TRAF family-generated poly-ubiquitin chains in NF- κ B signalling has been well-documented, particularly in the TLR pathways (84). Studies also demonstrate a role for TRAF6 in the activation of NF- κ B via the cGAS-STING pathway in response to dsDNA, whereby overexpression of TRAF6 in HEK293T cells results in enhanced NF- κ B promoter activity, likely through the ubiquitination of STING (9). Similarly, TRAF3 overexpression enhances non-canonical activation of NF- κ B via the NF- κ B inducing kinase (NIK)-IKK α axis (9).

While TRAF-family proteins are required in most known cases of NF- κ B activation, recent research by Fang et al. describes a mechanism by which the cGAS-STING system utilises TRIM32/56 generated poly-ubiquitin chains to activate the IKK complex via NEMO (82). However, lower levels of NF- κ B activation were reported in comparison to TRAF6-mediated signalling, and IKK β but not IKK α phosphorylation was required for this pathway. While the exact mechanisms surrounding STING-mediated NF- κ B activation remain unclear, the mechanism of STING-mediated IRF activation seems clear

and the robust activation of both by the cGAS-STING system appears to be essential to efficiently eliminate invading pathogens displaying dsDNA as a PAMP.

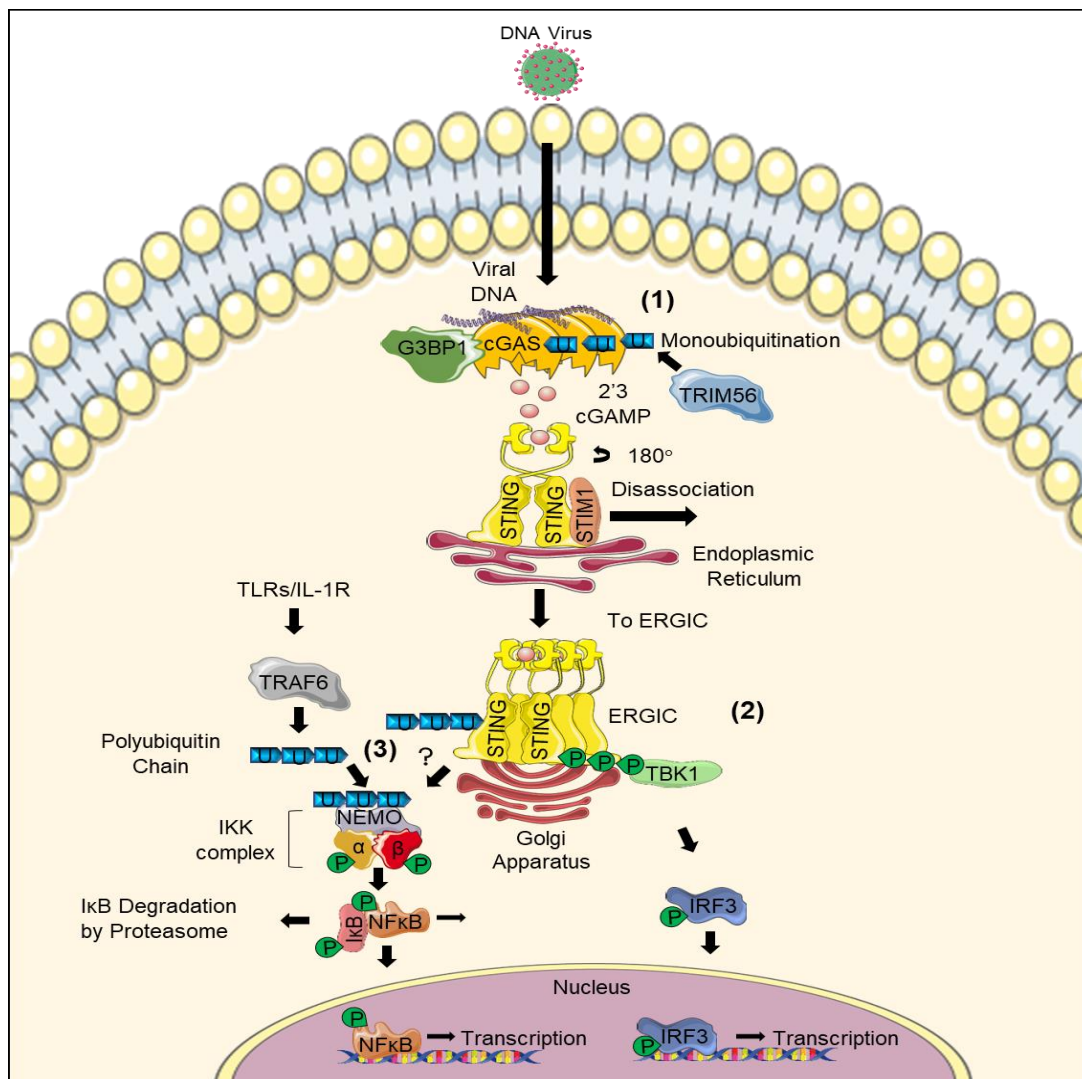


Figure 1.1.7 Sensing of DNA viruses by the cGAS-STING Pathway. When a DNA virus enters a cell, it un-coats, releasing the DNA genome. (1) The cytosolic DNA sensor cGAS binds to viral DNA and becomes mono-ubiquitinated by TRIM56. G3BP1 also interacts with cGAS to form large cGAS complexes. cGAS catalyses the conversion of ATP and GTP to form 2'3' cGAMP, a cyclic second messenger, which binds into a groove in the ligand binding domain between STING dimers. Ligand binding induces a 180° rotation of the binding domain resulting in STING activation. E3 ligases, such as TRAF6 or TRIM32, poly-ubiquitinate components in complex with STING. (2) Activated STING disassociates from STIM1 and translocates from the endoplasmic reticulum to the ERGIC where it oligomerizes and interacts with TBK1,

enabling phosphorylation of IRF3. Both TBK1 and IKK ϵ phosphorylate IRF3. Phosphorylated, dimeric IRF3 migrates into the nucleus where it can activate the transcription of genes like type I interferons. (3) The exact mechanism leading to STING-mediated NF- κ B activation remains unclear but may involve ubiquitin chains generated by specific E3 ligases, as is the case with TRAF6 in the TLR/IL-1R pathways. NEMO binds to ubiquitin chains triggering phosphorylation of IKK α and IKK β . IKK β phosphorylates the NF- κ B inhibitory protein, I κ B, leading to its proteasomal degradation. NF- κ B then migrates into the nucleus and activates transcription of target genes encoding proinflammatory cytokines. The forms of the individual proteins were adapted from Servier Medical Art (<https://smart.servier.com/>), licensed under a Creative Common Attribution 3.0 Generic License.

1.1.7 Anti-viral Effector Mechanisms

Whilst the previous sections demonstrate how pathogens can be sensed by the innate immune system; this section outlines how invading viral pathogens are eliminated following detection. In order for activated innate and adaptive immune cells to eradicate viral infections, appropriate effector mechanisms must be employed.

Type I IFNs, including IFN α/β , released from cells following PRR detection of viral pathogens bind to IFNAR receptors on neighbouring cells and immune cells, triggering activation of JAK-STAT signalling leading to the upregulation of hundreds of ISGs (50). ISG expression leads to a variety of anti-viral effects that make cellular environments unfavourable for replication. For example, ISG NCOA7 restricts viral entry by binding the vacuolar H⁺-ATPase to promote degradation of endocytosed cargo through acidification (85). ISGs can also promote increased expression of major histocompatibility complex (MHC) molecules on the surface of cells (86).

MHC-I molecules are found on all nucleated cells and present intracellular antigen to immune cells (87). NK cells are innate immune cells that can recognize low levels of

surface MHC-I caused by viral stress, leading to the release of cytotoxic substances such as granzymes and perforins which kill virally infected cells (88). As NK cells express CD16 receptor, they can interact with antibody coated cells and destroy them through a process known as antibody-dependent cellular cytotoxicity (89). They also produce IFN- γ which can activate macrophages (90) and plays a role in CD4⁺ T-helper cell differentiation during the adaptive immune response (91).

Apoptotic infected cells can be removed by macrophages in a process known as phagocytosis (92). Apoptosis is important for clearance of virally infected cells and is triggered by binding of TNF α , Fas ligand, or TNF related apoptosis-inducing ligand (TRAIL) to their respective receptor. They induce apoptosis through a signalling cascade that leads to the formation of the death-inducing signalling complex (DISC), resulting in the activation of executioner caspases such as caspase-3 and subsequent cell death (93, 94). The contents of phagocytosed infected cells are fused with lysosomes and degraded before being presented on the surface of macrophages in complex with MHC-II (95). Macrophages also secrete proinflammatory cytokines in response to detection of viral pathogens.

Proinflammatory cytokines and chemokines induced by innate immunity play a crucial role in the antiviral response and guide adaptive immune responses. CXCL8 released by infected cells is a major chemoattractant for neutrophils, another important class of innate immune cells that phagocytose, degranulate or produce neutrophil extracellular traps (NETs)(web-like structures composed of nuclear components and granular proteins), to destroy invading pathogens (96, 97). Proinflammatory cytokines, including TNF and IL-1, assist in leukocyte extravasation by promoting vasodilation and by increasing the number of adhesion molecules on endothelial cells (98, 99).

While both macrophages and dendritic cells (DCs) can present antigen to T cells, DCs are particularly adept at this as they can present phagocytosed viral peptides on MHC-I molecules in a process known as antigen cross presentation (100, 101). DCs then migrate to the lymph nodes to activate naïve CD8⁺ T cells. Mature CD8⁺ T-cells cells can subsequently travel to the site of infection and release a cytotoxic payload which induces cell lysis or apoptosis of infected cells (102, 103).

DCs can also present antigen via MHC-II to initiate a CD4⁺ T cell response. CD4⁺ T cells regulate various aspects of the adaptive immune response including promotion of CD8⁺ T cell activation and humoral immunity (104). Distinct subsets of CD4⁺ T cells (Th1, Th2, Th9, Th17, Th22, regulatory T cells, and follicular helper T cells) are polarized by innate immune cytokines (e.g. IL-12 and IFN- γ induce Th1 cells). These Th subsets release specific arrays of cytokines that can have either pro- or anti-inflammatory functions, survival or protective functions (105).

Humoral immunity plays a key role in eliminating viral pathogens. B cells are covered in one of a vast repertoire of membrane bound antigen specific receptors known as B cell receptors (BCRs). They internalize the extracellular antigen which is degraded and presented on the surface in complex with MHC-II for presentation to CD4⁺ T cells (106). Presentation of antigen to a CD4⁺ T cell results in B cell activation, and subsequent differentiation into either plasma or memory B cells. Plasma cells release antibodies into the extracellular environment, which can affect the pathogens in a variety of ways (107). The classical complement pathway can be activated by antibodies leading to the formation of membrane attack complexes and the subsequent lysis of infected cells (108). Antibodies can opsonise cells, thus aiding phagocytosis, and they can neutralize antigens by binding to their surface (109). Finally, they enable NK cells to carry out antibody-dependent cell-mediated cytotoxicity (89).

1.2 Poxviruses

1.2.1 Overview

Poxviruses are large viruses that contain a complex dsDNA genome, protected by enveloped capsid, and can only replicate within the cytoplasm of cells (110, 111). They are grouped into two subfamilies, Chordopoxvirinae and Entomopoxvirinae, which infect vertebrates and invertebrates, respectively. Chordopoxvirinae can be grouped into 10 genera (**Figure 1.2.1**): orthopoxviruses, molluscipoxviruses, leporipoxviruses, yatapoxviruses, cervidpoxviruses, capripoxviruses, avipoxviruses, suipoxviruses, parapoxviruses and crocodylipoxviruses (112).

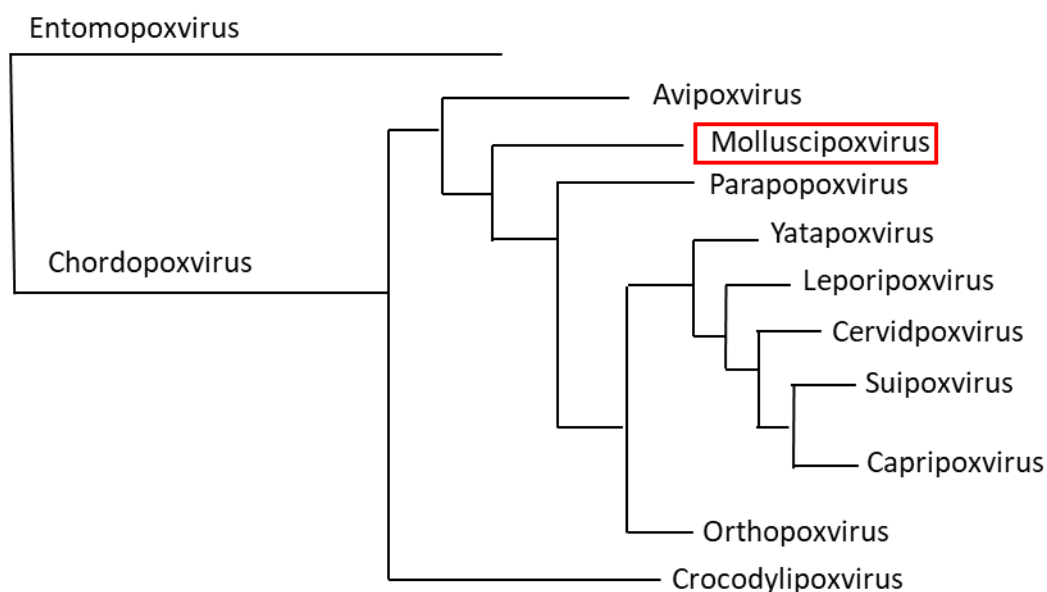


Figure 1.2.1 Phylogenetic tree of poxviruses. This phylogenetic prediction is based on analysis performed by Hendrickson et al. (113). The location of MCV within the tree is highlighted in red.

Orthopoxviruses have a formidable reputation, largely due to the devastation caused by the historic Variola virus (VARV), the causative agent of smallpox, which killed nearly half a billion people in the 20th century (114). However, the World Health Organisation

(WHO) led a global immunization campaign resulting in smallpox being declared eradicated in 1980 (115). This was achieved using another member of the orthopoxvirus family, Vaccinia virus (VACV), which provides cross-protection against VARV and Monkeypox virus (MPX) . In 2022, a resurgence of MPX, a disease that is zoonic in origin, sparked global fear. The decrease in vaccination efforts following the eradication of smallpox is believed to have contributed to this outbreak (116).

Molluscum contagiosum virus (MCV) accounts for most poxvirus infections in humans we see today. In 2010, over 122 million cases were recorded globally (117). However, infection by MCV is usually mild and self-limiting with treatment, in otherwise healthy patients, often only requested for cosmetic purposes due the ‘unsightly’ lesions this virus generates on the skin (118). Interestingly, this virus has a remarkable ability to evade human immunity. MCV infection usually persists for months, and in some cases years, before being cleared by the host immune response (119-121). This is largely because poxviruses encode vast arrays of immunomodulatory proteins (110, 122, 123). As MCV appears to have an exclusive tropism for human infection, it has become proficient in evading human immunity due to constant selective pressure. Investigating innate immune antagonists encoded by this human-adapted poxvirus may provide valuable insight into previously unknown mechanisms of human immunity and highlight novel targets for therapeutic intervention

1.2.2 Poxvirus Genome & Morphological Characteristics

Poxviruses are among the largest animal viruses known, ranging between 200nm-400nm in length and appear as oval or brick shaped (124). VACV is the most extensively studied member of the poxvirus family and as result, most knowledge about poxvirus characteristics is based on it. There are two types of virions produced by infected cells,

mature virions (MVs) and enveloped virions (EVs) (125). Poxvirus virions contain over 100 polypeptides and ultrastructural analysis using electron microscopy has revealed they are composed of four distinct structures: the core, lateral body, membrane (**Figure 1.2.2**), and envelope (124, 126). The core contains genomic material along with viral proteins, many of which are required for transcription (125). MVs have a lipoprotein bilayer membrane, approximately 50nm thick, wrapped around the nucleoprotein core. The outer surface of the membrane contains randomly arranged surface tubule elements, up to 100nm in length (124). Two amorphous lateral bodies flank the core, potentially serving as immunomodulatory delivery packets (127). The envelope is a second lipoprotein bilayer acquired by EVs, containing proteins distinct from MVs (A33, A34, A56, B5, K2, F13), and encapsulates the nucleoprotein core, associated lateral bodies and the membrane (124, 128). While the more abundant MVs appear to be involved in transmission between hosts, EVs appear to have a role in viral spread within the infected host (125).

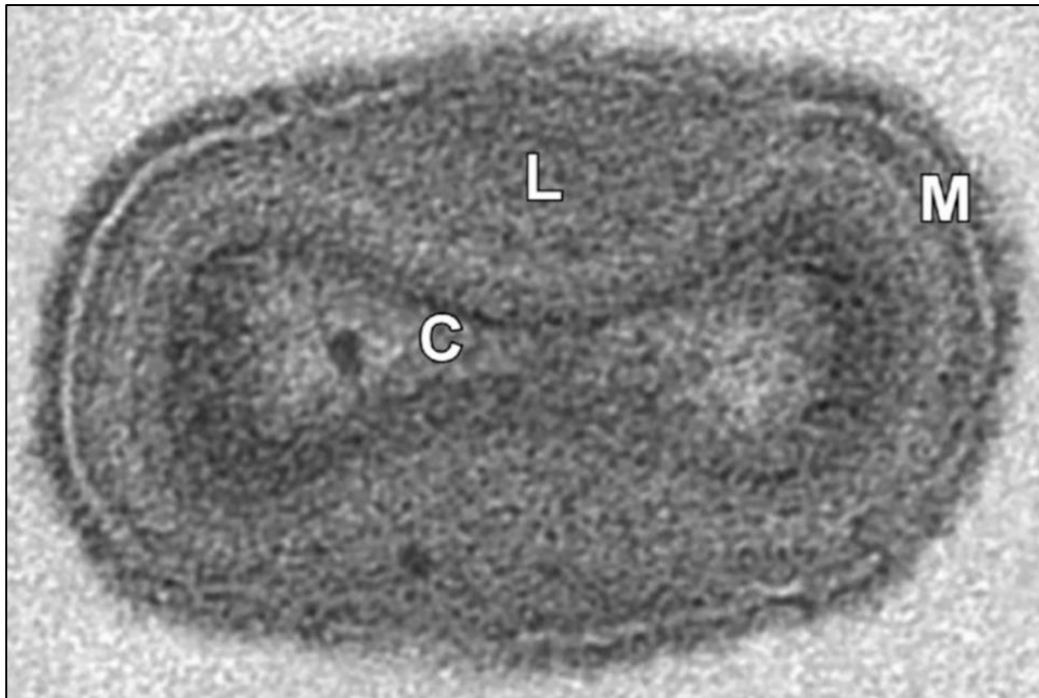


Figure 1.2.2 Electron micrograph of vaccinia virus. This image shows 3 subviral structures of vaccinia virus; the core (C), lateral bodies (L) and the membrane (M). This image was reproduced in accordance with a CC BY 4.0 licence. Original image by Bidgood SR, 2019 (129).

Poxvirus mutation rates are relatively slow compared to RNA viruses, hindering their ability to evade immune defences by antigenic variation (130). Consequently, slow mutation rates have enabled poxviruses to support much larger genomes and thus have been able to accumulate large numbers of genes that can evade and subvert almost every aspect of antiviral immune signalling. Poxviruses have a large DNA genome ranging from 130 to 360 kbp that can encode over 200 ORFs and contain an incompletely base-paired A+T rich hairpin loop at each end which connects the two DNA strands (130, 131). Around 50 of these genes are present in all sequenced poxviruses and approximately 90 genes are common to all Chordopoxviruses (131). These genes tend to be centrally

located in the genome and encode proteins involved in key processes such as replication, transcription and virion assembly (122, 131). In contrast, genes involved in perturbing host immune functions tend to be located at the terminal ends of the genome and generally have a lower sequence identity to orthologs in other poxviruses (131). The reason for this disparity in the localisation of genes is likely due to the fact that replication is initiated at the termini, and because repeat sequences in the termini encourage homologous recombination (132). Early research has shown that poxviruses can combine with non-homologous DNA sequences in vivo resulting in genetic variation (133), along with mutation of pre-existing viral sequences (134), both of which likely drive evolution of immunoregulators. Furthermore, poxviruses have been shown to transiently expand their genome in localized regions in response to selective pressure (135). The study provides evidence that genomic ‘accordions’ promote recombination and then subsequently contract (alleviating the toll of DNA replication on a larger genome), thus facilitating genetic drift and enabling rapid viral evolution in the absence of a high mutation rate.

1.2.3 Poxvirus Replication

Most of what we know about poxvirus replication is based on VACV studies. Whilst little is known about specific MCV replication, this virus shares 105 orthologs with VACV, many of which correspond to genes involved in replication (136). Therefore, it is expected that MCV replication occurs in largely the same way. Poxvirus replication occurs in the cytoplasm, and as such, the process begins with binding the target cell. Since MVs and EVs have distinct viral epitopes, they have different mechanisms for attachment to host cells (137). However, the exact mechanisms remain unclear. In VACV, cell surface attachment of MVs to glycosaminoglycans (GAG) is mediated by three proteins, H3, D8, and A27 (138-140). More specifically, H3 and A27 have been shown to bind to heparan sulphate, whereas D8 has been shown to bind chondroitin sulphate. GAG-

independent binding has also been described and is potentially mediated between the viral A26 protein and laminin, an extracellular matrix protein (141), or between the viral L1 protein and unknown target (142). Regarding EVs, little is known about cellular attachment factors. However, it has been demonstrated that the soluble protein growth arrest-specific 6 (Gas6) enhances EV infection while having little effect on MV infection, but the mechanism behind this has not yet been elucidated (143).

After attaching to the host cell, the virion can be taken up into the cytoplasm by macropinocytosis, a form of endocytosis involving changes in actin dynamics and extrusion of plasma membrane ruffles (144, 145). Cellular attachment of MVs induce a complex signalling pathway involving the GTPase Rac1, the kinase PAK1 and other factors, due to the expression of phosphatidylserine on the membrane, likely enabling the virion to make use of apoptotic mimicry to enter the cell (146). Another method of entry that has been described involves membrane fusion facilitated by lipid rafts, cholesterol-rich regions of the host cell membrane (147). Viral membrane fusion has been shown to require a multiprotein entry fusion complex made up of 11 proteins (A21, A28, G3, H2, O3, A16, F9, G9, J5, L1 and L5), embedded in the MV membrane (148).

Poxviruses are unique because they replicate exclusively in the cytoplasm of cells and as such, they encode all the necessary machinery for replication (111). The transcription system in poxvirus virions produces mRNAs that are capped, methylated, and polyadenylated. Regulation of transcription in poxviruses involves activation of early and late genes, which as their names suggest, are transcribed in a temporally distinct pattern (128). Upon entering the cytoplasm, the virus cores are liberated from the membrane and transported to transcription sites on microtubules, and within 20 minutes, mRNA production can be seen (128, 149). Transcription is initiated at an A/T rich core region - 13 to -25bp upstream of the RNA start site (150). Typically, transcription starts with a

purine 12 to 17 nucleotides downstream of the core region. The TTTTNT (5TNT) sequence (UUUUUNU or 5UNU when the non-template strand is transcribed to RNA) is present near the end of most VACV early mRNAs, resulting in termination of transcription (128).

A large number of early genes are transcribed shortly after infection, and these encode the genes necessary for genome expression and replication (128). Initiation of transcription is mediated by a number of proteins. RNA polymerase associated factor (RAP94) is required for transcription of early promoter templates and interacts the vaccinia early transcription factor (VETF) (151-153). Binding assays suggested that VETF may also be involved in the recruitment of RNA polymerase to the promoter (154). On DNA templates containing an early promoter and the T5NT sequence, transcription can be initiated and terminated by a complex of RNA polymerase, RAP94, VETF, capping enzyme, and nucleotide phosphohydrolase I (NPH I), with great precision (128). In an adenosine triphosphate (ATP)-dependent process, the capping enzyme and NPH I are required to release nascent mRNA with a U5NU sequence from the transcription complex (128, 155, 156). Capping and polyadenylation are crucial processes that help maintain the integrity of the viral mRNA transcripts, as in eukaryotic mRNAs. When the nascent RNA chains are around 30 nucleotides long, capping is mediated by a 127-kD capping enzyme heterodimer during the transcription process (128, 157, 158). This cap protects the mRNA against degradation by exonucleases (159). Polyadenylation, which makes the mRNA more stable through addition of a poly(A) tail (160), is achieved through an enzyme heterodimer composed of virus encoded 55 (VP55) and 39 (VP39) (161).

An intermediate class of genes are expressed following the early genes and peak approximately 2 hours post infection. The tetranucleotide TAAAT sequence, which like

in early gene transcription is contained in an A/T rich region, serves as the initiator element for intermediate genes (128). There are approximately 55 genes belonging to the intermediate class, and they mainly encode proteins involved in DNA binding/packaging and late transcription factors (162).

Late gene transcription follows directly after the immediate genes and continues until the end of the viral replication process. Again, the promoter region is A/T rich but differs from the early and intermediate promoters due to the presence of a core motif of approximately 20bp containing some consecutive T residues. This is separated by roughly 6bp from the conserved TAAAT sequence which enables initiation of transcription (128). The late genes encode proteins involved in morphogenesis, MV membrane proteins and early transcription factors (162).

Assembly of the virion is one of the last steps in the viral replication process. The early phases of virion assembly take place in dense cytoplasmic inclusions, originally called Guarnieri bodies, but now known as viral factories (163). Crescent-shaped cupules appear in these factories with the membrane consisting of a lattice scaffold formed by trimers of a 65kDa protein D13, interacting with the N-terminal of another viral protein, A17 (128, 164). While the exact events in immature virion (IV) formation remain to be elucidated, it has been demonstrated that before the crescents completely seal, a dense mass of nucleoprotein containing the genome enters the immature envelope (128). It has been shown that the ATPase, A32, the I6 telomere-binding protein, and the membrane protein, A13, all play a role in DNA packaging, as their absence leads to packaging defects (165-167). Following this, the lattice scaffold is lost leading to internal reorganisation and conversion to an oval or brick shaped particle (128). The D13 scaffold dismantling correlates with proteolytic cleavage of A17 by a viral protease I7, suggesting that these interactions play a key role in regulation of the disassembly process (128, 164). Once the

assembly process is complete the virion is referred to as a MV and is now able to infect new hosts following release from the cell.

Some single membrane MVs remain in the cytoplasm or in inclusions prior to release through cell lysis (148). Others acquire a double membrane derived from modified trans-Golgi or endosome to become wrapped virions. MVs use host microtubules to exit the viral factories and travel to these sites for wrapping, likely facilitated by A36 and F12 proteins which interact with microtubule motor proteins (128). Following wrapping, these virions are transported to the cell surface on microtubules where the viral membrane fuses with the plasma membrane of the host cell, releasing the EV. The more stable MVs are thought to facilitate transmission between hosts, whereas the more fragile EVs are specialised for exiting the intact host cell and facilitate transmission from one cell to another (148). The EVs can be released into the extracellular fluid or remain at the cell surface through association with the tips of actin-containing protrusions, with the latter being the predominant method of cell-to-cell transmission observed (148, 168, 169).

This is the general pattern of replication observed in poxviruses. However, this process is predominantly modelled on VACV replication. Identification of differences between MCV and other orthopoxviral replication systems remains hindered by the lack of an established MCV culture system (170). Future development of such a system would provide a platform for investigating unique differences in replication that lead to the narrow tropism of this virus.

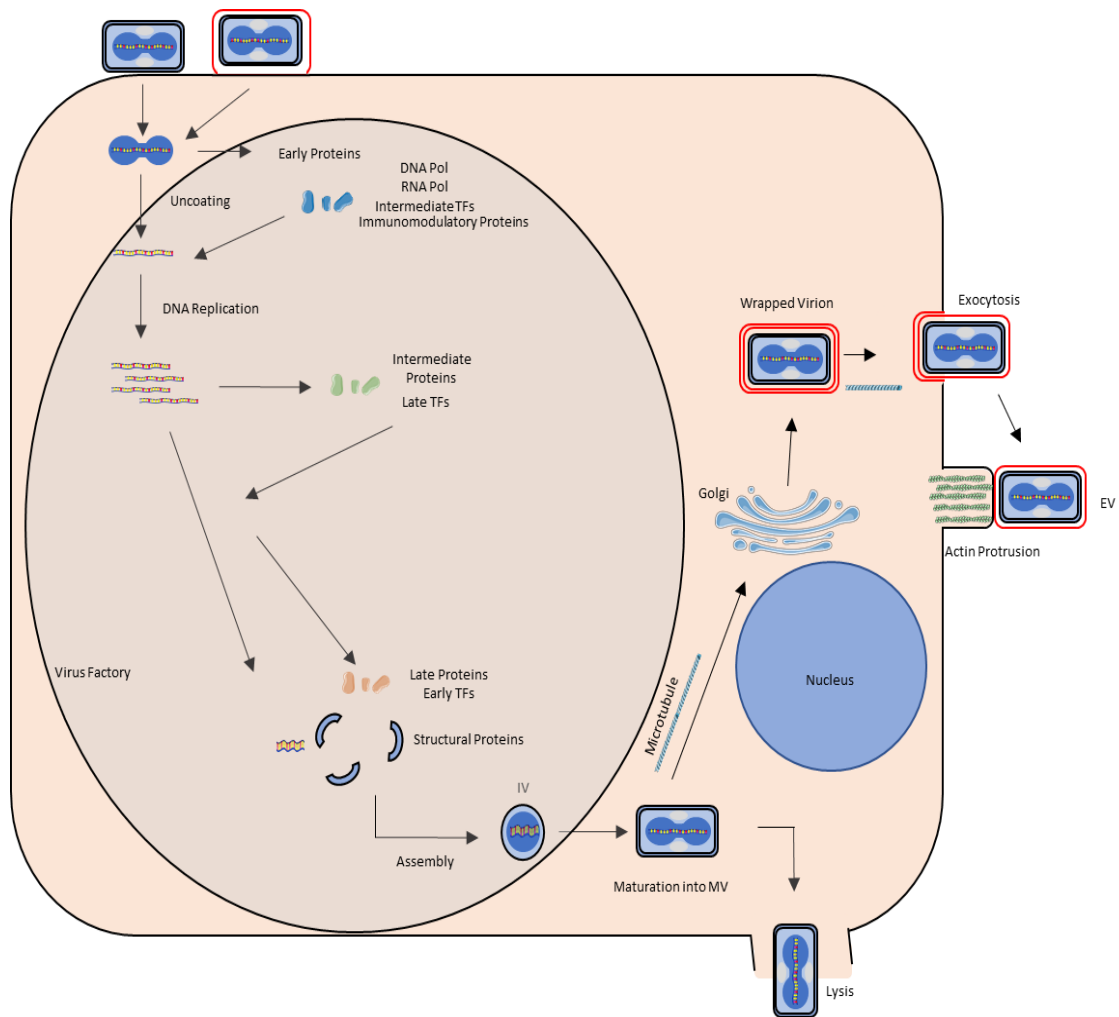


Figure 1.2.3 The Poxvirus Replication Cycle. Mature virions attach to the cell and are internalized through macropinocytosis. Enveloped virions uncoat and the inner membrane fuses with the cell surface. The nucleocapsid core is released into the cytoplasm. The viral core synthesises early mRNAs which are translated into proteins including host-modulating factors, core-associated enzymes, and factors required for DNA replication and intermediate transcription. The core uncoats and the DNA is replicated. The DNA is transcribed into mRNAs encoding proteins and late transcription factors. The late genes are then transcribed and translated into late proteins such as those involved in morphogenesis, along with early transcription factors. An assembled immature virion formed following packaging of concatemeric DNA and the closure of the crescent-shaped cupules. The virion matures and is now an infectious particle that can exit the cell through cell lysis. Alternatively, the MV is transported on microtubules where it can be wrapped by modified trans-Golgi and endosomal cisternae. The wrapped virion is then transported to the cell membrane

where it is exocytosed. The EV can associate with actin-containing protrusions to facilitate cell-to-cell transmission. The individual elements were obtained from Servier Medical Art (<https://smart.servier.com/>), licensed under a Creative Commons Attribution 3.0 Generic License.

1.2.4 Poxvirus Sensing

The innate immune response is the first line of defence against viral infection and also plays an important role in the establishment of an adaptive immune response. Poxviruses are recognized by DNA-sensing apparatus in cells such as cGAS, TLR9, AIM2, IFI16, DNA-dependent protein kinase (DNA-PK) and RNA polymerase III (171). Following sensing, immune signal transduction leads the production defence molecules including type I IFNs, proinflammatory cytokines and chemokines.

cGAS is a crucial sensor of cytosolic DNA in a wide variety of cells (63) and has been described in detail in section 1.1.6. Briefly, cGAS binds to dsDNA and catalyses the conversion of ATP and GTP to form a second cyclic messenger known as 2'3'-cGAMP. This binds to the adaptor molecule STING, inducing a conformational change leading to its trafficking through the ERGIC. STING recruits the kinase TBK1 which phosphorylates IRF3. Phosphorylated IRF3 can then migrate into the nucleus and activate transcription of type I interferons and establish an antiviral state (110).

TLR9 is the only known TLR DNA sensor. TLR9 recognizes unmethylated CpG DNA which is common to bacterial and viral genomes (26). It recruits the adaptor molecule MyD88 and induces NF- κ B activation through IRAK recruitment and TRAF6 generation of poly-ubiquitin scaffolds as described in detail in section 1.1.3 (29). TLR9 also activates IRF7 in plasmacytoid dendritic cells, a dendritic cell subset specialized in producing large amounts of type I interferon in response to TLR activation (172). In these cells IRF7 is constitutively expressed and forms a complex with MyD88, IRAK1 and IRAK4.

Following ligand binding to TLR9, IRF7 is phosphorylated by IRAK1, leading to its translocation into the nucleus and induction of type one IFNs (173).

The AIM2 inflammasome, a member of the PYHIN family, is present in a wide range of cell types and senses cytosolic DNA leading to the activation of the inflammasome pathway (174, 175). This pathway produces proinflammatory cytokines and aids the clearance of infected cells through pyroptosis (a lytic form of cell death). AIM2 binds to DNA through its HIN200 domain and recruits Apoptosis-associated speck-like protein containing a CARD (ASC) through its CARD domain leading to ASC oligomerization. ASC recruits and activates caspase-1 which can then cleave pro-IL-1 β and pro-IL-18 and gasdermin D into their active forms. Gasdermin D forms pores in the plasma membrane resulting in pyroptosis (176).

IFI16, another member of the PYHIN family, is mainly localized in the nucleus but can shuttle into the cytosol (177). DNA binding can lead to the activation of the inflammasome pathway through ASC and caspase-1 (178). It has also been shown to bind to a 70 bp dsDNA fragment from the VACV genome and induce IFN β (179). IFI16 has been shown to act in co-operation with cGAS to promote STING phosphorylation and translocation in human monocytes and keratinocytes (180, 181).

DNA-dependent protein kinase (DNA-PK) is a heterotrimeric complex that binds to dsDNA leading to an IRF3-dependent response. In VACV infected fibroblasts, DNA-PK induction of IRF3 was shown to rely on STING and TBK1 (182). However, an exact mechanism remains to be elucidated.

RNA polymerase III has also been described as a cytosolic DNA sensor, albeit in a more indirect manner. This enzyme uses AT-rich dsDNA as a template and transcribes it into

AU-rich dsRNA (183, 184). The dsRNA can then serve as a RIG-I agonist to activate IRF and NF- κ B signalling pathways, as described in section 1.1.4.

1.2.5 Poxvirus Immune Evasion

To assist evasion and subversion of the immune system, poxviruses encode arrays of immunomodulatory proteins that act on various signalling pathways in cells (**Figure 1.2.5**). Modified vaccinia virus Ankara (MVA) is an attenuated form of vaccinia virus that was developed to increase safety of the smallpox vaccine. Serial passaging of MVA in chick embryo fibroblasts resulted in deletions and mutations of various genes involved virulence and evasion, leading to a virus with replication restricted to avian cells (185). As a result of the deletion of many immunomodulators, it is no surprise that MVA induces cGAS-STING mediated type I IFN signalling in conventional dendritic cells. The VACV protein N1 is an immunomodulatory protein that is altered by a 4 amino acid frameshift in MVA, resulting in loss of the protein function (186, 187). N1 has been previously shown to inhibit TLR mediated NF- κ B signalling by targeting the IKK complex and IRF3 signalling by targeting TBK1 (188). Although the exact mechanism of inhibition remains to be elucidated, in light of earlier studies, the effect is likely due to the interaction between N1 and TBK1 along with its ability to inhibit at the level of the IKK complex. VACV proteins B14 and C4 also target NF- κ B signalling at the level of the IKK complex (122). B14 sterically hinders interaction between the kinase domains of IKK β , thus inhibiting trans-autophosphorylation and subsequent NF- κ B signalling (189). C4 has been shown to act at, or downstream of, the IKK complex (190). However, the mechanism remains unclear. Further downstream, the N1-related VACV protein N2, inhibits signalling at the level of nuclear IRF3. Inhibition of upstream signalling is observed in response to the presence of N2 but nuclear translocation of IRF3 is still able

to occur. However, reduced expression of CXCL10 indicates that this effect is due to N2-mediated inhibition of IRF3 (191).

The VACV protein C6 is yet another immunomodulator of downstream targets of antiviral signalling and specifically inhibits type I IFN production, while having no effect on NF- κ B activation. C6 was shown to interact with TBK1 and I κ B kinase- ϵ (IKK ϵ), both of which are upstream of IRF3/7 and required to phosphorylate and activate these IRFs. C6 associates with the adaptor proteins NF-Kappa-B-Activating Kinase-Associated Protein 1 (NAP1), TRAF family member-associated NF-kappa-B activator (TANK) and similar to NAP1 TBK1 adaptor (SINTBAD) which are constitutively associated with TBK1 and IKK ϵ , and appears to disrupt their function (192). The TBK1 and IKK ϵ level of signalling is a key point of convergence for a number of PRRs such as TLRs, RLRs, and cytosolic DNA sensors, so it is unsurprising that inhibition at this level has a profound effect on many innate virus sensing pathways (193).

The poxvirus protein F17 is conserved across poxvirus species and is involved in virion formation (194). Until recently, F17 has had no known function outside of its role as a structural protein, but it has now been reported to indirectly induce cGAS degradation by manipulating the mammalian Target of Rapamycin complexes (mTORC)1/2 (195). mTORC1 and mTORC2 are distinguished by the presence of their N-terminal regulators known as Regulatory associated protein of mTOR (Raptor) and Rapamycin-insensitive companion of mTOR (Rictor), respectively. These two regulatory proteins bind their target proteins antagonistically in relation to one another. mTORC1 activity occurs in response to mitogenic signals such as those induced by the phosphatidylinositol-3-kinase (PI3K)-protein kinase B (AKT) axis.

PI3K is an intracellular lipid kinase and AKT is a serine/threonine kinase, and these exert their effect by phosphorylating downstream targets such as mTORC1. This influences metabolic processes and increases protein synthesis. mTORC2 acts in a similar manner as it is responsive to mitogenic stimuli such as growth factors but differs in its regulatory function as it controls cytoskeletal formation and cell survival. Activity of mTORC1 leads to mTORC2 inhibition through another kinase p70S6K, meaning activities of both are finely balanced (195, 196).

VACV activates mTOR signalling during the early stages of infection allowing the virus to benefit from metabolic signalling and establish infection. F17 was shown to interact with both raptor and rictor, leading to hyperactivation of mTOR and enhanced phosphorylation of p70S6K and 4E-BP1, both of which are downstream targets of mTORC1. This dysregulated signalling of mTOR by F17 drove cGAS degradation leading to inhibition of downstream signalling. Furthermore, STING and mTOR cooperate to resolve stressed ER membranes as a result of STING activation which is required for optimal signalling in this system. These ER membranes are sequestered and degraded by autophagy induced by the inhibition of mTORC1 (197). Therefore, the persistent activation of mTORC1 by F17 likely leads to a build-up of ER stress thus dampening the IFN response (195).

Following its production by cGAS, cGAMP potently induces innate immune responses by activating STING, making it a key element of this DNA sensing pathway (198). Hence, one would assume that a strong selective pressure exists for viruses to target cGAMP directly. Until recently, the only known enzyme with the ability to degrade cGAMP is the phosphodiesterase ecto-nucleotide pyrophosphatase/phosphodiesterase ENPP1, an extracellular protein which is not found in the cytosol (199). However, a recent study revealed that poxviruses can also target cGAMP using poxvirus immune

nucleases (poxins) (200). In this work, an array of mammalian viruses was screened for nuclease activity against cGAMP resulting in the identification of the B2R gene. VACV B2R gene encodes poxin that destabilises cGAMP through hydrolysis of the canonical 3'–5' bond.

While many poxvirus studies aiming to identify host immune-evasion mechanisms focus on proteins found in VACV, relatively few studies have looked in detail at a human-adapted poxvirus. This will be discussed in the next section.

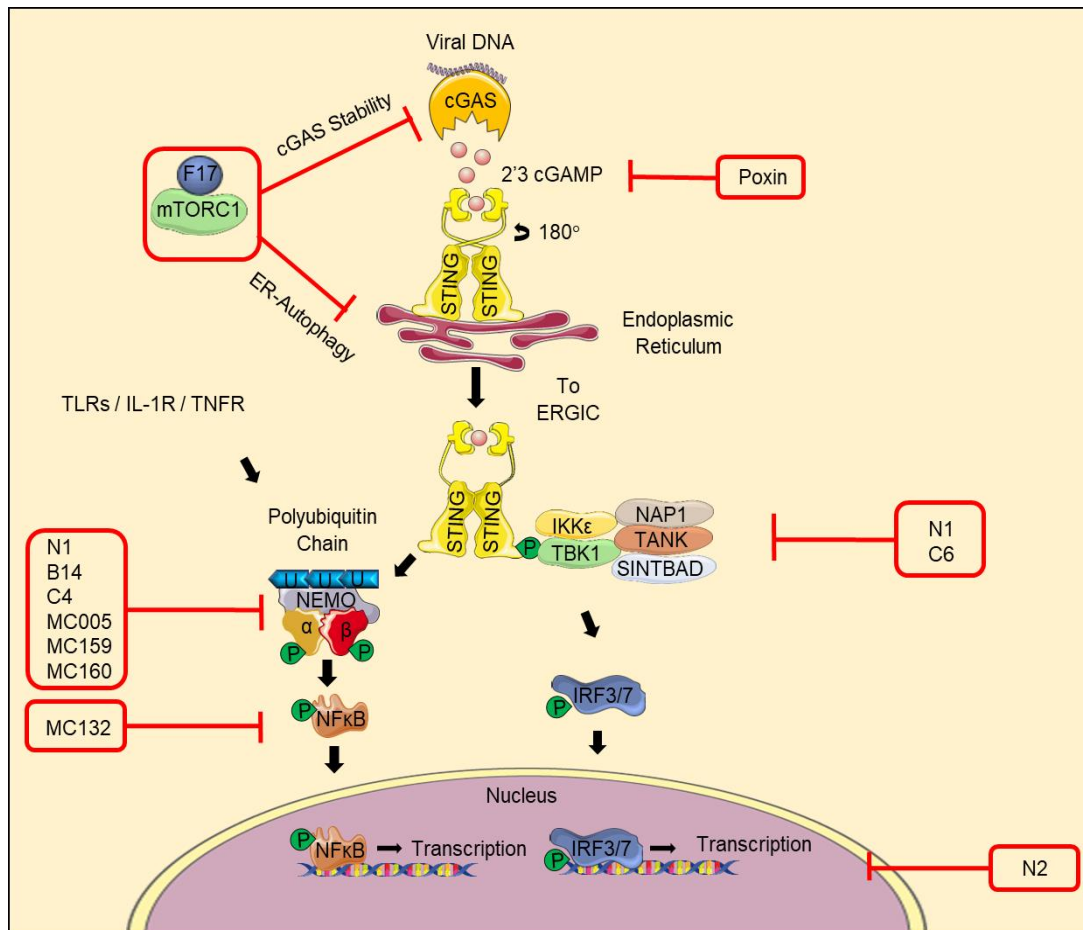


Figure 1.2.5 Inhibition of antiviral signalling by Poxvirus proteins. Poxvirus proteins inhibit antiviral signalling at various points in the pathway to prevent the activation of IRF3/7 and NF-κB. The forms of the individual proteins were adapted

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1.3 Molluscum contagiosum

1.3.1 Epidemiology and Clinical Features

MCV was first described by Thomas Bateman in 1817 (201). Interestingly, Edward Jenner, over 20 years earlier, recorded in his journal the observation of “hydatids on the skin” common in children (202). Jenner described a morphology and the release of white opaque fluid, characteristically similar to Batemans official description of MCV. However, he never published these findings, and it was only in the 20th century that his observations were revealed.

MCV is now known to be a large doubled-stranded DNA virus that is specifically adapted to unusually long-term human infection for a poxvirus (203). There are four types of MCV with MCV-1 being the causative agent in 98% of immunocompetent hosts (204). MCV types 2-4 are much less common, and infection is mainly limited to immunocompromised hosts, such as those suffering from human immunodeficiency virus (HIV). MCV infection is common throughout the developed world and is the primary poxvirus causing human disease, resulting in 122 million reported cases in 2010 (117). MCV mainly infects children, sexually active young adults, and the immunocompromised (203). It is usually spread through skin to skin contact but transmission can also occasionally occur through fomites such as towels (205). In 2005, a joint England and Wales study reported that 90% of new MCV cases were observed in children up to the age of 14 (170, 206). MCV infections are known to persist in immunocompromised patients, with an incidence rates of 5-8% observed in HIV infected individuals, rising as high as 18% in HIV infected children (170).

Infection occurs in the differentiating cells of the human epidermis, resulting in the formation of small umbilicated papules, which display very little to no inflammation

(203). These papules are approximately 3-5mm in diameter (**Figure 1.3.1A**) and contain grey-white matter when squeezed. Infected immunocompetent individuals usually present with < 20. However, > 100 papules have occasionally been observed (203, 207). Pruritus is thought to aid viral spread, as localized eczematous dermatitis is often found around lesions (208). MCV lesions usually affect the trunk and proximal extremities in children, and the pubic region, thighs, and trunk in adults; but, in all cases, infection can spread to other regions through autoinoculation. Molluscum lesions also tend to develop along the beard line in male HIV-positive patients (209). MCV diagnosis is usually possible through physical exam given the lesions are observably unique. However, if clinical diagnosis is difficult due to atypical presentation (e.g., inflamed lesions), a biopsy can be taken for histopathological analysis (**Figure 1.3.1B**). Under the microscope, MCV classically presents a cup-shaped lesion containing intracytoplasmic viral inclusions known as molluscum bodies/Henderson-Patterson bodies (210).

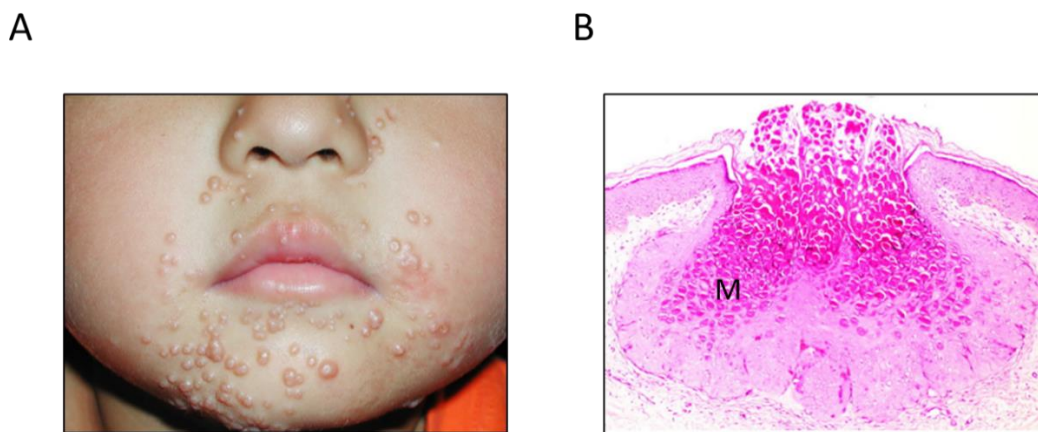


Figure 1.3.1. Typical MCV clinical presentation and histopathology of biopsy. (A) A child presenting with umbilicated papules typical of MCV around the nose and mouth. (B) Histopathological analysis of an MCV lesion displaying molluscum bodies (M) and a crateriform structure. Both images reproduced under a CC BY-NC 4.0 licence with modification to include a label on figure B. Images were obtained from Qian Y-T and Ma D-L, 2019 (211).

1.3.2 Treatment

While some forms of treatment are available for MCV, the self-limiting nature of the disease means the decision to treat or not is determined on a case-by-case basis. MCV treatments have been extensively reviewed (212-214). However, some of the more commonly used treatment options will be discussed here.

Cryotherapy has been effectively used to treat MCV, but potential for pain and scarring have resulted in its limited use on children. Nevertheless, one study of 74 children has shown clearance rates of 70.3% after 3 weeks and 100% after 6 weeks (215), with another study of thirty patients (1-24 years old) showing 93.3% clearance after 4 weeks of treatment (216). Both studies reported mild local side effects including hyperpigmentation. Another effective mechanical method of removal is curettage which usually involves scraping the skin lesion with a specialised tool called a curette. A study involving 1,878 children demonstrated a 70% cure rate after one session and a further 26% were completely cured after a second session (217). Pain and bleeding during this process is usually minimal.

Chemical methods have also been used to treat MCV lesions, albeit with less consistent results than the mechanical methods mentioned above. Nevertheless, these types of treatment tend to be less painful which is of particular benefit when treating cases in children. Cantharidin is an inhibitor of phosphodiesterase and is applied topically to the lesion, resulting in intraepidermal blister that rarely scars (218). Its use to treat MCV has been documented since the 1950s (219). However, reported clearance rates vary, ranging between 15.4% and 100% (220). Another common method of chemical is treatment with 10% potassium hydroxide, a strong alkaline compound that can dissolve keratin (221). Its effectiveness has been compared to cryotherapy with clearance rates of 86.6%

reported after 4 weeks of treatment (216). Chemicals including podophyllotoxin, trichloroacetic acid, salicylic acid have also been used to varying degrees of effectiveness (213).

Immunomodulatory methods have been used to treat MCV as they can engage a patient's immune system to resolve the lesion. One such example is a treatment with 5% imiquimod topical cream. While a study comparing this treatment strategy with cryotherapy demonstrated 91.8% clearance after 16 weeks (215), a more recent systemic review comparing imiquimod treatment with a placebo, demonstrated no significant difference between the two cohorts, making the efficacy of this treatment controversial (222).

1.3.3 MCV Genome

MCV is the sole member of the molluscipoxvirus genus, and the complete genome was first sequenced in 1996 (223). This study revealed that the MCV genome comprises of 190,289 base pairs (bp) and has a high guanine–cytosine content of 63.3% compared to 34% in VARV, resulting in a scarcity of stop codons and a concomitant large number of ORFs greater than 60 codons. While the authors originally noted 163 proteins, along with 103 orthologs in VARV, a more detailed subsequent analysis predicted 182 proteins and 105 orthologs with other poxviruses (136). Most of these are centrally conserved and encode proteins essential for structure, transcription, and replication. The remaining open reading frames are unique to MCV virus, and while many have yet to be investigated, we know some are immunomodulatory in nature. As is typical with poxviruses, unique MCV genes with cellular homologues or no identified homologues are largely located on the terminal ends of the genome and distributed between conserved genes (131).

1.3.4 MCV Immune Evasion

MCV does not cross the basement membrane, thereby reducing the likelihood of interaction with the host immune system, and it also employs an arsenal of inhibitory proteins that block specific events in cell signalling within infected regions (170, 224-227). As such, it can subvert the immune system's ability to clear the virus, leading to persistent infection. In contrast, infection with a non-human adapted poxvirus, such as VACV, induces painful inflamed lesions which usually heal within a few weeks (228) (**Figure 1.3.2**).

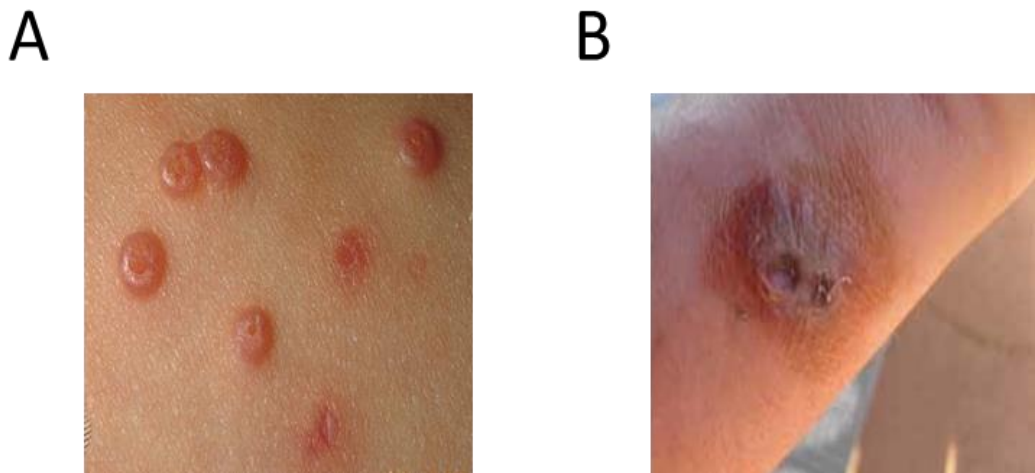


Figure 1.3.2 Comparison of human infection by Molluscum Contagiosum virus and Vaccinia virus. (A) Clusters of papules caused by an MCV infection in a human (B) Necrotic lesion on a human arm caused by a Vaccinia virus infection. Reuse of Image A was obtained from DermNet (229) and is licensed under a Creative Commons Attribution-Non-Commercial-NoDerivs 3.0 licence. Image B was obtained from Peres et al., 2016 (230).

Although much of the MCV genome remains to be studied, it has been shown to encode immunomodulatory proteins that interact with various elements of the canonical NF- κ B signalling pathway and key processes of the host immune response. The viral FLICE-like

proteins (vFLIPS) MC159 and MC160 both inhibit TNF α receptor-induced apoptosis. These can inhibit NF- κ B signalling by binding to NEMO and preventing IKK β phosphorylation and by inducing IKK α degradation, respectively (231, 232). MC159 has more recently been shown to competitively bind to the NEMO N-terminal region and prevent cIAP1 poly-ubiquitination of NEMO (224), thus inhibiting downstream NF- κ B activation. MC163 was shown to inhibit apoptosis by preventing mitochondrial membrane permeabilization (233). MC148 is a chemokine homolog and was shown to act as an antagonist for CXCL12 α by blocking the interaction with CXCR4 (234). MC053 and MC054 acts as IL-18 binding proteins which inhibit the gamma interferon-inducing activity of IL-18 (235, 236). MC080 was shown to disrupt MHC-I antigen presentation by targeting tapasin, a component of the peptide loading complex, for ubiquitination and subsequent degradation (237). MC007 targets the tumour suppressor retinoblastoma protein and sequesters it at the mitochondria to promote cell survival (238).

Work in the host lab has revealed 5 more inhibitors of innate immune signalling following a screen of the MCV genome, two of which have been reported to date. The MCV protein MC132 recruits the p65 subunit of NF- κ B to an elongin B/C Cullin 5 E3 ligase complex where it ubiquitinates it, tagging it for degradation by the proteasome (226). MC005 mediates its inhibitory effects by binding to NEMO and preventing conformational change required for the exposure of phosphorylation sites on IKK β (227). Likely many more inhibitors remain to be identified.

Thesis Aim

The aim of my PhD research was to investigate the MCV encoded MC008 protein, in order to understand its role in evasion of host inflammatory signalling. In doing so, we hope to lay the foundation for future development of a novel anti-inflammatory therapeutic derived from this protein. Furthermore, understanding how MCV targets and modulates this pathway at key nodes of activation offers the possibility to identify novel facets of signalling and, potentially, new rate-limiting steps for therapeutic intervention.

Chapter 2 Materials & Methods

2.1 Materials

Name	Product Code	Company
0.05% Trypsin-EDTA (1x), phenol red	25300054	Gibco
0.2µm Filter	83.1826.001	Sarstedt Ltd
0.25% Trypsin-EDTA (1x), phenol red	25200056	Gibco
0.45µm Nitrocellulose	15259804	Fisher Scientific
1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid	319945-25G	Merck
10cm cell culture dishes	CLS430167-500EA	Merck
15mL Tube	62.554.502	Sarstedt Ltd
20mL syringe	300613	BD
50mL Tube	62.547.254	Sarstedt Ltd
5ml Stripettes	10420201	Fisher scientific
96 well IMMULON ELISA plates	735-0465	VWR
96 well plate TC flat bottom	3596	Scientific Laboratory Supplies
Acetyl Coenzyme A lithium salt	A2181-100mg	Sigma-Aldrich
Adenosine 5'-Triphosphate Disodium Salt	A2383	Sigma-Aldrich
Alcohol burner wick	WHEA237071	VWR
Ammonium peroxodisulphate	ACRO201531000	VWR
Ampicillin Sodium Crystalline	A9518-5G	Sigma-Aldrich
Antibody-FLAG (M2)	F3165	Sigma-Aldrich
Antibody-FLAG M2 Affinity Gel	A2220-5ML	Sigma-Aldrich
Antibody-HA	901515	Bio-legend
Antibody-NEMO	2685	Cell Signalling Technology
Antibody-p65	D14E12	Cell Signalling Technology
Antibody-phospho-p65	93H1	Cell Signalling Technology
Antibody-β-actin	A5316	Sigma-Aldrich
Aprotinin	CAYM14716-25	VWR
Autoclave Tape	AAU630.10	Lennox Lab Supplies
Bacterial Spreader	12822775	Fisher Scientific
Benzonase	70746-3	Sigma-Aldrich
Beta glycerophosphate disodium salt	G9422-100g	Sigma-Aldrich
Bovine Serum Albumin	12659-1KG-M	Merck

Bright-Line™ Hemacytometer	Z359629	Merck
Cadmium Chloride	655198-5G	Sigma-Aldrich
Cardboard storage boxes	95.064.981	Sarstedt Ltd
Chamber Slides	16260661	Fisher Scientific
ChemiDoc MP blotting system with Image Lab software V6.1		Bio-Rad
Coelenterazine	10110-1	VWR
Copper sulfate	C8027-500g	Sigma-Aldrich
Corning Costar Flat Bottom Cell Culture Plates (6-well)	10146810	Fisher Scientific
Cryovials	72.380.002	Sarstedt Ltd
Digital Hotplate Magnetic Stirrer	H3770-HS-E	Benchmark Scientific
Dithiothreitol	10592945	Fisher Scientific
D-Luciferin Firefly	FL08607	Carbosynth
Doxycycline hyclate	D9891-1G	Merck
Dulbecco's Modified Eagle Medium	61965026	Gibco
Electrophoresis Kit (4 Gel)	1658006FC	Bio-Rad
Ethanol	ETH001	TCD Hazardous Materials
Ethylenediaminetetraacetic acid	15575020	Thermo Fisher Scientific
Fisherbrand SureOne P1000 Filter tips	11973466	Fisher Scientific
FLAG Epitope Tag L5 Antibody, Alexa Fluor 488 conjugate (L5)	MA1-142-A488	Fisher Scientific
FLAG Peptide	F3290-4MG	Sigma-Aldrich
Foetal Bovine Serum	F9665-500ML	Sigma-Aldrich
Gel casting stand	1658050	Bio-Rad
GeneJuice Transfection Reagent	70967-4	Merck
Glass gel plates (inner)	1653308	Bio-Rad
Glass gel plates (outer)	1653312	Bio-Rad
Glycerol	10336040	Fisher Scientific
Glycine	G8898-1KG	Sigma-Aldrich
GraphPad Prism (8.0.2)		GraphPad Software Inc.
Greiner Serological pipette 10ml sterile individually wrapped	G607180	Greiner
HA Antibody, Alexa Fluor 647 conjugate	26183-A647	Fisher Scientific
Hydrochloric Acid	15693640	Fisher Scientific
Hygromycin B Gold	ant-hg-1	InvivoGen
IGEPAL (NP40)	CA-630	Merck
IKK α / β (Ser176/180) rabbit monoclonal	16A6	Cell Signalling Technology

IL-6 ELISA kit (15-Plate)	DY206	R&D Systems
Incu-Shaker™ Mini	H1001-M-E	Benchmark Scientific
IRDye 680RD Goat anti-mouse IgG (H+L) 0.5mg	926-68070	LI-COR
Isopropanol	17140576	Fisher Scientific
IkBα mouse mAb	4814	Cell Signalling Technology
Kimwipes	Z188964-1PAK	Merck
Large Nitrile Gloves	GN003	TCD Hazardous Materials
LB Agar	11758902	Fisher Scientific
LB Broth	12801660	Fisher Scientific
Lecia SP8 Confoal microscope with LasX software version 1.4.3		Lecia
Lenti-X™ Packaging Single Shots (VSV-G)	631278	Takara-Bio
Li-COR IRDye 800CW Goat anti Rabbit IgG	926-32211	LI-COR
Lipofectamine 2000 Transfection Reagent	11668019	Thermo Fisher Scientific
Luminoskan Ascent with Skanit software (V2.6)		Biosciences/Thermo Fisher Scientific
Magnesium carbonate hydroxide pentahydrate	56378-72-4	Sigma-Aldrich
Magnesium sulfate heptahydrate	10034-99-8	Sigma-Aldrich
Maxi Prep Kit (25 preps)	A31231	Invitrogen
Methanol	MET001	TCD Hazardous Materials
Micro Tube 1.5ml	72.690.001	Sarstedt Ltd
Molecular grade ethanol	E7023-500ML	VWR
Mr Frosty	5100-0001	Biosciences
Multiskan FC plate reader	N07710	Thermo Fisher Scientific
Odyssey imaging system B446 with Image Studio lite software version 5.2		LI-COR
OptiPlex 5090 Micro Desktop (Windows 10)	210-AYRX	Dell
P10 Tips	11577442	Fisher scientific
P1000 Tips	11548442	Fisher scientific
P200 Tips	70.760.002	Sarstedt Ltd
Parafilm ®	ASE165.15	Lennox Lab Supplies
Paraformaldehyde Solution, 4% in PBS	15670799	Thermo Fisher Scientific
PBS, pH 7.4	10010015	Gibco
Penicillin Streptomycin	15140-122	Gibco
Petri dishes	82.1473.001	Sarstedt Ltd
Phenylmethanesulfonyl fluoride	P7626-5G	Sigma-Aldrich
Phosphoric Acid 85%	15686710	Fisher Scientific

PolarSafe® Cryogenic Storage Dewar, 47L, with 6 Round Canister Racks	WZ-44260-58	Cole-Parmer
Poly(dA:dT)	P0883-10UN	Sigma-Aldrich
Polybrene	TR-1003-G	Merck
Polyethylenimine	408727	Sigma-Aldrich
Potassium Chloride	7447-40-7	Sigma-Aldrich
Potassium phosphate monobasic	7778-77-0	Sigma-Aldrich
ProLong Gold Mounting media with DAPI	P36931	Invitrogen
Protein Ladder	26619	Thermo Fisher Scientific
ProtoGel 30% Solution	NAT1260	National Diagnostics
Reservoirs	SPL-23050	Medical Supply Co
Round Bottom 96 Well Plate	MICR-TPU	Scientific Laboratory Supplies
Scales	PCB 2500-2	Kern
Sodium Chloride	7647-14-5	Sigma-Aldrich
Sodium Dodecyl Sulphate (SDS)	L4509-500G	Sigma-Aldrich
Sodium Fluoride	GSK5130-1G	Merck
Sodium hydrogen phosphate, anhydrous	7558-79-4	Sigma-Aldrich
Sodium Hydroxide	1310-73-2	Sigma-Aldrich
Sorvall™ Legend™ Micro 17R Microcentrifuge, Ventilated	75002430	Biosciences
Subcloning Efficiency™ DH5α Competent Cells	18265017	Thermo Fisher Scientific
T175 Flask	83.3912.002	Sarstedt Ltd
3,3',5,5' – tetramethylbenzidine (TMB) Substrate	N301	Thermo Fisher Scientific
TNFα	570104	Bio-Legend
Transfer Kit (2-Gel)	1703930	Bio-Rad
Tricine	T0377-1KG	Sigma-Aldrich
TRIM32 siRNA	1027418	Qiagen
TRIM32 siRNA Control	1027281	Qiagen
Tris Base	10376743	Fisher Scientific
Triton X-100	SIGT8787-100ML	Sigma-Aldrich
Tube Roller	R3005	Benchmark Scientific
Tween-20	EC-607	Sparks Lab Supplies
Virkon® disinfectant cleaner	Z692158	Merck
Vortex Mixer	BV1003	Benchmark Scientific
Water bath Conditioner	11950218	Fisher scientific

Table 2. List of materials, reagents and equipment used during experimentation

2.2 Cell Culture Techniques

HEK293T, HeLa and HaCaT cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (GIBCO, 61965-026), supplemented with 10% foetal bovine serum (Sigma, F9665), and containing 1% penicillin streptomycin from (GIBCO, 15140-122). Cells were passaged by washing with sterile phosphate buffered saline (PBS) and were detached from the flask by adding prewarmed 0.05% or 0.25% trypsin-EDTA. For cell stimulations, 50ng/mL TNF α (BioLegend, 570104) or 1 μ g/mL poly(dA:dT) (Sigma, P0883) was used.

2.3 Plasmids and Oligonucleotides

Prior to PhD commencement, custom-synthesized MCV subtype 1 MC008L (GenScript) was subcloned into KpnI and NotI sites of the pCEP4 plasmid (Invitrogen, V04450) containing a C-terminal FLAG (DYKDDDDK) or HA (YPYDVPDYA) tag. MC008L was also cloned into these sites in the pMEP4 plasmid (Invitrogen) with a FLAG-tag for CdCl₂-inducible expression. All other plasmids were sourced as described previously (226, 227). MC008 truncations were generated previously by the host lab by PCR-amplifying indicated regions with KpnI and NotI site overhangs and sub-cloning into pCEP4 with a C-terminal FLAG tag.

2.4 Bacterial Transformation

Bacteria have a natural ability to take up foreign DNA from the environment in a process known as horizontal gene transfer (bacterial transformation) (239). This can be exploited to enable replication of specific plasmid DNA sequences. The plasmid of interest contains an antibiotic resistance gene (in this case ampicillin resistance) which enables the bacterial cell to survive in the presence of ampicillin when transformed,

while the rest of the untransformed cells die. This leaves a pure population of bacterial cells containing the plasmid of interest that can grow and divide (in ampicillin) in order to replicate the plasmid DNA.

LB agar (Fisher Scientific, 11758902) and LB broth (Fisher Scientific, 12801660) were reconstituted in dH₂O and sterilised through autoclaving. The LB agar was left to cool to ~50°C before the addition of selection antibiotic (50µg/mL ampicillin) and was subsequently poured into 10cm dishes and left to cool. 1µL of plasmid was added to 10µL of DH5α competent *E. coli* (ThermoFisher, 18265017) in a sterile microtube, then left on ice for 30 minutes. The cells were then heat-shocked by placing the tube into a water bath at 42°C for 45 seconds, before recovery on ice for a further 5 mins. 150µL of LB broth without antibiotic was added to the tube, under a flame, and left to shake in an incubator at 37°C for 1 hour. 50µL of cells were then added to a 10cm agar plate under a flame and spread using a sterile spreader. The plates were then incubated upside down at 37°C overnight in stationary incubator. The following morning, 5mL of sterile LB broth, with 50µg/mL ampicillin, was added to a sterile tube, along with a single bacterial colony for the pre-culture. The lid was then loosely closed with tape and left to shake for 8 hours at 37°C. 200mL LB broth, along with 2 mL of pre-culture, was added to sterile 250mL conical flasks covered with tinfoil and left to shake in an incubator for 16h at 37°C. The 200mL culture was subsequently divided into sterile 50mL tubes and centrifuged at 3500xg for 15 minutes. The supernatants were then discarded into Virkon® and left for 24h prior to disposal.

2.5 Plasmid Purification

Following the culture of bacteria containing the plasmid DNA of interest, the plasmid DNA needs to be isolated and purified from the bacterial cells before it can be used to

modify mammalian cells. This process involves bacterial cell lysis and removal of debris, followed by DNA binding to a column and subsequent elution. The DNA is then precipitated and stored in an antimicrobial buffer before use in mammalian cell experiments.

Plasmid DNA was purified from the transformed bacterial pellets using the PureLink™ Expi Endotoxin-Free Maxi Plasmid Purification Kit (Invitrogen, A31231), as described in the manual. All reagents (except molecular grade ethanol and isopropanol) were provided in the kit.

Four pellets were combined into one tube in 6mL of resuspension buffer. 6mL of lysis buffer was added to the tube, mixed by inversion, then left to incubate at room temperature for 5 minutes. 6mL of precipitation buffer was then added and the tube was mixed by inversion. The lysates were then poured into a clarification column in a provided 50mL tube, and subsequently centrifuged at 1000xg for 4 minutes. The columns were discarded and 2.5mL of endotoxin removal buffer was added to the clarified lysate and mixed by inversion. The clarified lysate was then poured into DNA binding columns and centrifuged at 1000xg for one minute. The flowthrough was discarded and 20mL of wash buffer was added to the columns and centrifuged at 1000xg for 1 minute. DNA binding columns were then inserted into new endotoxin-free 50mL tubes. 15 mL of endotoxin-free elution buffer was added, and the tubes were centrifuged at 1000xg for 1 minute. The DNA was then precipitated by adding 10.5mL of molecular grade isopropanol and centrifuging at 3500xg (max speed of centrifuge) at 4°C for 1 hour. The supernatants were then discarded, and the pellets were washed with 5mL of 70% molecular grade ethanol. The ethanol was then removed, and the pellets were left to air-dry for 10 minutes before the addition of 300μL of endotoxin-free TRIS-EDTA Buffer.

2.6 Reporter Gene Assays

Reporter gene assays enable the measurement of the regulatory ability of DNA. In this case, assays were used to measure promoter activation of key transcription factors involved in antiviral and inflammatory signalling, following stimulation and MC008 expression. Activated transcription factors bind to the promoter sequence of interest, upstream of the luciferase reporter gene construct, resulting in transcription and subsequent production of luciferase enzyme. Addition of substrate to the enzyme results in a measurable light reaction that is proportionate to the level of reporter gene activation.

HEK293T cells were seeded at 2×10^5 cells/mL in a flat bottomed 96-well plate with 200 μ L of DMEM/well. The cells were transfected 16 hours later using Gene Juice transfection reagent (Merck Millipore, 70967) with 80 ng of Firefly luciferase reporter gene constructs, 40 ng of pGL3-Renilla luciferase plasmid, and the indicated quantities of expression vector. The quantity of these constructs was adjusted to give a final volume of 220 ng of DNA/well using empty vector pCMV-HA. After 24 hours, the cells were lysed using passive lysis buffer (25 mM TRIS-phosphate (pH 7.8), 2 mM DTT, 2 mM 1,2-diaminocyclohexaneN,N,N',N'-tetraacetic acid, 10% glycerol, 1% Triton® X-100) as described in the Promega technical bulletin 281 (240). 20 μ L of lysates were added to two identical white microplates and luciferase assay substrate (20 mM tricine, 2.67 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 mM EDTA, 33.3 mM DTT, 530 μ M ATP, 270 μ M acetyl coenzyme A sodium salt, 420 nM D-luciferin, 5 μ M NaOH, 9.7 mM $((\text{MgCO}_3)_4\text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O})$ or coelenterazine (Biotium Inc, 10110-1) were added to induce the light reaction. The lysates were then analysed using a Thermo Scientific Luminoskan Microplate Luminometer with Ascent software v2.6. Transfection efficiency was controlled by normalizing Firefly luciferase activity to Renilla luciferase activity. For the Elk1 reporter

assay, the pathway was driven by RasVHa expression vector and 1 ng of the pFA-Elk1 expression vector and 80 ng of the pFR-luciferase reporter plasmid.

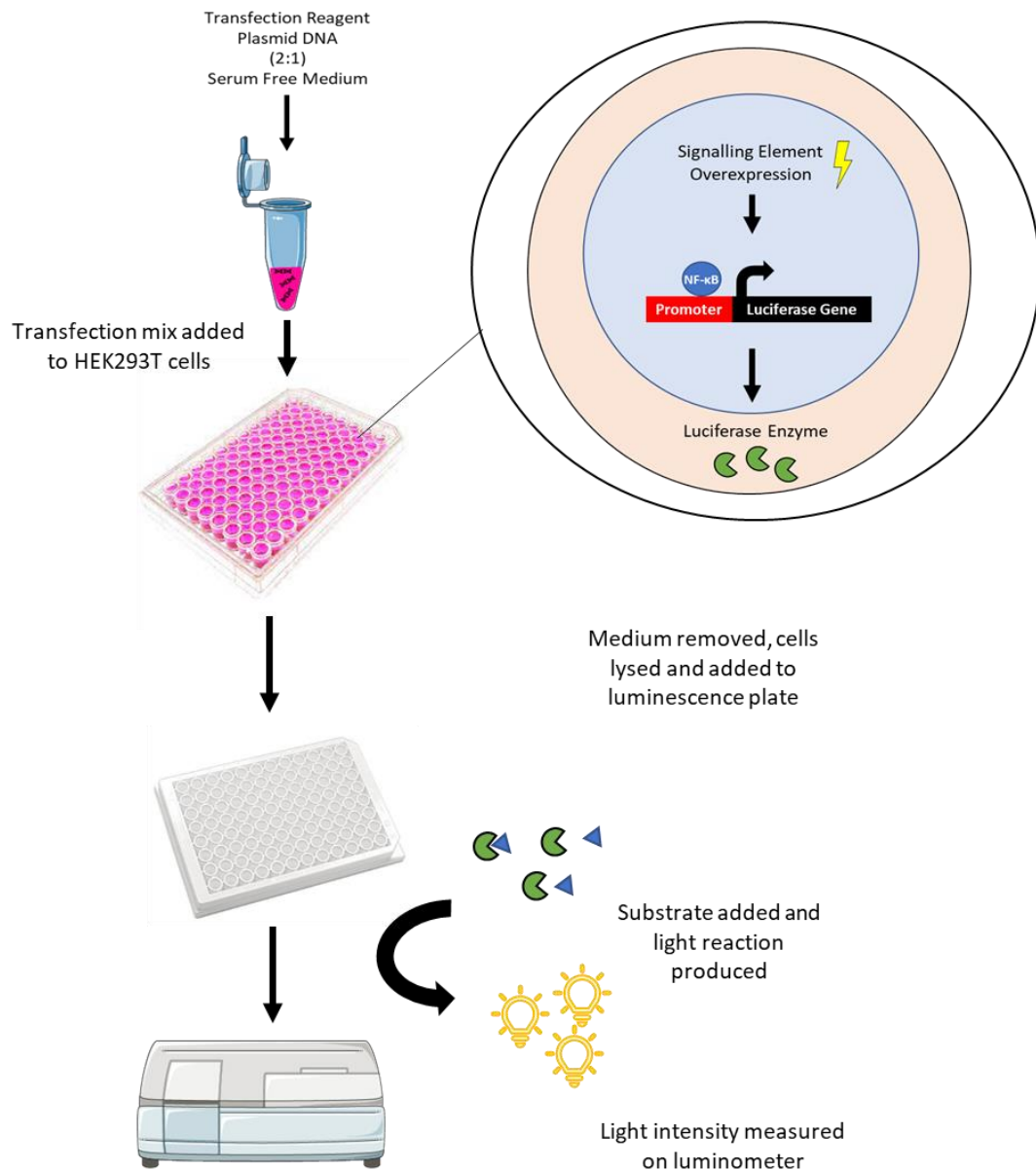


Figure 2.1 Overview of luciferase assay protocol. Plasmid DNA is transfected into HEK293T cells using by GeneJuice® transfection reagent. Activated NF-κB binds to the promoter sequence upstream of the luciferase reporter gene construct resulting in transcription and subsequent production of luciferase enzyme. Following media removal, the cells are lysed and added to white microplates. Substrate solution is then added to the microplate wells and the light intensity is measured using a luminometer.

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2.7 Antibodies

Anti- β -actin (Sigma-Aldrich, A5316), anti-FLAG (M2) (Sigma-Aldrich, F3165), anti-HA (Bio-Legend, 901515), Anti-I κ B α (CST, 4814), anti-phospho-p65 (CST, 93H1), anti-p65 (CST, D14E12), phospho-IKK α/β (Ser176/180) (16A6) rabbit monoclonal (CST), anti-NEMO (CST, 2685), anti-TRIM32 (Abcam, EP6355), were the primary antibodies used for immunoblotting. The secondary antibodies used for immunoblotting were IRDye 680LT anti-mouse (Li-Core, 926-68070) and IRDye 800CW anti-rabbit (Li-Core, 926-32211).

2.8 ELISA

ELISA's are a common immunological test used to measure substances such as antibodies, proteins, and antigens in a sample. The type of assay used here is known as a sandwich ELISA and was used to measure the effect of MC008 expression on the cellular release of proinflammatory cytokines, following the stimulation of inflammatory signalling pathways. This assay works on the principle that the antigen (in this case the cytokine IL-6) is bound between two layers of specific antibodies with different epitopes for the same antigen (capture and detection antibodies). The quantity of this antigen can be measured due to the presence of a biotinylated moiety on the detection antibody. This moiety interacts with the enzyme Streptavidin-HRP which produces a measurable signal in the presence of a substrate.

Cell culture supernatants were harvested and assayed for IL-6 using an enzyme linked immunosorbent assay (ELISA) kit (R&D systems, DY206) and stock reagents were diluted in accordance with the manufactures protocol.

Flat bottomed 96-well high binding plates (VWR, 735-0465) were coated with capture antibody and incubated overnight at 4°C. The capture antibody was subsequently removed, and the plates washed with 0.05% Tween-20 (Sparks lab supplies, EC-607) in PBS three times. Plates were then dried on lab tissue and 200µL of blocking solution containing 1% BSA (Merck, 12659-1KG-M) in PBS was added to each well for 2 hours at room temperature before repeating the washing step. Cytokine standards were serially diluted in triplicate in separate round bottom 96-well plates to create a 7-point standard curve, along with a negative blank. 100µL of each was then transferred into the coated plates. Supernatants were diluted 1:2 and 100µL of diluted sample was added to the plates in triplicate. The plates were then covered and incubated overnight at 4°C. The supernatants and standards were then discarded followed by three more rounds of washing and subsequent drying. 100µL of diluted Streptavidin-HRP (1:40) solution was added to each well and plates were covered and incubated at room temperature in the dark for 20 minutes. The washing step was repeated a further three times, the plates were dried and 100µL of TMB substrate (Thermo Fisher Scientific, N301) was added to each well. The plates were covered and incubated at room temperature in the dark for a minimum of 20 minutes, until the standard curve colour was appropriately developed. 50µL of 2M H₂SO₄ was then added to each well to stop the reaction. Absorbance values were immediately measured on a Multiskan FC plate reader (Thermo Fisher Scientific) at 450nm. Standard curves were then interpolated in GraphPad Prism software (Version 8.0.2) to determine the values of unknown samples.

2.9 Immunoblotting

Immunoblotting enables the separation of proteins by size which can then be specifically labelled to identify target proteins. This method was used to identify expression of MC008 and other co-expressed signalling proteins. This process involves SDS-PAGE separation of proteins whereby charged molecules travel along a polyacrylamide gel towards the electrode when placed in an electric field. Smaller molecules migrate along the gel at a faster rate than larger molecules, due to less resistance, enabling separation. The separated proteins are then transferred to a solid support membrane which can then be probed with specific antibodies to detect the target protein.

Cells were seeded at 6×10^5 cells per well in 6-well plates and transfected with 4 μ g/well of expression construct 16 hours later using GeneJuice® transfection reagent (Merck Millipore). After 24 hours, the cells were lysed using 200 μ L of sample buffer with 100 mM dithiothreitol (Fisher Scientific, 10592945) and 1 μ L/mL benzonase (Merck Millipore, 70746-3). Lysates were left on ice for 5 minutes, then boiled at 100°C for 5 minutes. 20 μ L of lysates were then loaded into 8-20% resolving gels and SDS-PAGE was performed. The separated proteins were then transferred onto nitrocellulose membrane (Fisher Scientific, 15259804) and blocked with 3% (w/v) bovine serum albumin (BSA) and Tween-20 0.1% (v/v) in PBS for 1 hour. The membrane was then probed with primary antibody (diluted 1:1000 HA, 1:7000 FLAG, 1:10000 β -actin in blocking buffer) overnight at 4°C on a tube roller. The membrane was subsequently washed x3 in PBS with 0.1% Tween-20 for 5 minutes, incubated with the secondary antibody at room temperature for 1 hour, before repeating the washing step. The membranes were then viewed using the Odyssey imaging system B446 (LI-COR Biosciences) or Bio-Rad ChemiDoc MP system and analysed with Image Studio Lite software v5.2 (LI-COR Biosciences) or Image lab software version 6.1, respectively.

2.10 Immunoprecipitation

Immunoprecipitation is a technique used to precipitate a protein out of solution using an antibody that is specific for that protein. This enables the isolation and concentration of a target protein from a sample containing other protein contaminants. In the experiments, proteins labelled with a FLAG tag were immunoprecipitated using agarose beads containing anti-FLAG monoclonal antibody, thus enabling observation of interactions between signalling proteins and MC008. The protein is eluted from the antibody using 3x FLAG peptide solution which outcompetes binding to the antibody. This method of elution is preferred over boiling as it reduces the likelihood of observing antibody light and heavy chain bands overlapping with target protein bands on the immunoblot. Isolated target protein and interacting proteins are then immunoblotted as described in section 2.9.

Cells were seeded with 4×10^6 cells/10cm dish and transfected 16 hours later using Gene Juice transfection reagent (Merk Millipore). After 24 hours, cells were then lysed on ice with ice-cold lysis buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 0.5% [vol/vol] NP-40, 30 mM NaF, 5 mM EDTA, 10% [vol/vol] glycerol, and 40 mM β -glycerophosphate containing the inhibitors 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 1% [vol/vol] aprotinin). Lysates were cleared by centrifuging for 10 minutes at 4°C and transferred into pre-cooled microtubes on ice. 10 μ L of anti-FLAG M2 affinity gel (Sigma, A2220) per sample was centrifuged and washed x3 with lysis buffer to equilibrate the beads. These were added to the cleared lysates and left rolling overnight at 4°C. The beads were then washed x3 with 1mL of ice-cold lysis buffer. 4 μ L (from 5mg/mL stock) of reconstituted 3X FLAG-peptide (Sigma, F3290) and 80 μ L of PBS was added to each sample and left rolling at 4°C for 30 minutes to elute the immunoprecipitated material. The samples were then separated from the beads and 40 μ L

of sample buffer was added to each sample and were subsequently immunoblotted for the proteins indicated.

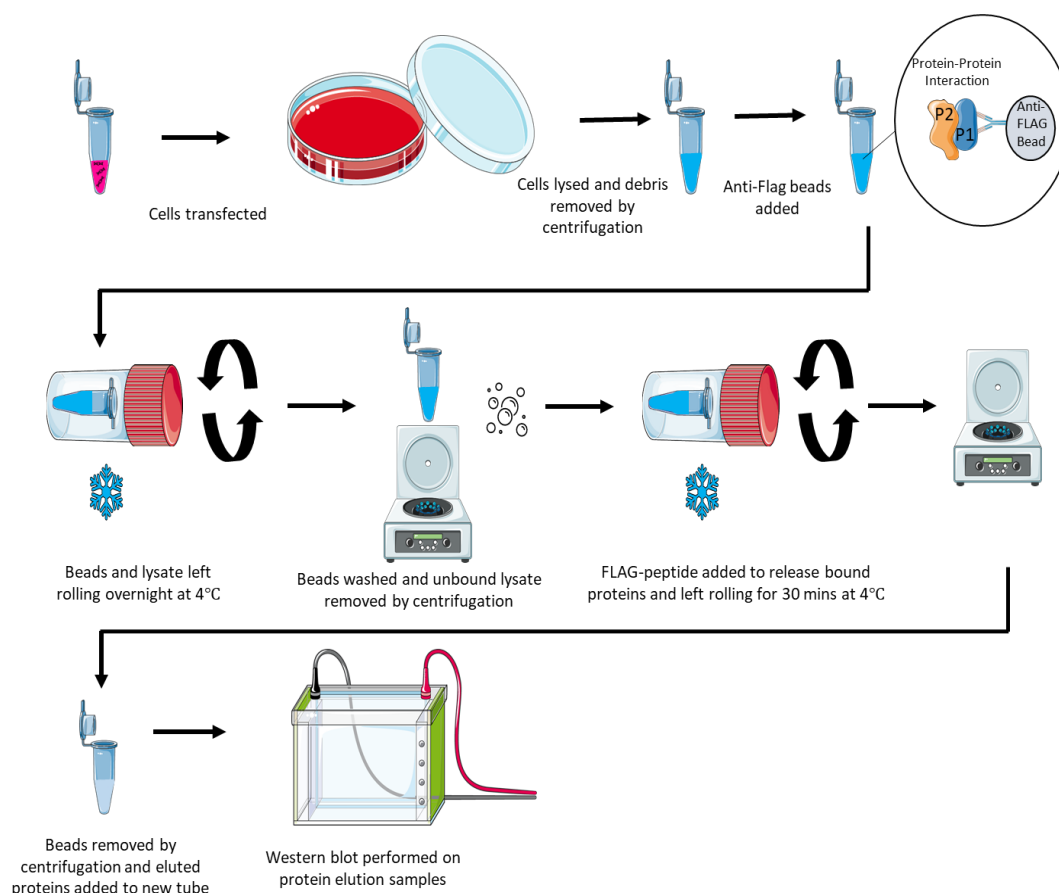


Figure 2.2 Overview of immunoprecipitation protocol. Cells are transfected in 10cm dishes and then lysed. Following removal of cell debris by centrifugation, equilibrated anti-FLAG beads are added to the lysates which are then left to roll overnight at 4°C. The lysate-bead mix is washed multiple times with lysis buffer to remove unbound material. FLAG-peptide is then added to elute the bound material and left to roll for a further 30 minutes at 4°C. Following elution, the purified immunoprecipitates are removed from the beads by centrifugation and transferred into a new microtube. Immunoblotting is then performed on the immunoprecipitates. The figure elements were obtained from Servier Medical Art (<https://smart.servier.com/>), licensed under a Creative Common Attribution 3.0 Generic License.

2.11 Generation of MC008-expressing Stable Cell Lines

Stable cell lines express a foreign gene that has been integrated into the host cell genome and are largely used to address inconsistencies associated with transient gene expression. Transfecting plasmid DNA into cells inherently triggers a DNA sensing response in the host cell. To circumvent this, stable cell lines provide an alternative means to transient expression, thus limiting transfection associated experimental artifacts. Briefly, producer cells (HEK293T cells) are transfected with a combination of plasmids encoding the structural and functional components of a lentivirus, along with a gene of interest under the control of an inducible promoter (tetracycline). These cells release lentiviral particles into the supernatant which can then be used to transduce target cells (HeLa cells). Following an antibiotic selection period, the surviving cells stably express the gene of interest and expression can be induced by stimulating with doxycycline.

Doxycycline-inducible stable cell generation employed the pLVX-TetOne-Puro expression construct with cloning of MC008L into the EcoRI and AgeI sites (performed by Prof. Gareth Brady). Lentivirus was packaged with the Lenti-X™ Tet-One™ Inducible Expression System (Takara, 631844 and 631847), as described in the manual.

4×10^6 HEK293T cells were seeded in 10cm dishes containing 8mL of DMEM for 16 hours. 7µg of pLVX-TetOne-Puro plasmid containing the MC008-FLAG sequence was diluted in sterile dH₂O with a final volume of 600µL and then vortexed. The 600µL volume was then added to Lenti-X Packaging Single Shots (VSV-G), which contain pre-mixed transfection reagent, and vortexed for 20 seconds. The transfection mix was then incubated for 10 minutes at room temperature and centrifuged for 2 seconds to remove residue from the cap. 600µL of the transfection mix was added dropwise to the cells. 16h later the transfection medium was replaced with 10mL of fresh DMEM. After 48 hours,

the lentivirus containing supernatant was harvested and centrifuged at 500xg for 10 minutes to remove debris. The presence of lentivirus in the supernatant was confirmed using Lenti-X™ GoStix™ Plus (Takara, 31280). The lentivirus stock was then aliquoted and stored at -80°C.

HeLa cells were seeded in 6-well plates at 2×10^5 cells/well. After 16 hours, the medium was replaced with a 1:4 dilution of lentivirus stock in DMEM containing 4µg/mL of polybrene. The plates were then centrifuged at 1200xg for 60 minutes at 32°C to enhance infection efficacy. Cells were transduced for 24 hours, and the viral containing medium was removed. The cells were subsequently trypsinized and split into 10cm dishes. Stable cells were selected for 1-week with 2.5µg/mL puromycin (Invivogen, ant-pr-1) and reduced to 1 µg/mL puromycin thereafter for maintenance.

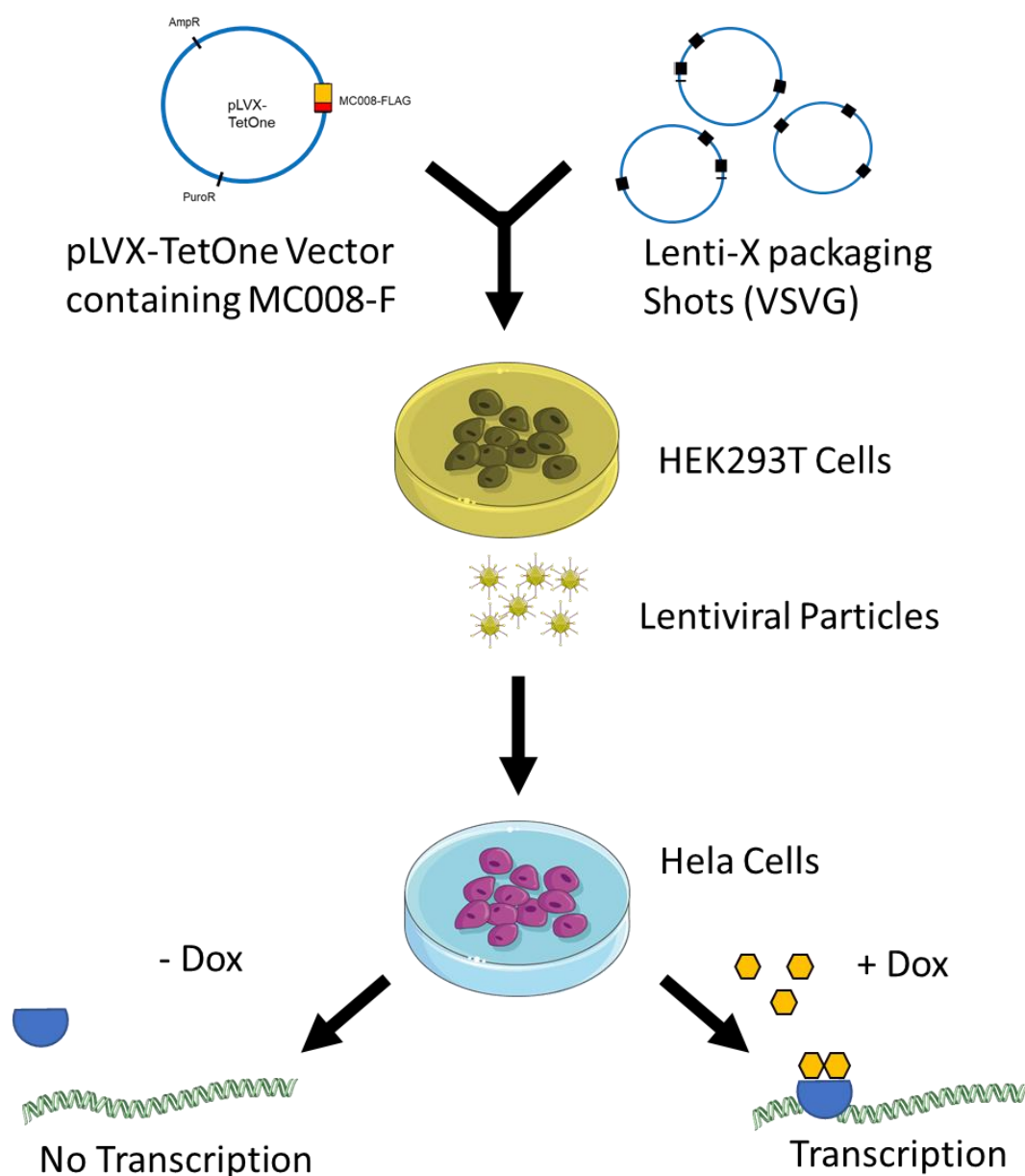


Figure 2.3 Overview of method used to generate MC008 stable HeLa cells using lentiviral transduction. Lentivirus stocks were generated by transfection of pLVXTetOne vector containing MC008-FLAG into HEK293T cells using Lenti-X™ Packaging Single Shots (VSV-G). The lentiviral stocks were then used to transduce HeLa cells which were subsequently selected for puromycin resistance. Doxycycline (Dox) was then added to the cells to induce MC008 expression. The figure elements were obtained from Servier Medical Art (<https://smart.servier.com/>), licensed under a Creative Common Attribution 3.0 Generic License.

2.12 Confocal Microscopy

Confocal microscopy is a high-resolution optical imaging technique. The microscope laser focuses on the sample at a defined region and depth leading to the emission of fluorescent light which enters the photomultiplier tube through a pinhole aperture, cutting-off out of focus signals. The light is converted to electrical signals by the photomultiplier tube and the computer builds up an image pixel by pixel (241). This method was used to visualize cellular localization of MC008 and colocalization with NEMO. By probing cells with fluorophore conjugated antibodies that emit light at specific wavelengths when excited by a laser, along with the appropriate use of filters, a multi-colour image can be produced.

Cells were seeded at 7×10^4 cells/well on 8-well chamber slides (Fisher Scientific, 1626066) and transfected 16 hours later. The next day, cells were washed in PBS and fixed with 4% (wt/vol) paraformaldehyde for 12 minutes. Cells were then permeabilised for 15 minutes with 0.5% (wt/vol) Triton X-100 in PBS and blocked for 1h in 5% (wt/vol) BSA-0.05% (vol/vol) Tween 20 in PBS. Cells were incubated overnight with anti-FLAG Alexa fluor 488 (Fisher Scientific, MA1-142-A488) and anti-HA Alexa fluor 647 (Fisher Scientific, 26183-A647) at 1:2000 and 1:500 in blocking solution, respectively. The slides were subsequently washed with 0.05% PBS Tween 20 and mounted in ProLong™ Gold Antifade Mountant with DAPI (Invitrogen, P36931). Images were obtained using a Leica SP8 confocal microscope with a 40x oil immersion objective.

2.13 Statistical Analysis

The data obtained from triplicate experiments is presented as the mean $\pm$ the standard deviation. Mean differences were compared to the positive control using two-way ANOVA with correction for multiple comparisons using Dunnett's test or Holm-Šidák

test on GraphPad Prism software version 8.0.2. Statistical significance is indicated by
* <0.05 , ** < 0.01 , *** <0.001 , and **** <0.0001 .

Chapter 3 Investigation of MC008 Targeting of TRIM32

MCV open reading frames (ORFs) were originally screened by the host laboratory for their ability to inhibit inflammatory signalling using a high throughput luciferase assay-based screening system and identified MC008 as a specific inhibitor of NF- κ B activation for further investigation prior to the beginning of this PhD.

MC008 is a 176-amino-acid protein (predicted 19 kDa) with an ORF located in the left-hand terminus of the MCV genome. Until recently, it was believed to be unique to MCV. However, an MC008-like sequence has been discovered in the recently described equine mollusum contagiosum-like virus with a 56% sequence identity to MCV subtype 1 MC008 (242).

After MC008 was identified by the host laboratory as an inhibitor of PRR and proinflammatory cytokine activation of NF- κ B, unbiased protein-protein interaction analysis by mass spectrometry also identified that MC008 associates with the E3 ligase TRIM32 which had previously been described as a regulator of the cGAS-STING DNA sensing pathway (1.1.6). Additionally, previous work indicated that MC008 inhibited TRIM32-mediated STING poly-ubiquitination (unpublished data). This chapter describes the investigation into MC008 targeting of TRIM32 as a potential mechanism of its inhibition of NF- κ B activation.

3.1 Lab-made PEI and Passive Lysis Buffer are Viable Alternatives to Commercial Reagents

Due to the large number of transfections and luciferase assays required for this project, we initially sought more cost-effective alternatives to commercial transfection reagents. GeneJuice® transfection reagent was compared to sterile filtered 1mg/mL lab-made polyethylenimine (PEI) (**Figures 3.1.1A-B**) by transfecting HEK293T cells with 25ng of cGAS and STING plasmids, parsing activation of NF- κ B or ISRE reporter constructs. As

MC008 was the focus of this project, the MC008-FLAG construct was included to ensure its specific inhibitory effects on NF- κ B remained consistent. We observed that the constructs transfected using GeneJuice® resulted in activation and subsequent inhibition of NF- κ B and potent activation of ISRE. Constructs transfected using PEI failed to drive NF- κ B and ISRE reporter activation to a meaningful extent, indicating that PEI was not suitable for small-scale transfections. However, when scaled up to 6-well plates, we observed similar expression levels of MC008 following transfection using either PEI or GeneJuice® (**Figure 3.1.1C**).

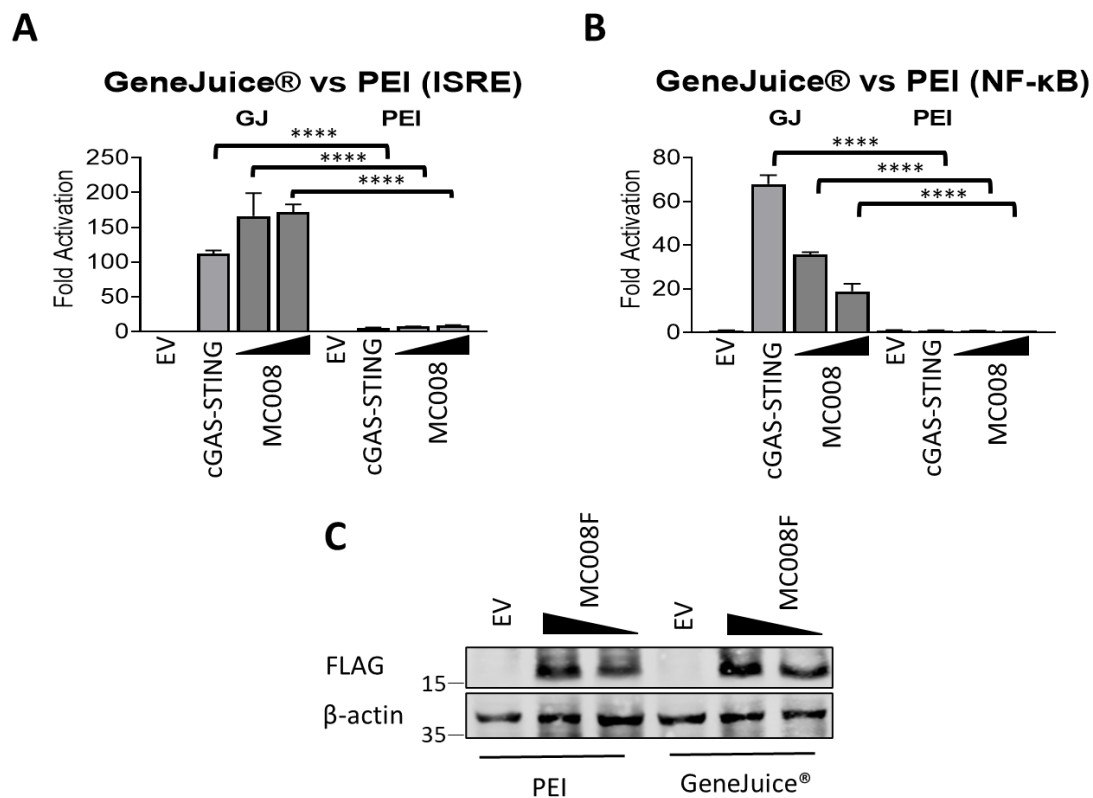


Figure 3.1.1 Assay validation and optimisation of lab-made versus commercial transfection reagents. (A & B) 2×10^5 /mL HEK293T cells were seeded for 16 hours and transfected with the reporter gene constructs indicated, 25ng of both cGAS-FLAG and STING-FLAG expression constructs, 25 or 50 ng of MC008 expression construct (indicated by wedge) and 25 or 50ng of empty vector (EV) expression construct. Transfection was carried out using GeneJuice® or 1 μ g/mL PEI. After 24 hours, NF-

κ B and ISRE reporter activity was measured to compare transfection efficacy of the two transfection reagents. (C) HEK293T cells were seeded in 6-well plates at 6×10^5 /well for 16 hours and transfected with EV expression constructs and at 2 and $4 \mu\text{g}$ of MC008-FLAG expression constructs using GeneJuice® (2:1 GJ:DNA) or 1mg/mL stock-PEI (3:1 PEI:DNA) for 24 hours. Cell lysates were then immunoblotted to compare the efficacy of the two transfection reagents. These data are the combined mean $\pm$ the standard deviation of triplicate samples ($n = 1$) and were analysed using two-way ANOVA with correction for multiple comparisons using the Holm-Šídák test. Statistical significance is indicated by * < 0.05 ** <0.01 , *** <0.001 and **** <0.0001 .

Passive lysis buffer is another costly reagent when sourced commercially, so we investigated an inexpensive, lab-made alternative. We compared this to the commercial reagent (**Figure 3.1.2**) by transfecting 12.5ng, 25ng, and 50ng of an activator (TRIM32 expression construct) along with the NF- κ B reporter gene construct and found that while there is a statistical difference between the two, the standard deviation of the increase in NF- κ B activity in each set remains similar (SD=5.3 for commercial and SD=6.6 for the lab-made). These results identify both PEI and lab-made passive lysis buffer as viable, inexpensive alternatives to GeneJuice® and commercial passive lysis buffer, respectively.

Commercial vs Lab-made Passive Lysis Buffer

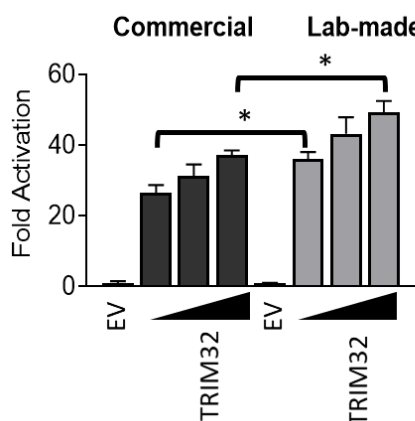


Figure 3.1.2 Assay validation and optimisation of lab-made versus commercial lysis reagents. 2×10^5 /mL HEK293T cells were seeded for 16 hours and transfected with the NF- κ B reporter gene construct, 12.5, 25 or 50ng of TRIM32-HA and EV expression constructs. After 24 hours, commercial or lab-made passive lysis buffer was used to lyse the cells and NF- κ B reporter activity was measured to compare the efficacy of both. This data is the combined mean $\pm$ the standard deviation of triplicate samples ($n = 1$) and was analysed using two-way ANOVA with correction for multiple comparisons using the Holm-Šídák test. Statistical significance is indicated by * < 0.05 **<0.01, ***<0.001 and **** <0.0001.

3.2 Establishing a Negative Control

To investigate the effects of MC008 under controlled conditions, we needed to establish a suitable negative control to be used in subsequent experiments. We tested a variety of MCV proteins that were shown to have little to no effect on inflammatory signalling during the initial screening process, alongside known published inhibitors MC132 and MC005 (226, 227). Both MC020 and MC052 expression consistently had no significant effect cGAS-STING induced NF- κ B and ISRE activity at all three concentrations (**Fig 3.2.1A-B**). MC014 and MC063 demonstrated statistically significant inhibition of NF- κ B and ISRE activity at higher concentrations of plasmid (**Fig 3.2.1C**). MC020 demonstrated a higher p-value in 5 out of 6 measurements when compared with MC052 (**Fig 3.2.1C**). Thus, MC020 was chosen as the negative control for subsequent MC008 experimentation.

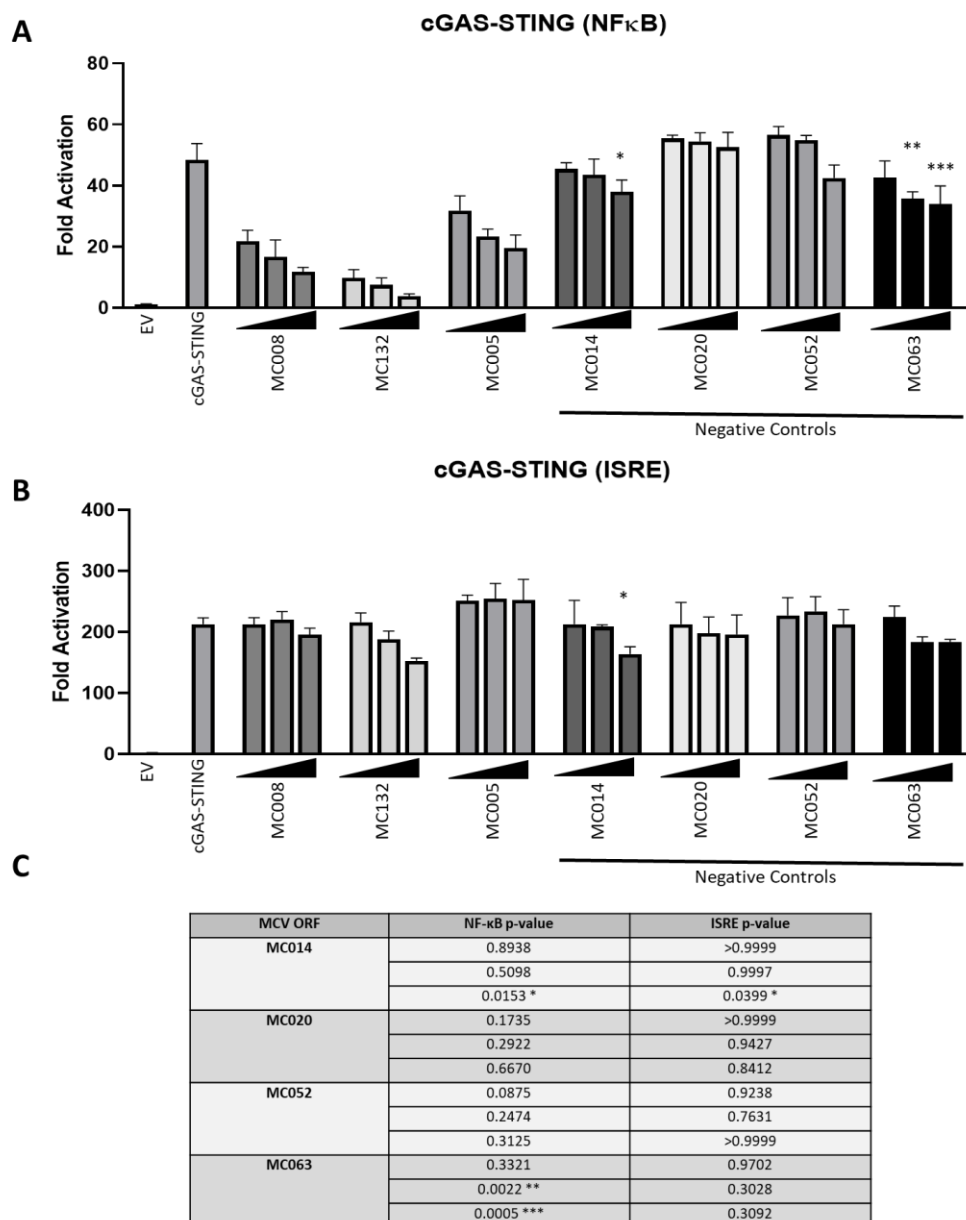


Figure 3.2.1 MC020 is a suitable negative control. (A & B) HEK293T cells were seeded at 2×10^5 cells/mL for 16 hours and transfected with 80ng of NF- κ B or ISRE luciferase reporter gene constructs and 40ng of TK Renilla reporter gene construct, along with EV and 10, 25 or 50ng of the MCV expression constructs indicated for 24 hours. cGAS (25ng) and STING (25ng) expression constructs were used to activate the pathways. The cells were subsequently lysed after 24 hours and assayed for NF- κ B and ISRE reporter activity. (C) This table indicates the two-way ANOVA p-values with correction for multiple comparisons using Dunnett's test for the measurements in A & B versus the positive control. Data is presented as fold activation and is the combined

mean $\pm$ the standard deviation of triplicate samples ($n = 1$). Statistical significance is indicated by * < 0.05 , ** < 0.01 , *** < 0.001 and **** < 0.0001 .

3.3 MC008 Associates with TRIM32

In the early stages of the investigation into MCV inhibitors of host immunity, affinity purification mass spectrometry was performed on MC008 immunoprecipitates and revealed an interaction with the E3 ligase TRIM32 and the E1 ubiquitin activating enzyme UBA1 (**Figure 3.3.1**) (collaboration with Andreas Pichlmair, TUM, Germany).

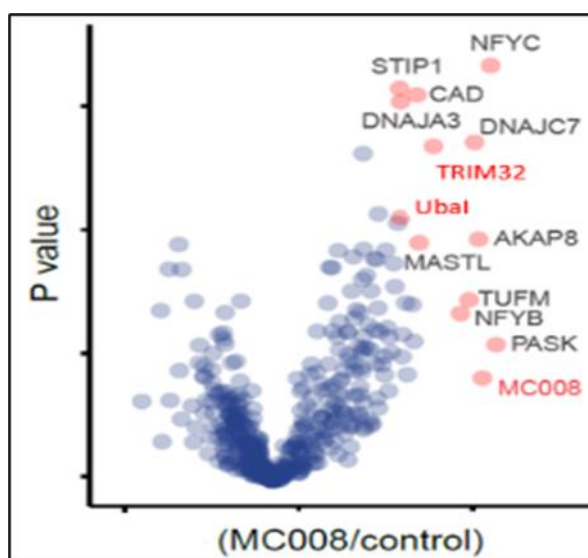


Figure 3.3.1 A volcano plot of affinity purification mass spectrometry results on MC008 immunoprecipitates. This plot indicates potential interactions between MC008 and various cellular proteins. The X axis indicates the fold change of protein immunoprecipitating with MC008 and the Y axis indicates the significance of the fold change based on the two-sample t test with the Welch correction. TRIM32 and UBA1 are highlighted (red) as they have been shown to have a regulatory role in the immune response following viral sensing. This data was obtained by Dr. Andreas Pichlmair in collaboration with Prof. Gareth Brady and has been included in this thesis to give context to the initial course of investigation.

Since we observed that MC008 inhibited cGAS-STING driven NF- κ B but not ISRE activity in sections 3.1 and 3.2, and an interaction between TRIM32 and MC008 was identified through mass spectrometry, we investigated the role of TRIM32 in more detail. TRIM32 was previously shown to play a regulatory role in the cGAS-STING system by driving poly-ubiquitination of STING (73). A later report demonstrated that TRIM32 generates ubiquitin chains that can bind to NEMO and drive NF- κ B signalling (82). Therefore, we hypothesised that TRIM32 is responsible for NF- κ B induced signalling in this pathway and that MC008 was disrupting this function.

The previously reported MCV proteins MC005 and MC132 were used as control inhibitors for subsequent luciferase assays. These proteins target NEMO and NF- κ B p65, respectively (226, 227). MC020 was used as a negative control as previously validated in Figure 3.2. We tested expression of MC008 at concentrations similar to those used in luciferase assay-based screening for MC005 and MC132 plasmids. All MCV proteins expressed to approximately equivalent levels at the upper concentration (**Figure 3.3.2A**). We observed that MC008, MC005 and MC132 inhibited NF- κ B activity following expression of TRIM32 (**Figure 3.3.2B**).

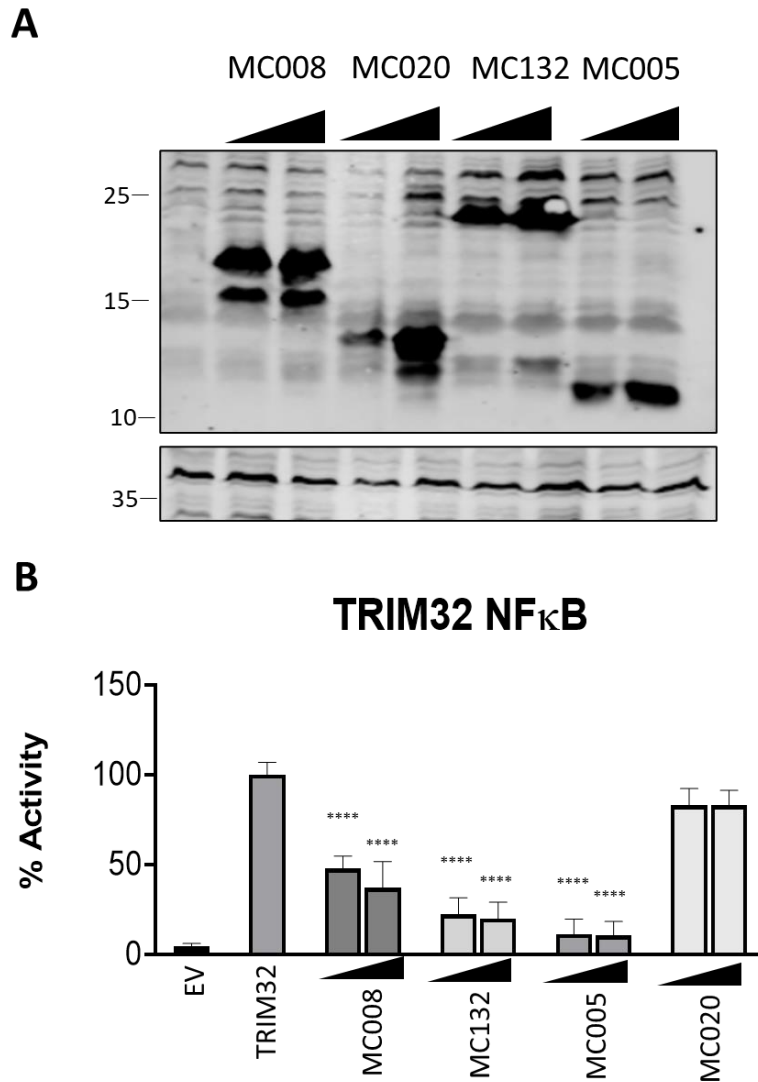


Figure 3.3.2 MC008 co-immunoprecipitates with TRIM32 and inhibits TRIM32-driven NF- κ B activity. (A) 6×10^5 HEK293T cells were seeded in 6-well plates for 16 hours then transfected with 2 μ g or 4 μ g of MC008-FLAG, MC005-FLAG, MC020-FLAG and MC132-FLAG expression constructs. After 24 hours, an immunoblot was performed on the lysates followed by probing with anti-FLAG and anti- β -actin antibodies. (B) HEK293T cells were seeded in 96-well plates at 2×10^5 cells/mL and transfected with 80ng of NF- κ B reporter gene construct and 40ng of TK Renilla reporter gene construct, along with EV and 25 or 50ng of the MCV expression constructs indicated for 24 hours. TRIM32 expression was used to activate the pathway. The cells were subsequently lysed and assayed for NF- κ B reporter activity. Data is presented as the percentage of reporter activation compared to the positive control and are the combined mean $\pm$ the standard deviation of triplicate samples from

representative experiments (n = 3). Statistical significance was determined using two-way ANOVA with correction for multiple comparisons using Dunnett's test and is indicated by * < 0.05, ** < 0.01, *** < 0.001 and **** < 0.0001.

Interestingly, whilst TRIM32 potentially activated NF- κ B in HEK293T cells, we did not observe ISRE activation, suggesting a specific role for this protein in NF- κ B activation which MC008 inhibits (**Figure 3.3.3**).

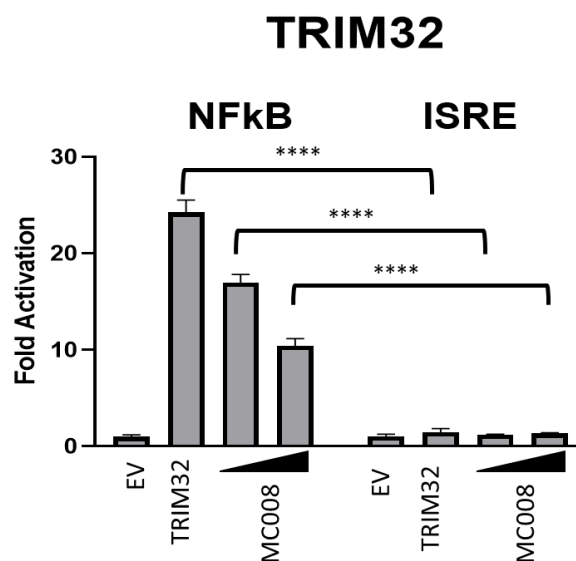


Figure 3.3.3 TRIM32 does not activate ISRE activity in HEK293T cells. HEK293T cells were seeded at 2×10^5 cells/mL for 16 hours and transfected with 80ng of NF- κ B or ISRE reporter gene constructs and 40ng of TK Renilla reporter gene constructs, along with EV and 25 or 50ng of MC008 expression constructs for 24 hours. TRIM32 expression construct was used to activate the pathway. The cells were subsequently lysed and assayed for NF- κ B and ISRE reporter activity. Data is presented as fold activation and are the combined mean $\pm$ the standard deviation of triplicate samples from representative experiments (n = 3). Data was analysed using two-way ANOVA with correction for multiple comparisons using the Holm-Šídák test. Statistical significance is indicated by * < 0.05, ** < 0.01, *** < 0.001 and **** < 0.0001.

To confirm that MC008 is targeting TRIM32, we investigated the ability of MC008 to co-immunoprecipitate with this protein. We observed an interaction between the two proteins, providing further evidence that TRIM32 is a target of MC008.

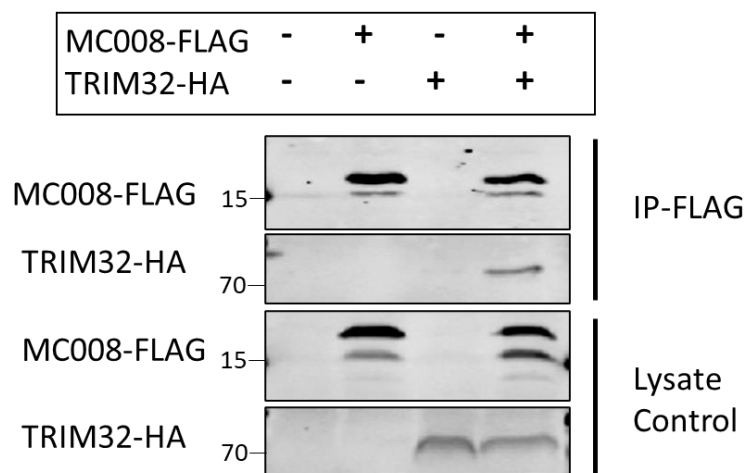


Figure 3.3.4 MC008 co-immunoprecipitates with TRIM32. 4 μ g of MC008 and 4 μ g of TRIM32 expression constructs were transiently expressed in 4×10^6 HEK293T cells per plate. The cells were subsequently lysed using ice-cold lysis buffer with a cocktail of phosphatase and protease inhibitors and co-immunoprecipitation was performed. The resulting immunoprecipitants were then immunoblotted with anti-HA and anti-FLAG antibodies. A representative blot (n = 3) is shown.

3.4 Investigating MC008 Inhibition of STING-activated Signalling Through Targeting TRIM32

We next investigated the mechanism by which MC008 exerts its inhibitory effects on TRIM32. In the previous section, we observed that MC008 interacts with TRIM32. Since TRIM32 is an E3 ligase, and it has been shown to poly-ubiquitinate STING (73), we co-expressed MC008, STING and TRIM32 in HEK293T cells and performed co-immunoprecipitation (**Figure 3.4.1**). However, we did not observe a consistent increase

in STING poly-ubiquitination, and on multiple occasions (n = 5), TRIM32 did not immunoprecipitate with STING.

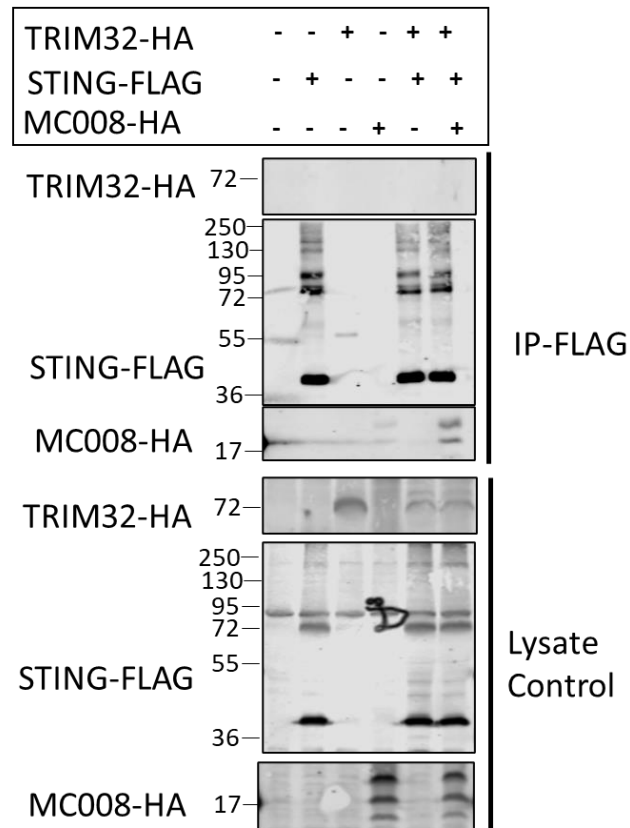


Figure 3.4.1 TRIM32 does not interact with STING when TRIM32 is solubilised at low levels. 4×10^6 HEK293T cells per plate were seed in 10cm dishes. 16 hours later, the cells were transfected with 3 μ g of MC008-HA, STING-FLAG and TRIM32-HA expression constructs. The cells were subsequently lysed using ice-cold lysis buffer with a cocktail of phosphatase and protease inhibitors, followed by co-immunoprecipitation. Representative experiment (n = 5).

As expression levels of TRIM32 appear relatively low compared to other proteins, we titrated the concentration of both TRIM32 and STING in an attempt to address this issue (**Figure 3.4.2**). It was noted that when TRIM32 was introduced, there was a minor increase in STING poly-ubiquitination which correlated with increases in TRIM32

expression levels. However, since this was performed by directly lysing in sample buffer which solubilises all but the most insoluble proteins, and we achieved higher levels of TRIM32 expression than in the co-immunoprecipitation experiments, this indicated that the lysis buffer used for these experiments was insufficiently stringent.

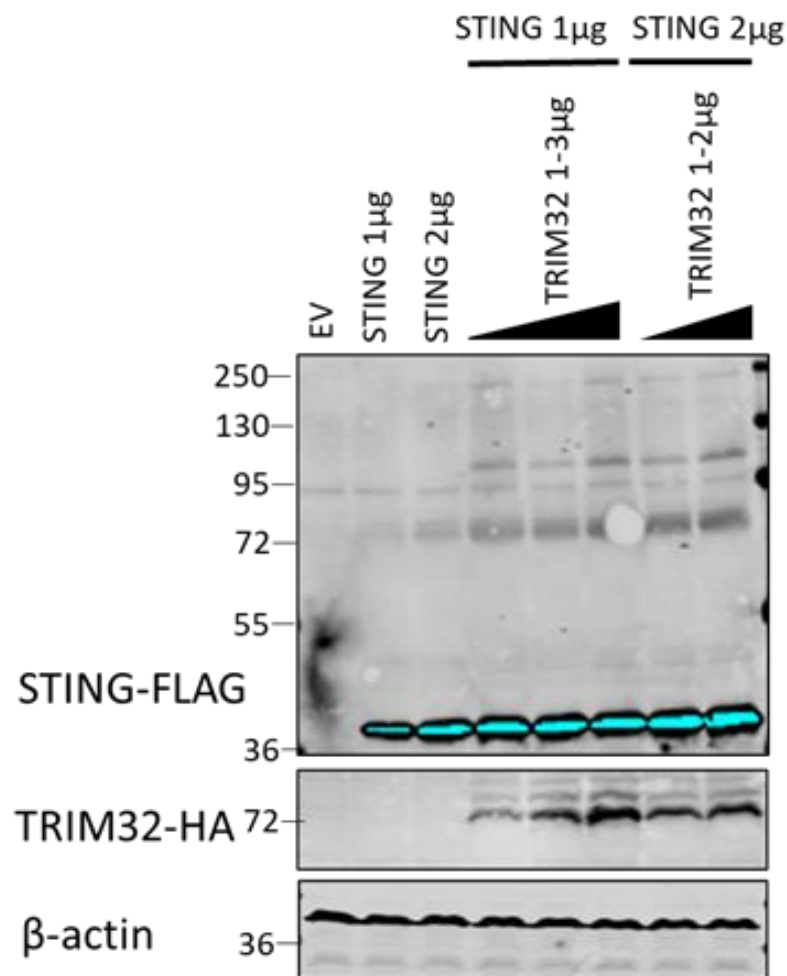


Figure 3.4.2 TRIM32 and STING plasmid titration. HEK293T cells were seeded in 6-well plates at 6×10^5 /well and transfected with EV, 1 or 2 μ g of STING-FLAG expression construct, or 1-3 μ g of TRIM32 expression construct for 24 hours. Cell lysates were then immunoblotted and probed for FLAG, HA and β -actin with their respective antibodies. Representative experiment (n = 1).

Since salt increases the ionic strength of buffer solutions and detergents are required to solubilise intracellular compartments, we titrated NaCl and detergent in the lysis buffer.

We found that increasing NaCl concentration to 600mM, but not the detergent, approximately doubled the level of TRIM32 expression observed (**Figure 3.4.3**). Hence, this NaCl concentration was used for subsequent co-immunoprecipitation experiments involving TRIM32.

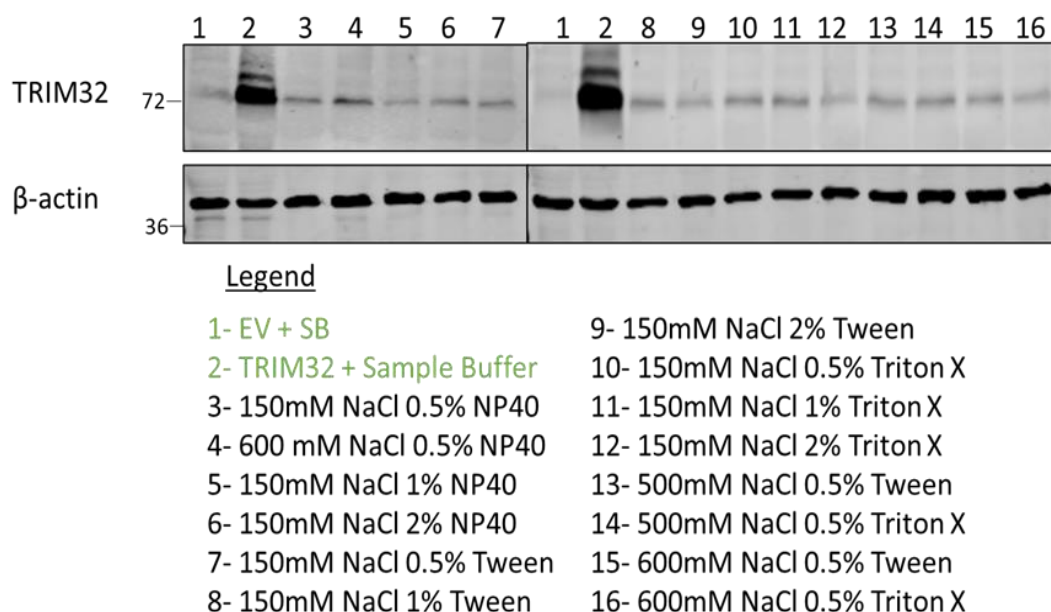


Figure 3.4.3 NaCl and detergent titration of immunoprecipitation lysis buffer for TRIM32 solubilisation. 1.5×10^5 HEK293T cells/well were seeded in 24-well plates. 16 hours later 500ng of TRIM32 or EV expression constructs were transfected into each well as indicated. 24 hours later, cells were lysed with immunoprecipitation lysis buffer containing the indicated quantities of NaCl and detergent (NP-40, Tween-20, and Triton X-100) or sample buffer. Cell lysates were then immunoblotted and probed with anti-HA and anti- β -actin antibodies. Representative experiment (n = 2).

Following this, we once again co-expressed MC008, STING and TRIM32 in HEK293T cells and performed co-immunoprecipitation, demonstrating higher levels of TRIM32 expression than in figure 3.4.1. When higher levels of TRIM32 were solubilised, we observed an interaction between TRIM32 and STING (**Figure 3.4.4**). However, we did

not observe any notable increase to STING poly-ubiquitination after TRIM32 introduction or inhibition following MC008 introduction.

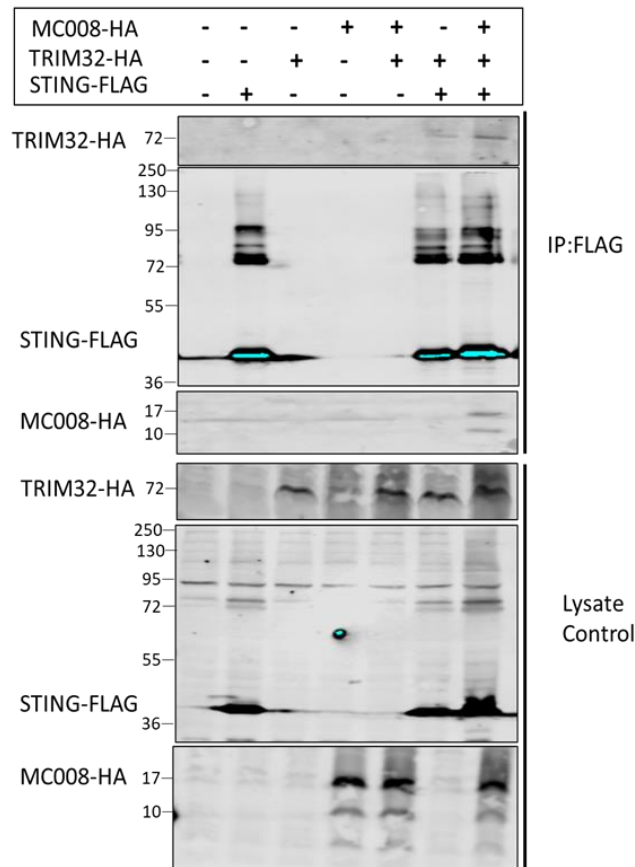


Figure 3.4.4 TRIM32 does not significantly increase STING poly-ubiquitination.

Similar to 3.4.1, 4×10^6 HEK293T cells per plate were seeded in 10cm dishes. 16 hours later, the cells were transfected with $3 \mu\text{g}$ of MC008-HA, STING-FLAG and TRIM32-HA expression constructs. The cells were subsequently lysed using ice-cold lysis buffer containing 600mM NaCl, followed by co-immunoprecipitation. Representative experiment ($n = 3$).

To boost the potential level of STING ubiquitination by TRIM32, we investigated the consequences of adding ubiquitin-HA and titrating both STING and TRIM32 (**Figure 3.4.5**). While increased ubiquitination was observed in all lanes containing ubiquitin-HA, we did not observe any significant effect on STING poly-ubiquitination in the presence

of TRIM32. Furthermore, we did not observe any inhibitory effect in lanes expressing MC008.

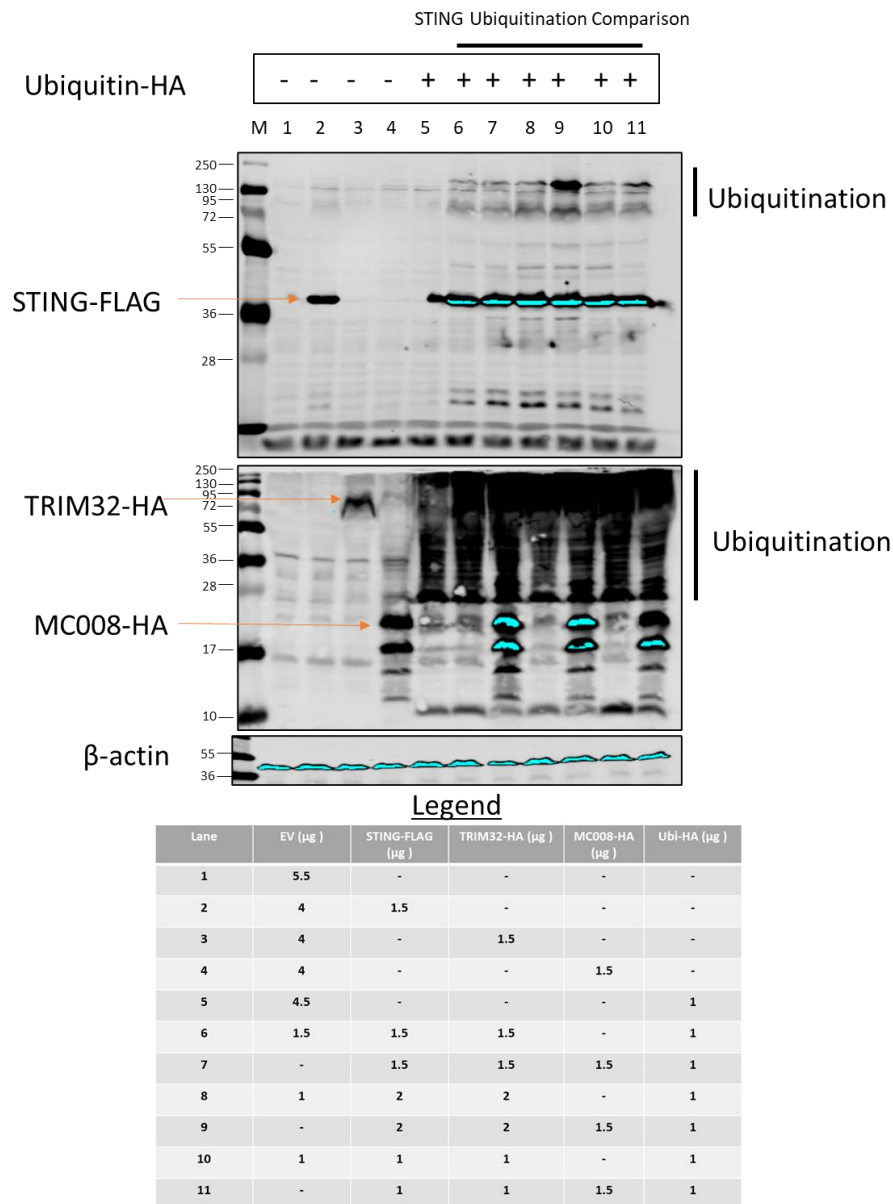


Figure 3.4.5 Addition of Ubiquitin-HA does not boost TRIM32-driven poly-ubiquitination of STING. HEK293T cells were seeded in 6-well plates at 6×10^5 /well for 16 hours and transfected with EV, STING-FLAG, TRIM32, MC008 or ubiquitin-HA expression constructs, according to the table, for 24 hours. Cell lysates were then immunoblotted and probed for FLAG, HA and β -actin with their respective antibodies. Representative experiment (n = 1).

3.5 Investigating MC008 Inhibition of the NEMO-TRIM32 Axis

It was previously reported that TRIM32-generated ubiquitin chains were required for poly-ubiquitination of NEMO following activation of the cGAS-STING pathway (82). Therefore, we hypothesised that MC008 might be preventing these TRIM32-generated ubiquitin chains from binding to NEMO, thus inhibiting inflammatory signalling. To investigate this, we co-expressed MC008, NEMO and TRIM32 in HEK293T cells and performed co-immunoprecipitation on the lysates (**Figure 3.5.1**). Similar to the previous experiments, we did not observe any obvious increase in NEMO poly-ubiquitination, but we did observe an interaction between TRIM32 and NEMO. Furthermore, baseline ubiquitination of NEMO was reduced in the presence of MC008, suggesting a potential inhibitory effect at this point in the pathway.

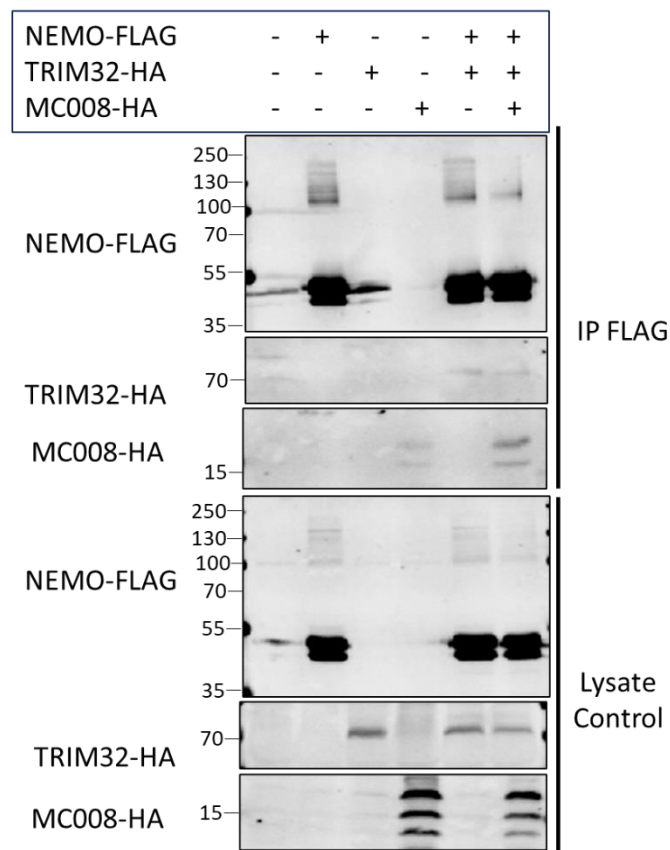


Figure 3.5.1 TRIM32 does not induce poly-ubiquitination of NEMO but MC008 appears to inhibit baseline ubiquitination of NEMO. 3µg of MC008-HA and TRIM32-HA expression constructs, along with 2µg of NEMO-FLAG expression construct were transiently expressed in 4x10⁶ HEK293T cells per plate. The cells were subsequently lysed using ice-cold lysis buffer with a cocktail of phosphatase and protease inhibitors and co-immunoprecipitation was performed. Blots were then probed with anti-FLAG and anti-HA antibodies. Representative experiment (n = 3).

HaCaT cells are keratinocytes (for which MCV has a tropism) and have an intact DNA sensing system (unlike HEK293T cells), thus making them an ideal cell line to investigate the effects of MC008 on an endogenous system. Since we observed an interaction between TRIM32 and NEMO, we aimed to investigate the effect of knocking down TRIM32 on the ability of MC008 and NEMO to interact. To optimise the knockdown, we transfected a TRIM32 small, interfering RNA (siRNA) and a control siRNA into HaCaT cells (**Figure 3.5.2**). We observed what appeared to be a reduction in TRIM32 expression when using 60nmol of siRNA for 24 hours. However, upon repeating this experiment over both 24 and 48 hours, we did not observe any significant difference between TRIM32 expression in the TRIM32 siRNA lanes and the control lanes.

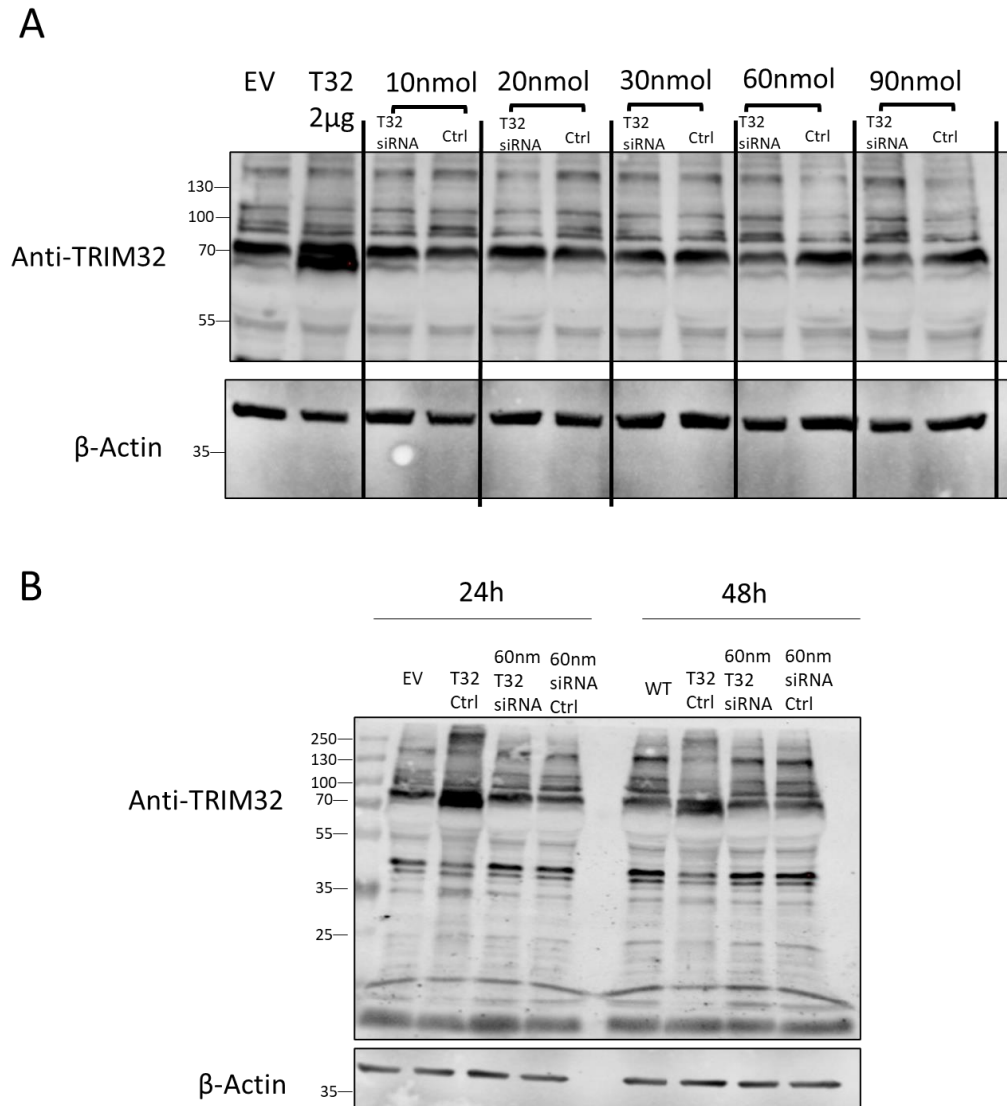


Figure 3.5.2 Optimisation of TRIM32 siRNA. (A) 3×10^5 HaCaT cells/well were seeded in 6-well plates for 16 hours. Cells were then transfected with $2 \mu\text{g}$ of TRIM32 expression construct or a range of concentrations of TRIM32 siRNA and control siRNA, as indicated, for 24 hours. Cells were subsequently lysed and immunoblotted. The blots were probed with anti-TRIM32 or anti- β -actin antibodies. (B) Similar to A but the cells were transfected with 60nmol of siRNA for either 24 or 48 hours. Representative experiments ($n = 1$).

3.6 Discussion

3.6.1 Commercial and Lab Reagents

While the use of costly commercial reagents is the mainstay of most molecular research, some of these reagents can be made in the lab with relative ease and have a similar efficacy to their commercial counterparts, thus making them suitable for certain types of research. A commonly used transfection reagent is GeneJuice® which provides high levels of transfection. However, GeneJuice® can cost upwards €250-€300/mL from Merck Millipore which makes it an expensive reagent, particularly when considering the large volumes required for transfecting 10cm dishes during co-immunoprecipitation experiments. Branched PEI is a less expensive alternative, with 100mL containers available from SIGMA-Aldrich for approximately €73. The use of PEI to deliver DNA intracellularly has been described since the mid-90's (243). This effect is largely due to its ability to condense the negatively charged phosphate backbone of DNA with its highly dense positive charge, enabling the DNA to bind to the surface of cells and be taken up via endocytosis (243-245). While PEI was unsuitable for small-scale transfections, on larger scales it is sufficient to observe effective levels of plasmid expression in cells, making this reagent an appropriate and cost-effective option. This result is supported by the report by Hsu and Uludag (244) which describe that the small volume of PEI generally results in the sporadic toxicity of cells due to unstable DNA aggregates. The surface area of a well in 6-well plate is ~30 times greater than that of a 96-well plate. However, the final volume of media per well is only 10 times greater in 6-well plate (2mL) compared to a 96-well plate (200µL). In the 96-well plate there is a higher concentration of DNA/PEI which can lead to an increased frequency of intermolecular interactions, given

the smaller volume. This may lead to improper DNA condensation, resulting in aggregate formation (244).

Passive lysis buffer is another relatively expensive reagent, and when sourced from Promega, it can cost upwards of €80 for 30mLs of 5X concentrated buffer (Promega, E194). An online search resulted in the identification of a technical bulletin from Promega (240) describing a lab-made version of this passive lysis buffer. Following this protocol, we lysed the cells with both the commercial and lab-made versions of the passive lysis buffer and performed a luciferase assay. There was a notable increase in the fold activation of NF- κ B activity in the cells lysed with the lab-made lysis buffer. This may be the result of a difference in detergent concentration between the lysis buffers, as it was noticed when lysing that there was increased foaming in the wells containing the lab-made version. However, a similar pattern between the commercial and lab-made lysis buffers was observed, suggesting that this buffer was suitable for future experiments.

3.6.2 MC020 is a Suitable Negative Control

Establishing a suitable negative control for the luciferase assays was another necessary step prior to large scale pathway mapping. While MC014 was used in past publications as a negative control (226, 227), expression of this protein consistently led to low levels of inhibition for both NF- κ B and ISRE signalling during this investigation. We were unable to determine the cause of this phenomenon and since multiple other negative controls were readily available, we limited our investigation due delays already incurred. Nevertheless, we overcame this issue by screening a variety of MCV proteins that demonstrated a lack of effect on inflammatory signalling, thereby identifying MC020 as a suitable negative control for subsequent MCV experiments.

3.6.3 MC008 Targeting of TRIM32

Preliminary mass spectrometry data suggested that TRIM32 was an MC008 binding partner. TRIM32 is a ubiquitous protein that plays a role in a variety of pathophysiological processes such as innate immunity (73, 82), neuronal development and differentiation (246), and tumorigenesis (247). TRIM32 consists of an N-terminal Really Interesting New Gene (RING) domain, a B-box domain, and a coiled-coiled domain, along with 6 NCL-1, HT2A, LIN-41 (NHL) repeats in the C-terminus (248). The RING domain is responsible for the E3 ligase activity (249). It interacts with the E2 ubiquitin conjugating enzyme along with the target protein substrate, thus bringing the two into close proximity and initiating the transfer of a ubiquitin molecule. Genetic mutations to TRIM32 NHL repeats can result in muscle disorders such as limb-girdle muscular dystrophy (250).

While we observed that MC008 can inhibit TRIM32-driven NF- κ B activation, and that MC008 can interact with this protein during co-immunoprecipitation, the exact mechanism of how TRIM32 drives this pathway remains poorly understood. Zhang et al. report activation of both NF- κ B and ISRE signalling in response to TRIM32 over expression (73). However, we only observed activation of NF- κ B. The discrepancy here is likely due to the use of HEK293T cells which do not possess a competent cGAS-STING system (63, 251), whereas Zhang et al, used the parental HEK293 cells which do express endogenous cGAS and STING (73, 251, 252).

Following the identification of TRIM32 as an MC008 binding partner by mass spectrometry, further preliminary evidence from the host lab prior to commencement of this PhD suggested a potential role for MC008 as an inhibitor of STING poly-ubiquitination (data not shown). We aimed to replicate this by overexpressing TRIM32,

STING and MC008. However, this was unsuccessful following multiple optimisation attempts. These attempts included titrating the plasmid concentrations, altering the salt and detergent levels in the immunoprecipitation lysis buffer, using different batches of HEK293T cells and remaking all reagents, including plasmid preparations from various stocks. Higher levels of expression of TRIM32 evident in the samples lysed with sample buffer indicated that even with the increased ionic strength of 600mM NaCl, much of the protein is lost in the cell debris. The enhanced efficacy of the sample buffer at lysing this protein is likely due to the 2% SDS content which completely solubilises membrane proteins amongst other hard to solubilise proteins (253). Unfortunately, using SDS as a detergent in co-immunoprecipitation experiments is not recommended as it would likely inactivate the antibodies attached to the anti-FLAG beads (254).

While overexpression of TRIM32 has been shown to correlate with a boost in STING poly-ubiquitination (73), it was also shown in a later study that TRIM32 does not specifically poly-ubiquitinate STING, but likely targets other elements in the STING complex (72). Nevertheless, we didn't observe this enhanced poly-ubiquitination of STING after one round of co-immunoprecipitation, which was shown in both studies.

3.6.4 MC008 Targeting of the TRIM32-NEMO Axis

Fang et al. (82) reported that TRIM32 generated free ubiquitin chains in HEK293T cells that can bind to NEMO following activation of the cGAS-STING system. However, we did not observe increased poly-ubiquitination of NEMO when the two proteins were overexpressed. We did, however, observe an interaction between TRIM32 and NEMO, along with a reduction in baseline levels of NEMO ubiquitination following introduction of MC008. Another possible mechanism by which TRIM32 is driving NF- κ B activation is through protein inhibitor of activated STAT γ (PIAS γ). Increased expression of

TRIM32 leads to degradation of PIAS γ and subsequent upregulation of NF- κ B activity associated with limb-girdle muscular dystrophy (255). However, the molecular role of TRIM32 in NF- κ B activation remains to be fully elucidated.

Attempts to knockdown TRIM32 were also unsuccessful. However, given the significant number of siRNAs available with subtly different sequences, it's possible that the chosen siRNA was not compatible with TRIM32 in the cells. It is also unlikely that knocking down TRIM32 would have a profound effect on inflammatory signalling. Fang et al. (82) report TRIM32 CRISPR-Cas9 knockout cells has no effect on DNA stimulated IKK activation. IKK activation was only perturbed when both TRIM32 and TRIM56 were knocked out, suggesting redundancy of these two proteins in the pathway.

3.7 Conclusion

In this chapter we confirmed that PEI and lab-made passive lysis buffer are viable alternatives to commercial transfection reagents and lysis reagents, respectively. We were also able to identify MC020 as an effective negative control for subsequent reporter gene assays. While we showed that MC008 interacts with TRIM32 and inhibits TRIM32-driven NF- κ B activity, we encountered technical difficulties relating to TRIM32 extraction, ubiquitination and knockdown, along with uncertainties surrounding the role of TRIM32 in innate immune signalling based on the reports by Fang et al. (82). This led us to change our focus towards the IKK protein, NEMO, since we observed an inhibitory effect on NEMO ubiquitination and an interaction between TRIM32 and NEMO. This coupled with the fact NEMO has a well-defined role and is a convergence point for many pathways involved in inflammatory signalling, make this protein an attractive target for further exploration.

Chapter 4 Investigation of MC008 Inhibition of Innate Signalling Pathways

*This chapter contains data submitted to the Journal of Virology and has been **accepted for publication**.
Phelan T, Lawler C, Pichlmair A, Little M, Bowie A, Brady G. Molluscum Contagiosum Virus Protein
MC008 targets NF- κ B Activation by Inhibiting Ubiquitination of NEMO.*

In the previous chapter, we showed the association of MC008 with TRIM32, an E3 ligase known to have a role in regulating the cGAS-STING signalling pathway. We also demonstrated MC008 inhibition of NF- κ B activation following TRIM32 overexpression. However, despite our analysis of several events in signalling thought to involve this protein, we were unable to attribute MC008 inhibition of proinflammatory signalling to targeting of TRIM32. In this chapter we aim to locate where the inhibitory effects of MC008 on NF- κ B signalling are bypassed, thereby directing us toward the target region of pathway.

4.1 MC008 Inhibits DNA-induced Proinflammatory Cytokine Release

We generated a tetracycline-inducible stable MC008-expressing line using HeLa cells which possess an intact DNA sensing system (82), as detailed in section 2.11 of the methods. These cells were chosen as we were unable to obtain stable integration of MC008 in HaCaT cells. The concentration of doxycycline required to induce optimal MC008 expression was titrated from 100ng to 2 μ g (**Figure 4.1.1**). As we observed similar levels of MC008 expression at all concentrations tested, we therefore performed subsequent experiments using the lowest concentration (100ng/mL).

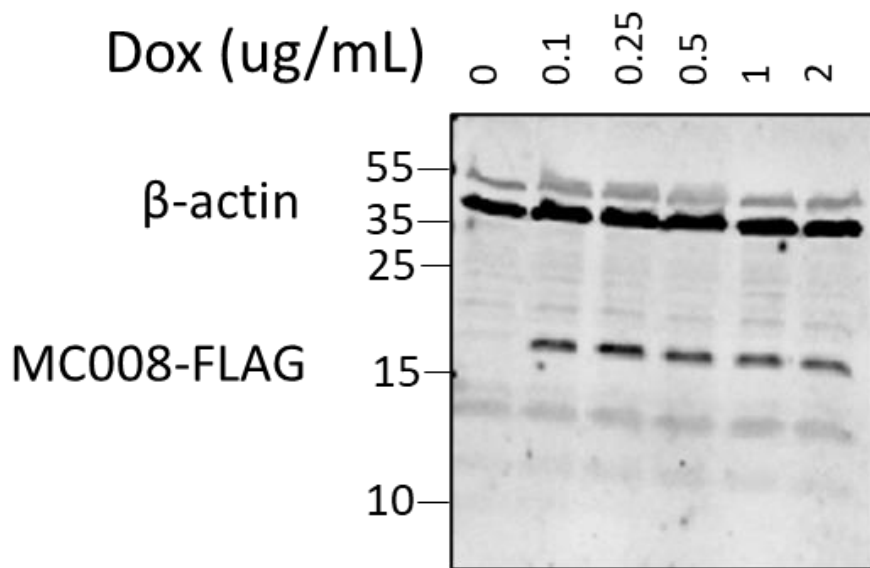


Figure 4.1.1 Doxycycline titration in stable HeLa cells. HeLa cells were transduced with lentivirus containing lenti-X tet-inducible MC008-FLAG. Following selection, 3×10^5 cells/well were seeded in a 6-well plate for 16 hours. Cells were then stimulated with the concentrations of doxycycline indicated for 24h to induce MC008 expression. Cells were subsequently lysed 24h later, followed by immunoblotting (n = 1).

To test the effect of MC008 expression on proinflammatory cytokine production, we measured IL-6 levels in cell supernatants. IL-6 levels were measured in both induced and uninduced cells following stimulation by transfection of poly(dA:dT), and MC008 expression resulted in a significant decrease in IL-6 release (**Fig 4.1.2A-B**).

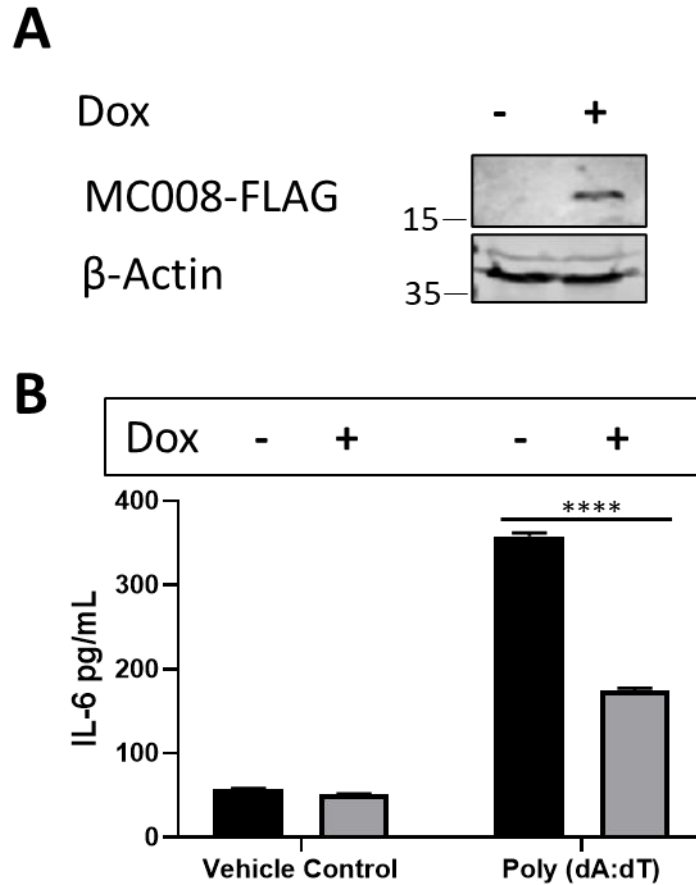


Figure 4.1.2 MC008 inhibits proinflammatory cytokine production. (A) 1×10^5 stable HeLa cells/mL were stimulated with 100ng/mL doxycycline and subsequently transfected with 1ug/mL poly(dA:dT) for 24h following MC008 induction. Cell lysates for the poly(dA:dT) transfection were immunoblotted and probed with anti-FLAG and anti- β -actin antibodies (B) Cell supernatants were subsequently harvested, and IL-6 levels were measured using ELISA. Serum-free medium and lipofectamine 2000 was used as the vehicle control (VC). Representative experiment shown (n = 3). Statistical significance was determined using two-way ANOVA with correction for multiple comparisons using the Holm-Šídák test and is indicated by * < 0.05, ** < 0.01, *** < 0.001 and **** < 0.0001.

4.2 Analysis of MC008 Localisation Using Confocal Microscopy

Next, we examined where MC008 was localising in cells by transiently transfecting HEK293T cells with MC008-HA, followed by analysis using laser scanning confocal microscopy. Confocal analysis revealed that MC008 localises throughout HEK293T cells with strong staining in the nucleus (**Figure 4.2.1**). However, low levels of MC008 can also be observed in the cytoplasm.

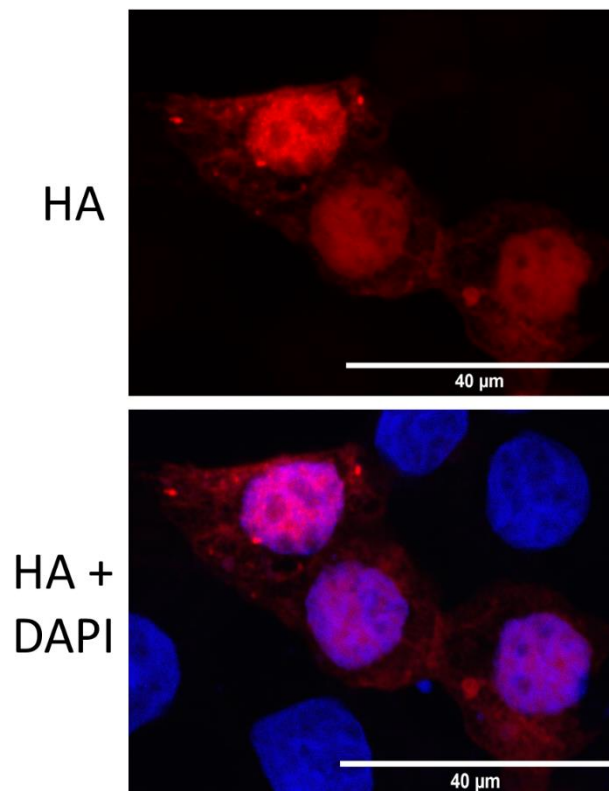


Figure 4.2.1 MC008 is predominantly nuclear. Localisation of MC008 in HEK293T cells. Cells were transfected with 100ng of pCEP4-MC008-HA expression construct and were fixed 24h later. Cells were subsequently stained with DAPI (blue) or probed with anti-HA (red) for MC008 expression. Representative experiment shown (n = 3).

4.3 Luciferase Pathway Mapping of MC008 Inhibitory Effects

To identify where MC008 effects were bypassed, we employed a luciferase assay-based screen, driving pathway activation with ligands or overexpressing key elements of signalling pathways to simulate activation at distinct points, parsing effects on activation using NF- κ B and ISRE (IRF-inducible) reporters. Both MC008 and MC020 proteins expressed to approximately equivalent levels (**Figure 4.3.1A**).

The cGAS-STING pathway, a key DNA sensing pathway, can be reconstituted in HEK293T cells by co-expressing cGAS and STING constructs as they are not usually detectable in these cells (256). Whilst MC008 expression potently inhibited activation of NF- κ B by this system, it had no effect on cGAS-STING-induced IRF activation (ISRE and IRF3-GAL4 reporters) (**Figure 4.3.1B-D**). The RIG-I pathway has been reported as an indirect poxvirus DNA sensing system in HEK293T cells, enabled by the presence of RNA polymerase III which transcribes AT-rich DNA into an RNA ligand (257). As such, both Poly(dA:dT) and mitochondrial antiviral-signalling protein (MAVS) expression were used as activators of the RIG-I signalling pathway. As with DNA sensing signalling, MC008 was unable to inhibit activation of ISRE by this pathway but potently inhibited NF- κ B activation (**Figure 4.3.1E-G**).

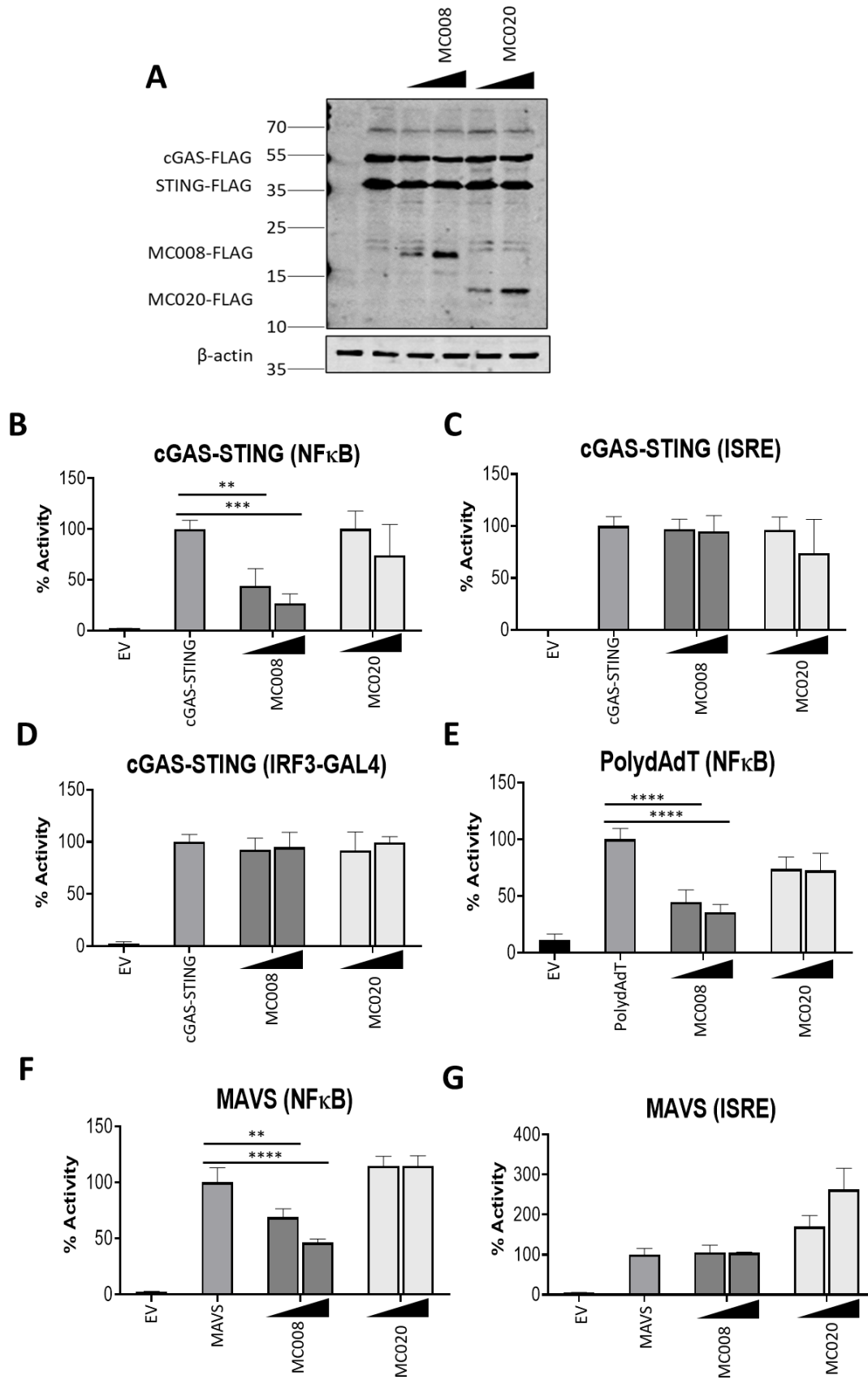


Figure 4.3.1 MC008 inhibits cytosolic nucleic acid sensing pathway activation of NF- κ B. (A) HEK293T cells were seeded at 2×10^5 cells/mL and transfected with 80ng of NF- κ B and 40ng of TK Renilla reporter gene construct, along with empty vector (EV) and 25 or 50ng of the MC008-FLAG and MC020-FLAG expression vectors for 24 hours. Cell lysates were subsequently harvested, and a western blot was performed. (B-G) Similarly, HEK293T cells were seeded at 2×10^5 cells/mL and transfected with 80ng of NF- κ B, ISRE or pFR-luciferase reporter gene constructs and 40ng of TK Renilla reporter gene construct, along with EV and 25 or 50ng of the MCV expression constructs indicated for 24 hours. cGAS (25ng) and STING (25ng), 10ng of MAVS, 5ng of IRF3-GAL4 expression constructs or 1 μ g/mL of poly(dA:dT) were used to activate the pathways. The cells were subsequently lysed after 24 hours and assayed for NF- κ B and ISRE or IRF3-GAL4 reporter activity. Data is presented as the percentage of reporter activation compared to the positive control and are the combined mean $\pm$ the standard deviation of triplicate samples from representative experiments (n = 3). Statistical significance was determined using two-way ANOVA with correction for multiple comparisons using Dunnett's test and is indicated by * < 0.05, ** < 0.01, *** < 0.001 and **** < 0.0001.

Next, we examined the effect of MC008 on TLR-driven signalling by overexpressing a constitutively active CD4 fusion of TLR3 and two downstream adaptor proteins used in TLR signalling, its adapter TRIF, and MyD88, an adapter utilised by all TIR-domain containing receptors except TLR3 (258). Consistent with our other observations, MC008 inhibited NF- κ B activity in CD4TLR3, MyD88 and TRIF-driven signalling (**Figure 4.3.2A-C**). While TRIF activates both NF- κ B and ISRE, MC008 was again unable to inhibit the ISRE-activating arm of the response in these contexts (**Figure 4.3.2D**). Together, these data suggested that MC008 is a specific inhibitor of NF- κ B activation and targets at a downstream point common to all innate sensing pathways we investigated.

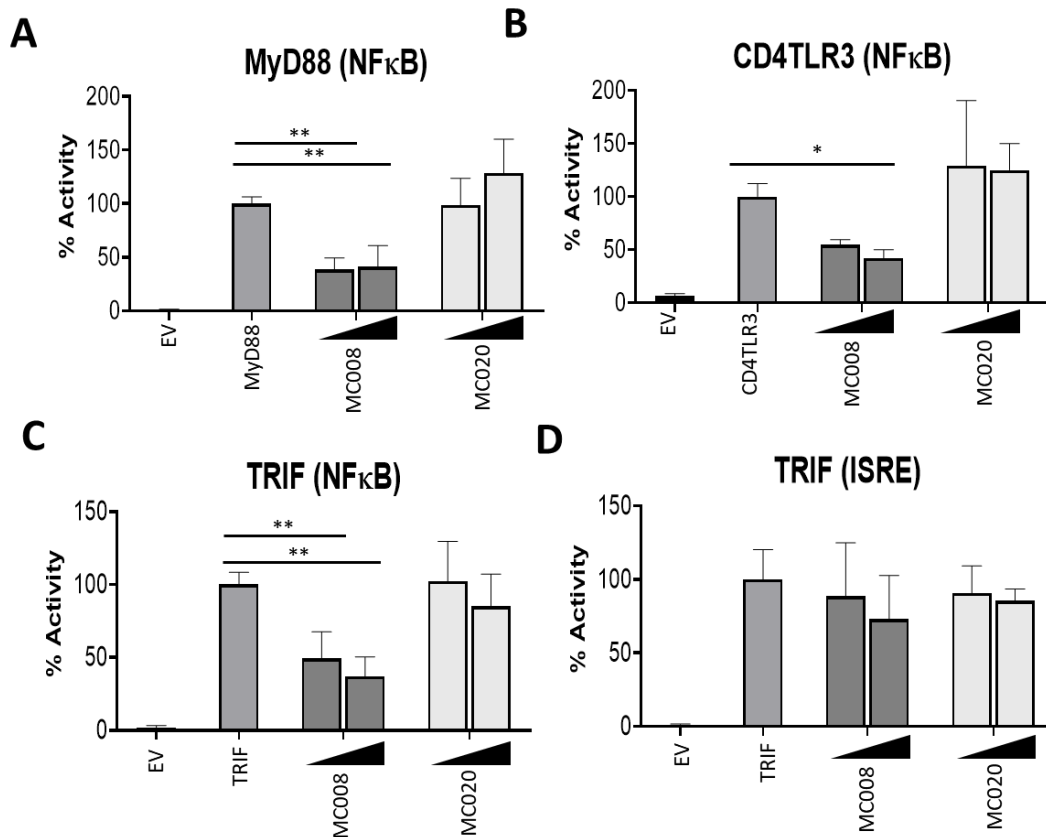


Figure 4.3.2 MC008 inhibits TLR pathway activation of NF- κ B. (A-D) HEK293T cells were seeded at 2×10^5 cells/mL and transfected with 80ng of NF- κ B, ISRE or pFR-luciferase reporter gene constructs and 40ng of TK Renilla reporter gene construct, along with EV and 25 or 50ng of the MCV expression constructs indicated for 24 hours. 50ng of myD88, CD4TLR3, or TRIF expression constructs were used to activate the pathways. The cells were subsequently lysed after 24 hours and assayed for NF- κ B and ISRE reporter activity. Data is presented as the percentage of reporter activation compared to the positive control and are the combined mean $\pm$ the standard deviation of triplicate samples from representative experiments ($n = 3$). Statistical significance was determined using two-way ANOVA with correction for multiple comparisons using Dunnett's test and is indicated by * < 0.05 , ** < 0.01 , *** < 0.001 and **** < 0.0001 .

4.4 MC008 inhibits NF- κ B Activation Induced by Proinflammatory Cytokines

While upregulation of type I interferons is key for the antiviral response, upregulation of proinflammatory cytokines is another crucial aspect of anti-viral immunity. Inflammation triggers the presence of infection to the wider immune system linking innate and adaptive immunity through activation and recruitment of various immune cells to facilitate the emergence of a specific adaptive response (122). After we determined that MC008 specifically inhibits NF- κ B activation without affecting IRF activation by upstream elements of nucleic acid sensing pathways, we next investigated whether NF- κ B activity induced by proinflammatory cytokines was similarly inhibited.

We stimulated the MC008 stable-inducible Hela cells with TNF α and again found a significant decrease in IL-6 release (**Figure 4.4.1A**). We then measured NF- κ B activation in HEK293T cells and found that MC008 inhibited NF- κ B activity driven by IL-1 β and TNF- α (**Figure 4.4.1B-C**). To further investigate where in these pathways MC008 was exerting its effect, we next examined the effect of MC008 on signalling induced by the E3 ligases, TRAF2 and TRAF6, which play key regulatory roles in TNF- and IL-1/TLR/Sensing pathway-activated signalling, respectively (259). Again, in line with our previous observations, MC008 but not the control MC020, inhibited NF- κ B activation (**Figure 4.4.1D-E**). Specificity of MC008 inhibition of NF- κ B signalling was further indicated by the lack of inhibition on the Ras-Elk1 mitogenic signalling pathway where RasVHa activation of Elk1 is monitored using an Elk-1-GAL4 DNA-binding domain fusion that drives activation of a GAL4 promoter luciferase reporter construct (**Figure 4.4.1F**).

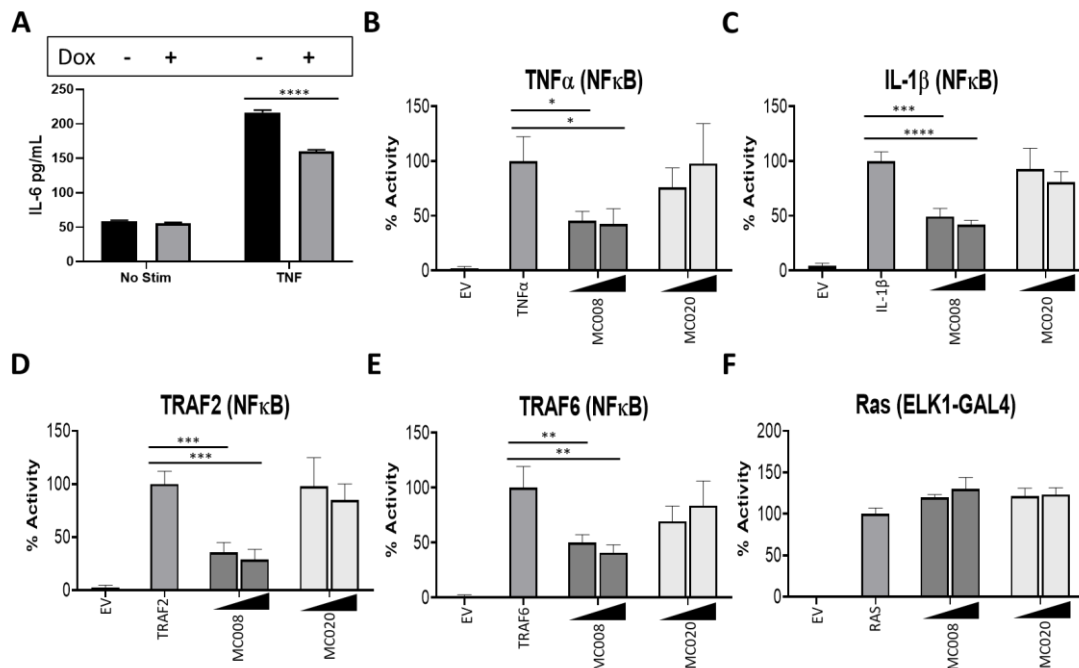


Figure 4.4.1 MC008 inhibits $\text{TNF}\alpha$ and $\text{IL-1}\beta$ -stimulated NF- κ B activation. (A) 1×10^5 HeLa stable cells/mL were stimulated with 50ng/mL of $\text{TNF}\alpha$ for 24h following MC008 induction with doxycycline and IL-6 levels were measured using ELISA. (B-C) The effect of MC008 on TNF and IL-1 -stimulated NF- κ B activation was examined. 2×10^5 cells/mL were seeded for 16 hours and transfected with 80ng of NF- κ B reporter gene construct and 40ng of TK Renilla reporter gene construct, along with 25 or 50ng of the MCV expression constructs indicated for 24 hours. Cells were then stimulated with 50ng/mL $\text{TNF}\alpha$ or $\text{IL-1}\beta$ for 8 hours and subsequently lysed and assayed for NF- κ B reporter activity. (D-E) Similar to B-C but 50ng of TRAF2 and TRAF6 were overexpressed to drive NF- κ B signalling. (F) The activation of Elk1 by Ras was measured by transfecting 80ng of the pFR-luciferase reporter gene construct along with 50ng of Ras, 5ng of pFA-Elk1 expression constructs and the indicated MCV expression constructs (25 and 50ng). Data is presented as the percentage of reporter activation compared to the positive control and are the combined mean $\pm$ the standard deviation of triplicate samples from representative experiments ($n = 3$). Statistical significance was determined using two-way ANOVA with correction for multiple comparisons using Dunnett's test and is indicated by * < 0.05 , ** < 0.01 , *** < 0.001 and **** < 0.0001 .

4.5 MC008 Inhibits NF- κ B Activation at the Level of the IKK Complex

Due to the ability of MC008 to inhibit a wide range of pathways and stimuli that induce NF- κ B (**Figure 4.5.1A**), this suggested that MC008 exerts its effects at a point common to all of these pathways. We investigated the effect of MC008 on the IKK complex, now including two inhibitors which were previously reported by the host lab to inhibit NF- κ B signalling at distinct points, MC005 (which targets NEMO) and MC132 (which drives NF- κ B p65 degradation), as inhibitory reference points (226, 227). TAB2 operates proximal to the IKK complex and functions as a ubiquitin-binding adaptor protein required for the recruitment of TAK1 (260). We observed that MC008, MC005 and MC132 inhibited NF- κ B activity following expression of TAB2 (**Figure 4.5.1B**). Ectopic expression of wild-type NEMO does not drive signalling, presumably due to an absence of the ubiquitin-induced conformational change required to activate downstream signalling. Thus, we used a constitutively active K277A mutant form of NEMO which simulates the active state of NEMO after ubiquitin-mediated activation to drive NF- κ B at this pathway point (227, 261). All three inhibitors potently inhibited NF- κ B activation by the NEMO K277A mutant (**Figure 4.5.1C**).

However, when we activated the system with IKK α and IKK β , the inhibitory effect of MC008 and MC005 on NF- κ B activity were greatly diminished whilst MC132 inhibition was maintained (**Figure 4.5.1D-E**). This indicated that inhibition by MC008, like MC005, was at a level proximal to NEMO, unlike MC132 which targets NF- κ B p65 downstream. Likewise, MC008 and MC005 inhibition was similarly bypassed by driving reporter activation with low amounts of p65 whereas MC132 still retained the capacity to inhibit its direct target protein (**Figure 4.5.1F**). Thus, these data indicated that MC008, like MC005, exerted its inhibitory activity at the level of the IKK complex, most likely by targeting NEMO.

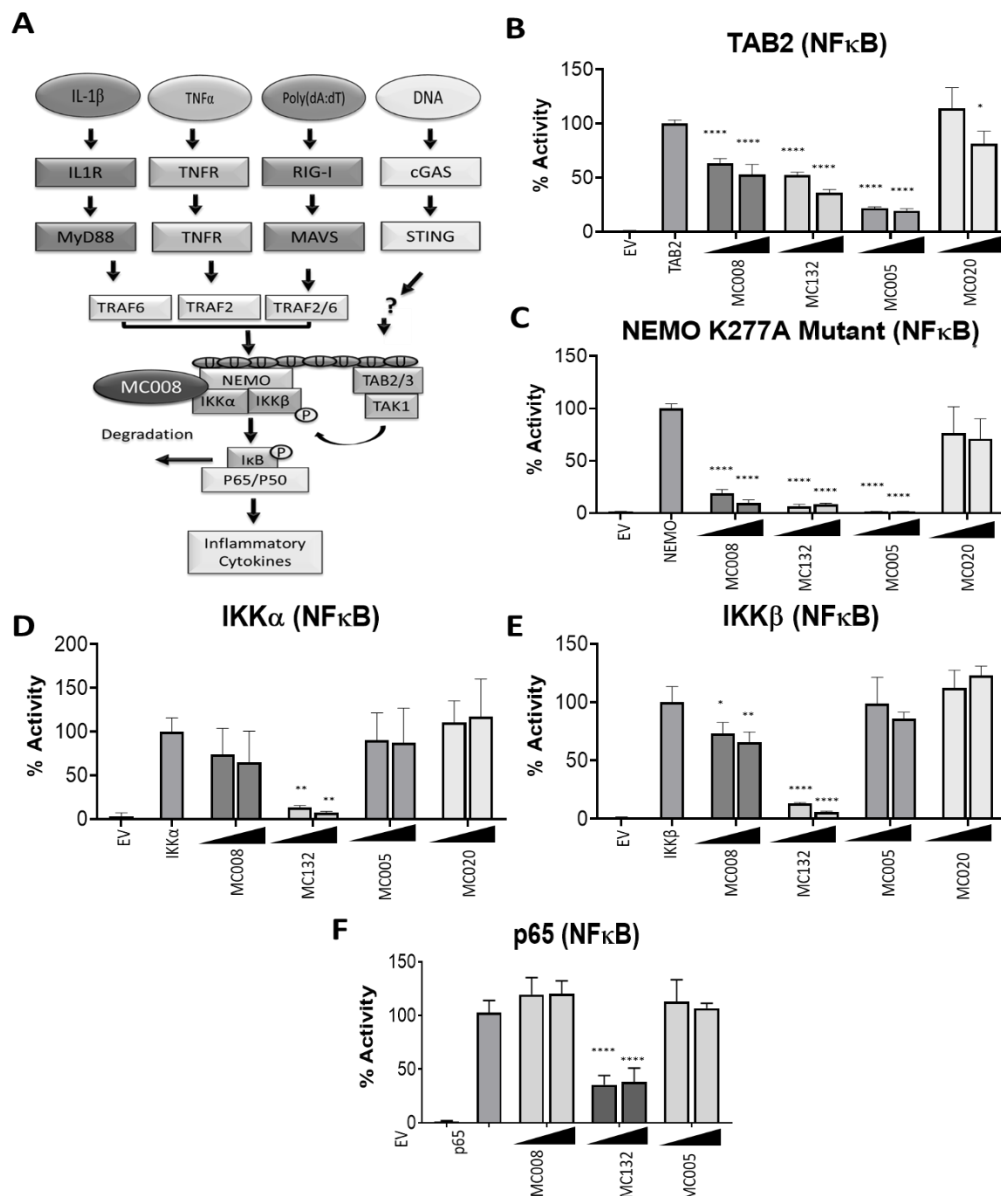


Figure 4.5.1 MC008 inhibits at a point proximal to the IKK complex. (A) Schematic showing multiple signalling pathways involved in NF-κB activation expected to be triggered by poxvirus infection. All of these pathways are perturbed by MC008 expression, as demonstrated above. (B-F) Comparison of MC008, MC005 and MC132 inhibition of signal transduction surrounding IKK complex activation. HEK293T cells were seeded at 2×10^5 cells/mL and transfected with 80ng of NF-κB and 40ng of TK Renilla reporter gene constructs, along with EV and 50ng of TAB2, NEMO K277A mutant, IKKα, or 10ng of IKKβ, or 1ng of p65 and 25 expression

constructs or 50ng of the indicated MCV expression constructs. The cells were subsequently lysed after 24h and assayed for NF- κ B reporter activity. Data is presented as the percentage of reporter activation compared to the positive control and are the combined mean $\pm$ the standard deviation of triplicate samples from individual experiments (n = 3). Statistical significance was determined using two-way ANOVA with correction for multiple comparisons using Dunnett's test and is indicated by * < 0.05, ** < 0.01, *** < 0.001 and **** < 0.0001.

4.6 Discussion

PRRs of the innate immune system, such as cytosolic nucleic acid sensors and TLRs, are a crucial means for detecting invading viral pathogens, leading to type I IFN activation. This, coupled with the production of proinflammatory cytokines such as IL-1 β and TNF production, which induce potent inflammatory signalling, help orchestrate emergence of an adaptive immune response, resulting in virus clearance (262). Similar to our previous observations for MC005 and MC132, MC008 inhibited PRR and inflammatory cytokine-induced NF- κ B activation and proinflammatory cytokine production without affecting IRF activation in any of these pathways or Ras-Elk-1 control pathway activation.

Using a process of sequential activation of key signalling pathway components, we mapped the effect of MC008 to the IKK complex and found that its effects are largely bypassed downstream of the core regulatory component, NEMO, which translates upstream ubiquitin-generating stimuli to the activation of the associated IKKs and thus NF- κ B. Given that the IKK complex plays a central regulatory role in inflammatory signalling and multiple pathways converge at this point, elements of this complex are common targets for viruses seeking to evade and subvert the immune response (110, 263).

Whilst our knowledge of the IKK complex advances, a precise understanding of its multi-layered mechanism of activation is continually emerging. However, it is clear that

ubiquitination plays a key role in this process. Following upstream activation by various stimuli, E3 ligases such as TRAF2, TRAF6, cIAP, and the LUBAC complex produce ubiquitin chains that bind to NEMO, among other signalling proteins (84, 264). K63-linked ubiquitin chains that bind to NEMO serve as a scaffold to bring proteins such as the TAB/TAK complex into close proximity to IKK α/β , thus enabling their phosphorylation (265). Furthermore, both K63-linked and Met-1-linked ubiquitin chains induce conformational changes in the IKK complex that allow this phosphorylation event to occur, ultimately leading to the release of NF- κ B into the nucleus (39, 61, 266). Since ubiquitin binding is a requirement for NEMO activation, we used a NEMO (K277A) mutant which has a point mutation that simulates a ubiquitin-bound state, as previously described (227). Interestingly, MC008, like MC005, potently inhibited activation of NF- κ B by this constitutively active mutant form of NEMO. This NEMO residue was based on a corresponding mutation to a lysine residue in murine cells. Replacing the positively charged K270 residue with an alanine (a physiochemically innocuous amino acid) enhances UBA1 homophilic interactions, inducing a conformational change that simulates a ubiquitin-bound state (261).

4.7 Conclusion

Our investigation into MCV ORFs that disrupt inflammatory signalling reveal that MC008 potently inhibits PRR- and cytokine-induced NF- κ B signalling, and this effect is bypassed downstream of the central regulator NEMO. Since the IKK complex is a key point of convergence for a number of PRR signalling pathways, it is unsurprising that many viruses target this complex.

Chapter 5 Investigation of MC008 Targeting of NEMO

*This chapter contains data submitted to the Journal of Virology and has been **accepted for publication**.
Phelan T, Lawler C, Pichlmair A, Little M, Bowie A, Brady G. Molluscum Contagiosum Virus Protein
MC008 targets NF- κ B Activation by Inhibiting Ubiquitination of NEMO.*

In the previous chapter we mapped the inhibitory effect of MC008 on NF- κ B signalling to the IKK complex using luciferase reporter gene assays. Since the inhibitory effect of MC008 on NF- κ B signalling was bypassed downstream of NEMO, this protein was considered a likely target. Furthermore, given the established nature of NEMO regulation by ubiquitin, this suggests a potential mechanism by which MC008 can perturb its function. In this chapter we aimed to confirm the specific target of MC008 in the IKK complex and discern a mechanism for the observed NF- κ B inhibition.

5.1 MC008 Interacts with NEMO

To confirm that MC008 is targeting at the level of the IKK complex, we first investigated MC008 co-immunoprecipitation with the signalling components TRAF2 and TRAF6. We observed no interaction between MC008 and TRAF2 or TRAF6 (**Figure 5.1.1**). However, we did observe an interaction in the positive control lane between NEMO and TRAF6.

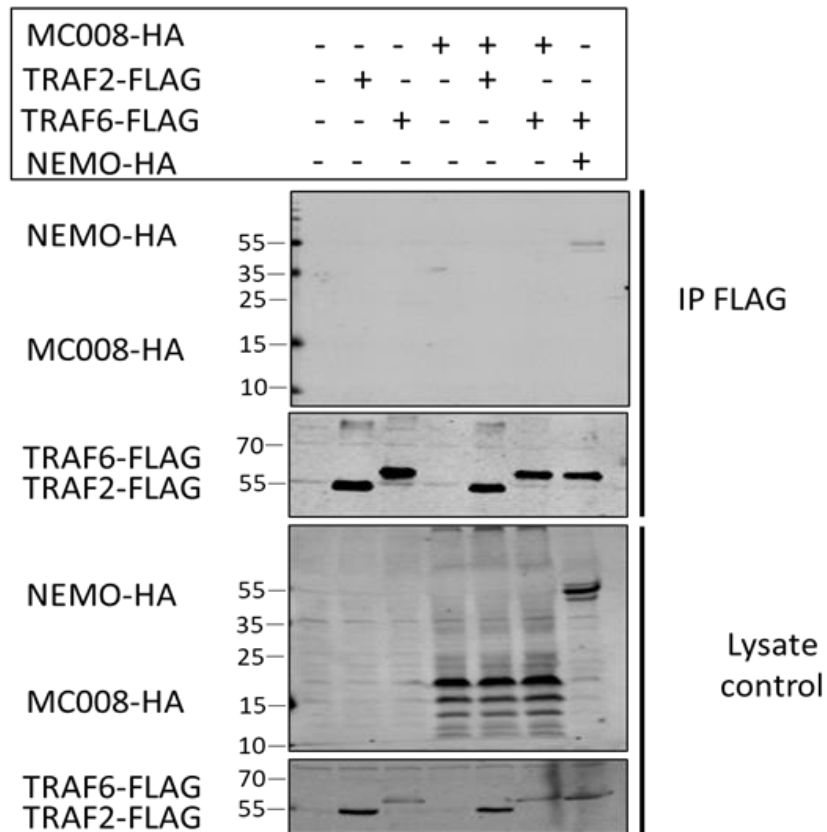


Figure 5.1.1 MC008 does not immunoprecipitate with TRAF2 or TRAF6. 4µg of MC008-HA expression vector and 4µg of TRAF2-FLAG, TRAF6-FLAG and NEMO-HA expression vectors were transiently expressed in 4×10^6 HEK293T cells per plate, as shown. The cells were subsequently lysed using ice-cold lysis buffer with a cocktail of phosphatase and protease inhibitors and co-immunoprecipitation was performed. The resulting immunoprecipitants were then immunoblotted with the antibodies indicated. Representative blots (n = 4) are shown.

Conversely, we observed an interaction between NEMO and MC008 (**Figure 5.1.2**) but not between MC008 and p65.

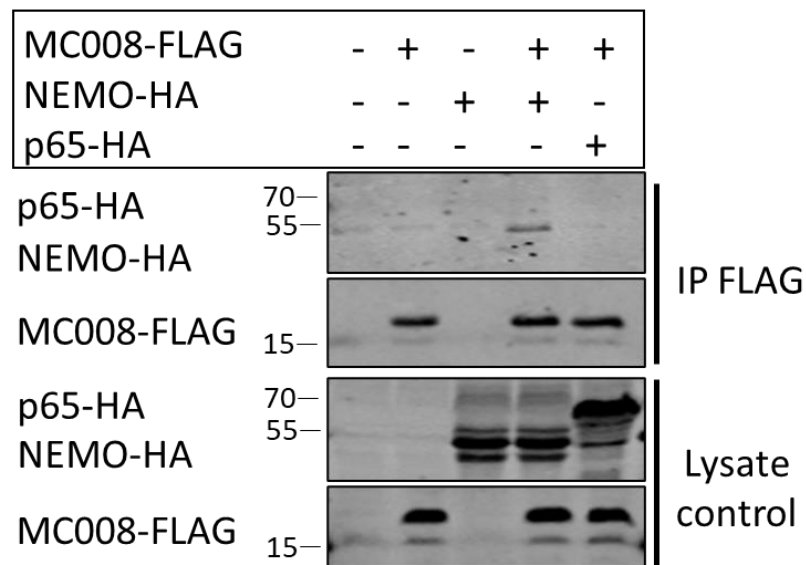


Figure 5.1.2 MC008 co-immunoprecipitates with transiently over-expressed NEMO. 4µg of MC008 and 4µg of the shown signalling elements were transiently expressed in 4×10^6 HEK293T cells per plate for 24 hours. The cells were subsequently lysed using ice-cold lysis buffer with a cocktail of phosphatase and protease inhibitors and co-immunoprecipitation was performed. The resulting immunoprecipitants were then immunoblotted with the antibodies indicated. Representative blot (n = 4) is shown.

Next, by using transiently transfected MC008 expression construct in HaCaT cells, we confirmed this interaction with endogenous NEMO but not p65 (**Figure 5.1.3**).

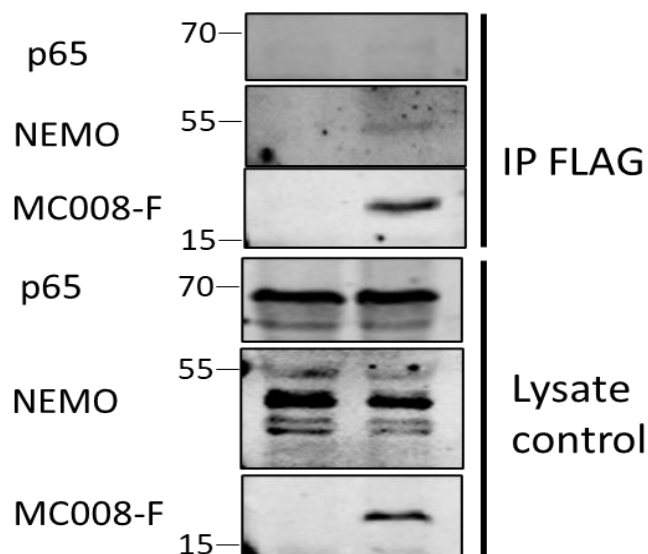


Figure 5.1.3 MC008 interacts with endogenous NEMO in HaCaT cells. 2×10^6 HaCaT cells per plate were transiently transfected with 4 μ g of MC008 expression construct for 24 hours. Co-immunoprecipitation was performed and interaction with endogenous levels of NEMO was confirmed using a NEMO specific antibody during immunoblotting. An antibody probing endogenous p65 was used as a negative control for the pulldown. Representative blot (n = 3) is shown.

Interestingly we also observed a weaker interaction between MC008 and over-expressed IKK α and IKK β , which associate with NEMO in the IKK complex (**Figure 5.1.4**).

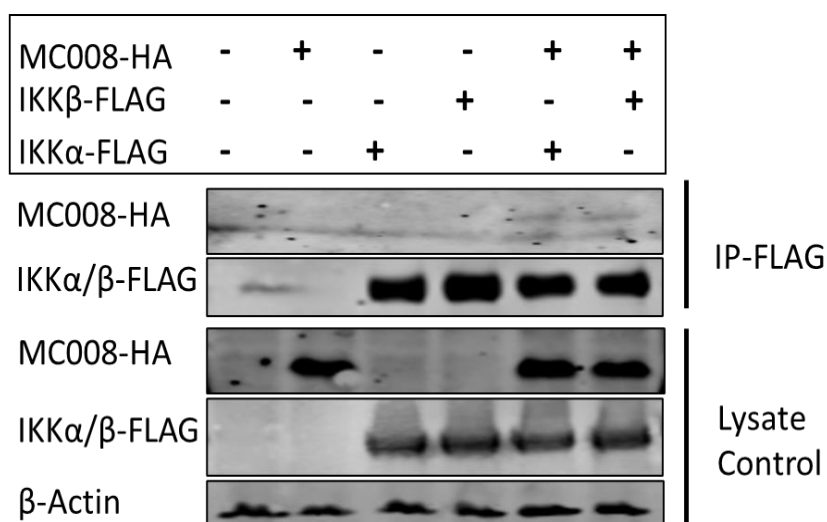


Figure 5.1.4 MC008 interacts weakly with IKK α/β . 4 μ g of MC008 expression construct and 4 μ g of expression construct encoding the shown signalling elements were transiently expressed in 4x10⁶ HEK293T cells per plate for 24 hours. The cells were subsequently lysed using ice-cold lysis buffer with a cocktail of phosphatase and protease inhibitors and co-immunoprecipitation was performed. The resulting immunoprecipitants were then immunoblotted with the antibodies indicated. Representative blot (n = 3) is shown.

Furthermore, even though MC008 is predominantly nuclear, we observed enrichment in areas of the cytoplasm over-expressing NEMO using confocal microscopy, demonstrating colocalization in both HEK293T and HaCaT cells (**Figure 5.1.5**).

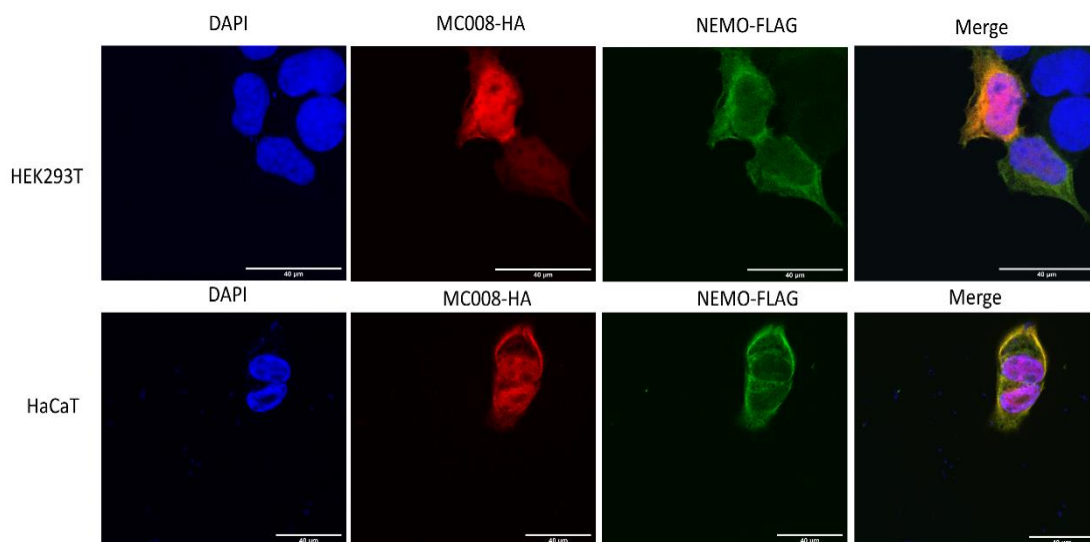


Figure 5.1.5 MC008 colocalises with NEMO in HEK293T and HaCaT cells. HEK293T and HaCaT cells were seeded at 7x10⁴ and 3x10⁴ cells per well in 8-well chamber slides, respectively. 24h later they were transfected with 100ng of expression constructs for MC008-HA and NEMO-FLAG. Cells were then fixed 24h later and stained with DAPI (blue), FLAG (green) and HA (red). Representative experiment shown (n = 3).

5.2 MC008 Requires a 64 Amino-acid Region to Inhibit NF- κ B Signalling

To identify the region of MC008 required for the inhibition of NF- κ B signalling, we used a series of truncations (previously made in the host lab) expressing specific regions of MC008. We found that deletion of large sections of the C terminus perturbed the ability of MC008 to inhibit TRAF6-induced NF- κ B activation in HEK293T cells (**Figure 5.2.1A**). Since all truncations inhibited to some extent, we based our significant level of inhibition on the full length MC008 positive control. Truncations that inhibited more than 60% were considered to be of significance and have been highlighted in red. Interestingly, the ability of these truncations to interact with NEMO, in most cases, correlated with an ability to inhibit NF- κ B signalling (**Figure 5.2.1B**). We identified the smallest truncation from the series [112-176], that gave more than 60% NF- κ B inhibition at both 25ng and 50ng construct concentrations, was a section of the C-terminal region of the protein (**Figure 5.2.1C**).

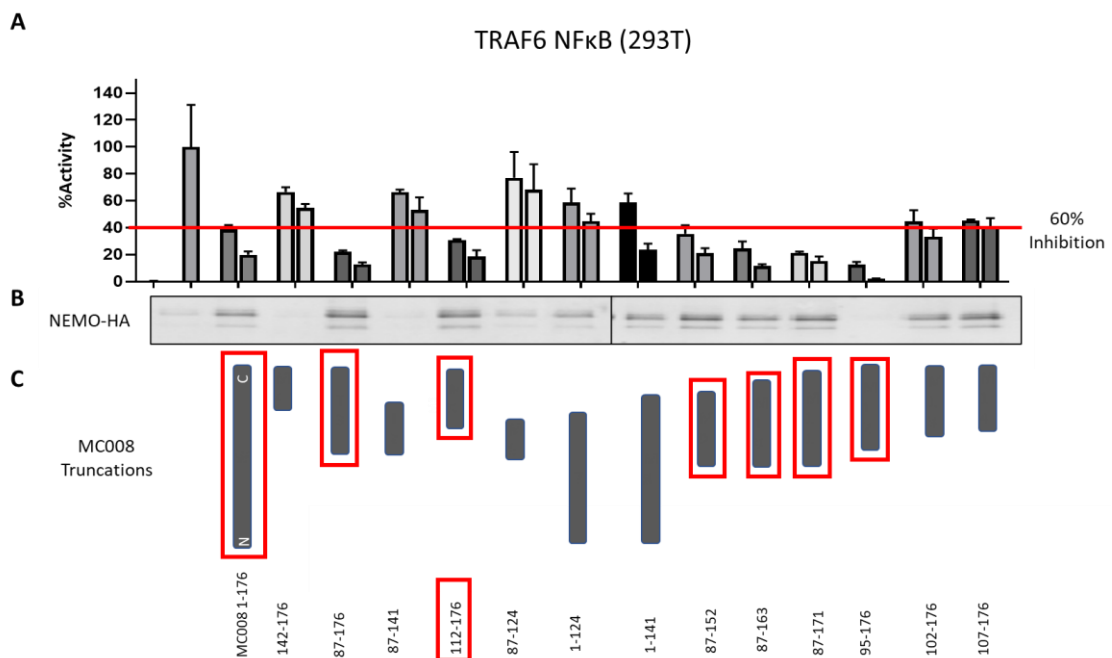
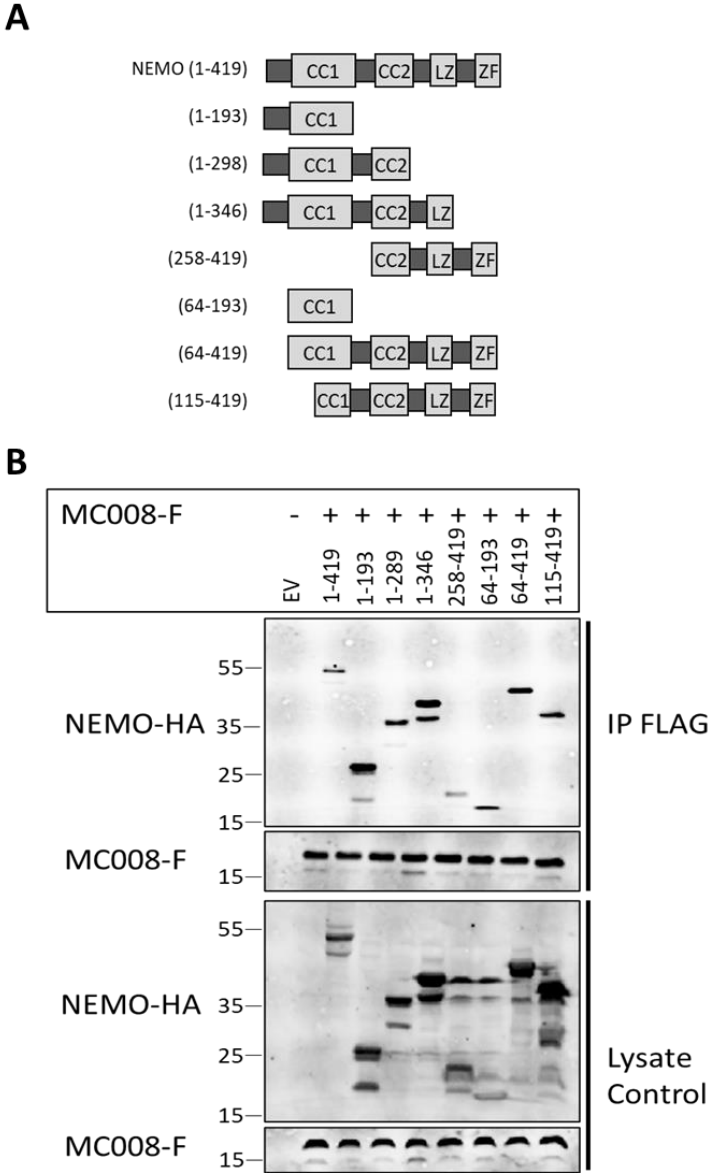


Figure 5.2.1 A 64-amino-acid region is required for MC008 inhibition of NF- κ B activation. (A) Constructs expressing MC008 were used to identify a 64-amino acid region required for NF- κ B inhibition. HEK293T cells were seeded at 2×10^5 cells/mL and transfected with 80ng of NF- κ B reporter gene construct, 40ng of TK Renilla reporter gene construct, and empty-vector along with 50ng of TRAF6 and 25ng or 50ng of pCEP4 plasmid expressing MC008 and truncations. The cells were subsequently lysed and assayed for NF- κ B reporter activity. (B) Interaction of MC008 and truncations with NEMO. 4×10^6 HEK293T cells per plate were seeded for 16 hours and 4 μ g of MC008/truncation-FLAG expression constructs and 4 μ g NEMO-HA expression construct were transiently expressed for 24 hours. The cells were subsequently lysed and co-immunoprecipitation with anti-FLAG antibody-coated beads was performed. The resulting material was then immunoblotted with anti-FLAG and anti-HA antibodies. (C) schematic of MC008 truncations generated. Constructs expressing the MC008 truncations were generated by truncation in the positions indicated. Data is presented as the percentage of reporter activation compared to the positive control and are the mean $\pm$ the standard deviation of triplicate samples from a representative experiment (n = 3). Truncations highlighted in red indicate inhibition of NF- κ B activity by more than 60%.

5.3 MC008 Inhibits NEMO Mono- and Poly-ubiquitination

To identify the region of NEMO required for MC008 interaction, we employed truncations corresponding to the functional domains of NEMO generated in previous research in the host lab (227) and investigated their ability to interact with full length MC008 (**Figure 5.3.1A-B**). Interestingly, MC008 association was maintained to varying extents across all NEMO truncations. While MC008 maintained a relatively strong interaction with truncation [64-193], the isolated CC1 domain, there was a notable reduction in the association with truncation [258-419], a region lacking the CC1 domain. Furthermore, the interaction with the CC1 domain is likely between residues [115-193] as residues [1-115] appear dispensable for the interaction. This data indicates that the

CC1 domain is important for interaction with MC008, and that, unlike MC005, this viral protein likely interacts with more than one region of NEMO.



5.3.1 MC008 interacts with all NEMO truncations but has a reduced association with the truncation lacking the CC1 domain. (A) NEMO truncations were generated as per the schematic. (B) 4×10^6 HEK293T cells per plate were seeded for 16 hours and $4 \mu\text{g}$ of MC008-FLAG expression construct and $4 \mu\text{g}$ of NEMO expression construct or the indicated truncation constructs were transiently expressed for 24 hours. The cells were subsequently lysed and co-immunoprecipitation was performed. The resulting

immunoprecipitants were then immunoblotted with anti-FLAG and anti-HA antibodies. A representative blot (n = 3) is shown.

To probe the consequences of inhibition at the point of NEMO, we investigated key signalling events in NF- κ B activation by stimulating inducible MC008 stable HEK293T cells with TNF- α and probing phosphorylation of IKK α / β and p65, along with I κ B α degradation, events which are thought to be necessary for NF- κ B activation. Interestingly, while MC008 inhibited IKK α / β and p65 phosphorylation, it caused only a slight but consistent delay in I κ B α degradation at 30 minutes (**Figure 5.3.2**).

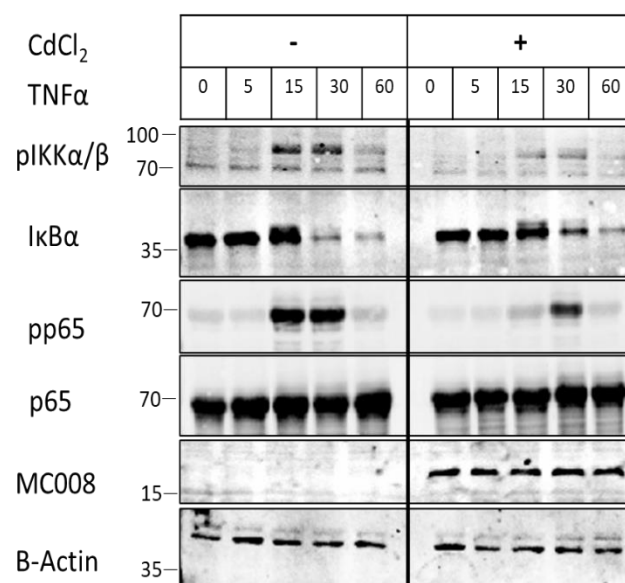


Figure 5.3.2 MC008 inhibits key NF- κ B signalling events. HEK293T cells, stably expressing pMEP4-MC008-FLAG, were seeded at 6×10^5 /well into six-well dishes for 16 hours and were left untreated (-) or treated (+) with 1 μ M CdCl₂ to induce MC008 expression. Cells were stimulated 24h later with 50ng/mL of TNF- α for the times indicated and lysates were immunoblotted with the antibodies indicated. A representative blot (n = 3) is shown.

We next investigated the mechanism of MC008 inhibition of NEMO. First, we examined the effect of MC008 on NEMO oligomerization through co-immunoprecipitation with FLAG and HA-tagged NEMO. The IKK complex unit is believed to consist of four NEMO molecules and two of each IKK α/β which are constitutively assembled even in the absence of upstream signalling (267). We observed no difference in the ability of NEMO to homo-oligomerise when MC008 was introduced, suggesting that MC008 does not interfere with NEMO assembly within the IKK complex (**Figure 5.3.3**).

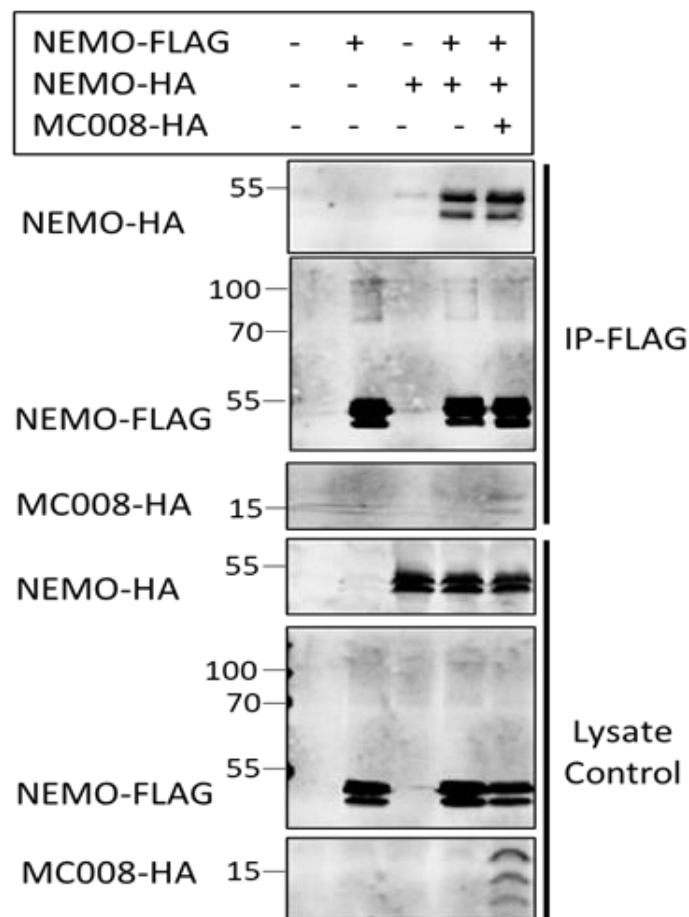


Figure 5.3.3 MC008 does not disrupt NEMO homo-oligomerization. 4×10^6 HEK293T cells per plate were seeded for 16 hours and 2 μ g NEMO-FLAG, 2 μ g-NEMO-HA and 3 μ g MC008-HA expression constructs were transiently expressed for 24 hours. The cells were subsequently lysed and co-immunoprecipitation was performed.

We then examined whether MC008 was perturbing interactions between NEMO and IKK α/β which are indispensable components of the multimeric IKK complex. Again, we observed no effect on association of NEMO and the IKKs (**Figure 5.3.4**).

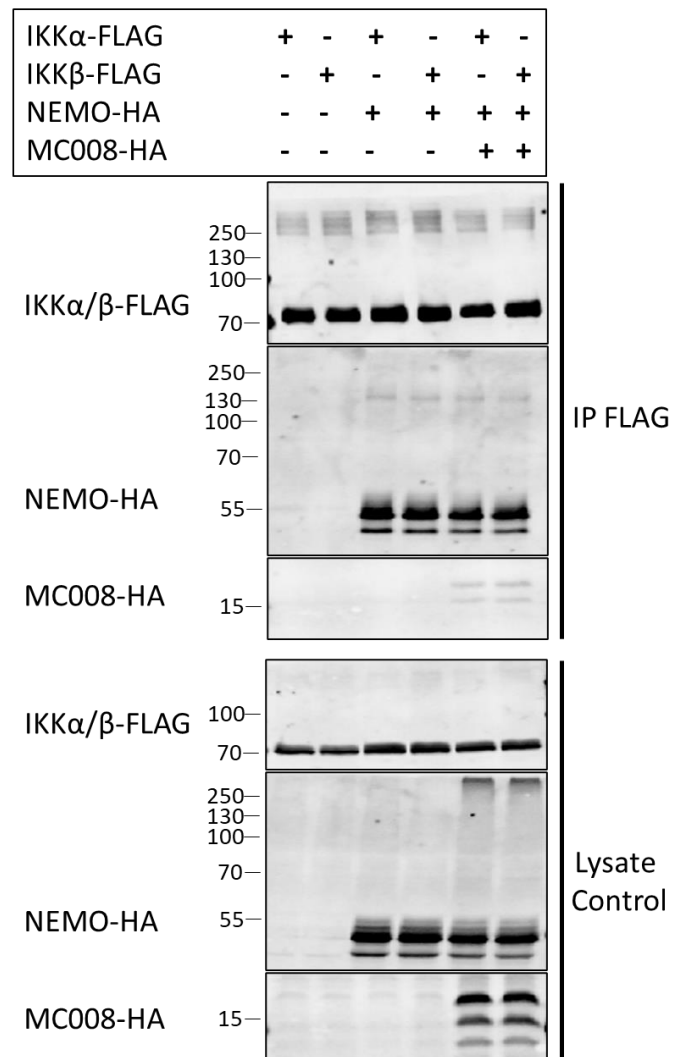


Figure 5.3.4 MC008 does not disrupt NEMO association with IKK α/β . 4×10^6 HEK293T cells per plate were seeded for 16 hours and $3 \mu\text{g}$ of IKK α -FLAG or IKK β -FLAG expression constructs, $2 \mu\text{g}$ -NEMO-HA and $3 \mu\text{g}$ MC008-HA expression constructs were transiently expressed for 24 hours. The cells were subsequently lysed and co-immunoprecipitation was performed.

Given the central role of ubiquitination in NEMO activation, we next examined the effect of MC008 on its regulation by ubiquitin. We observed both mono-ubiquitination and poly-ubiquitination of NEMO when ubiquitin is overexpressed (**Figure 5.3.5**). These are key modifications in NEMO activation (39, 61, 266) with initial mono-ubiquitination shown to be required for further poly-ubiquitination (268, 269). Interestingly, MC008 disrupted both forms of ubiquitin (**Figure 5.3.5**). Thus, this data suggests that MC008 inhibits NF- κ B signalling by preventing mono- thus poly-ubiquitination of NEMO, key regulatory steps required for downstream signalling. This notably differs from MC005 which does not affect NEMO ubiquitination or association with ubiquitin chains and instead inhibits the conformational consequences of these events impacting on IKK accessibility for phosphorylation.

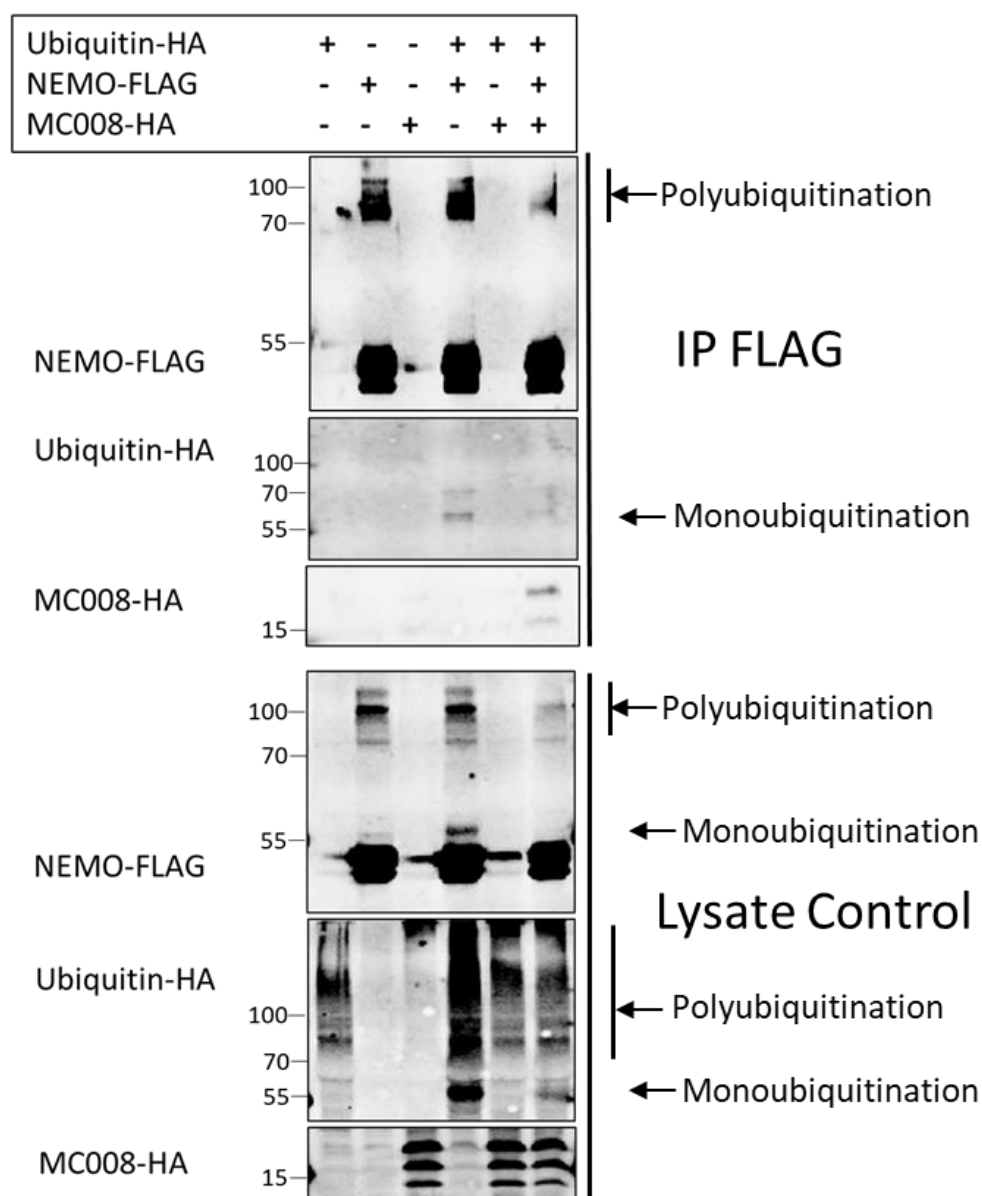


Figure 5.3.5 MC008 inhibits NEMO ubiquitination. 4×10^6 HEK293T cells per plate were seeded for 16 hours and 2 μ g NEMO-FLAG, 1 μ g ubiquitin-HA and 3 μ g MC008-HA expression constructs were transiently expressed for 24 hours. The cells were subsequently lysed and co-immunoprecipitation was performed. Black lines indicate areas of ubiquitination. Representative blots ($n = 3$) are shown.

Taken as a whole, these data indicate that MC008 specifically inhibits NF- κ B activation by preventing ubiquitination of NEMO, thereby inhibiting phosphorylation of IKK proteins with specific effects on subsequent NF- κ B activation.

5.4 Discussion

Poxvirus immune evasion research to date has largely centred around Orthopoxviruses, in particular, vaccinia virus (122, 262). The Molluscipoxvirus MCV is an understudied yet globally endemic human-specific poxvirus that causes a clinically mild infection in immunocompetent individuals forming distinct papular skin lesions of long duration (214). The typical absence of inflammation around these lesions in deference to other poxviral skin infections in humans has been of long-standing interest (203, 214, 228, 270). Well-characterised orthopoxviruses like vaccinia virus have long been known to produce broad arrays of inhibitors during infection that aggressively target NF- κ B activation in particular, at various levels (122, 171). Although distantly related to, and highly divergent from, well-characterised poxviruses, Molluscipoxviruses likely possess their own arrays of inhibitors which have evolved during their residence as human-specific poxviruses.

Immune evasion by MCV may be partially attributable to the keratinocyte tropism of the virus and restricting infection to the upper layers of the epidermis (170). While localization of the virus likely reduces interaction with the immune system to some extent, we and others have continued the process of systematically characterising MCV-specific immunomodulatory proteins that target key elements of innate immune signalling pathways in this historically understudied virus. One restricting factor on MCV research is the ongoing absence of an animal and cell-culture model of infection (271).

Nevertheless, the lab previously showed that MC132 recruits the p65 subunit of NF- κ B to an E3 ligase complex leading to its degradation by the proteasome and that MC005 targets NEMO to prevent a priming event causing disruption to downstream NF- κ B activation (226, 227). Other researchers have shown that MC159 and MC160 are viral FLICE-like proteins which prevent TNF-induced apoptosis as well as inhibit IKK β through binding to NEMO and degrading IKK α respectively (231, 232, 272). MC163 was shown to inhibit apoptosis by preventing mitochondrial membrane permeabilization (233). MC080 was shown to disrupt MHC-I antigen presentation by targeting tapasin, a component of the peptide loading complex, for ubiquitination and subsequent degradation (237). Given that at least three MCV proteins appear to target NEMO in distinct ways (**Table 3**), this is indicative of the importance of NEMO in anti-viral immunity against this virus. Likely many more inhibitors remain to be identified given the density of such inhibitors in the genomes of well-characterised poxviruses like VACV and Variola Virus (122, 188-190, 273).

Effect	MC005 (227)	MC008	MC159 (224, 231)
Inhibits NEMO-NEMO Interaction	No	No	Unknown
Region of NEMO interaction	Second half CC1/before CC2	N- and C-Terminus	N-Terminus 1-250
Inhibits NEMO-IKK Interaction	No	No	Unknown
Inhibits NEMO Mono-ubiquitination	No	Yes	No
Inhibits NEMO Poly-ubiquitination	No	Yes	Yes
Inhibits NEMO activation post UBAN-Ubiquitin activation	Yes	Yes	Unknown

Table 3. Comparison of three MCV proteins that target NEMO.

Using serial truncations of MC008 (previously generated by the lab), we demonstrated that the truncation corresponding to a C-terminal 64-amino acid region [112-176], was the minimal inhibitory region from the series required for more than 60% inhibition of NF- κ B activity. This truncation also retained the ability to interact with NEMO during co-immunoprecipitation. The last 24 amino acids of the C-terminus appear to be dispensable for this inhibition, as C-terminal truncations lacking these residues [87-152] maintained the ability to inhibit NF- κ B activation more than 60% at both plasmid concentrations.

We were unable to detect the smaller proteins below 64-amino acids (or ~7kDa) in the lysate control. We did observe some level of inhibition with the smaller truncations, suggesting that they were indeed expressed, thus mediating an effect. However, we cannot fully rule out the possibility of non-expression. Interestingly, the truncation corresponding to residues [95-176] was cytotoxic to the cell, possibly due to protein misfolding, accounting for the inhibition and lack of pull-down observed.

We also used a truncation-based approach to determine the region of NEMO required for MC008 interaction. MC008 interaction was maintained across all truncations. However, there was a reduced association with a truncation lacking the coiled-coil-1 (CC1) domain suggesting this region is important in its inhibition. MC008 binding to this region does not impair NEMO dimerization or its ability to associate with IKK α or IKK β . However, we did observe a significant reduction in NEMO mono-ubiquitination and poly-ubiquitination when MC008 was introduced. The CC1 domain of NEMO has shown to be a common target for MCV proteins; MC159 has been previously shown to competitively bind to the NEMO N-terminal region (1-250), which contains this CC1 domain, and prevent cIAP1 poly-ubiquitination of NEMO (224).

The lab previously demonstrated that MC005 interacts with the CC1 domain of NEMO and prevents an important ubiquitin induced conformational change required for downstream signalling (227). The beginning of the CC1 domain interacts with the C-terminus of IKK proteins (34, 274). This domain has also been shown to auto-inhibit NEMO in its resting state by preventing ubiquitin binding to the UBAN domain, and this resting state is only overcome by long ubiquitin chains (36). The CC2 domain and leucine zipper form this UBAN domain. This region also regulates the conformational change that allows phosphorylation of IKK β by TAK1 on serine-177 and -181 in the activation loop, leading to IKK activation (39, 40). The C-terminal zinc finger has also been shown

to be another ubiquitin binding domain (275). Given that MC008 maintains the ability to interact with multiple NEMO domains and it potently inhibits initial mono-ubiquitination and subsequent poly-ubiquitination, we propose that MC008 binding to NEMO prevents ubiquitin chains from disrupting its auto-inhibited state, thereby preventing ubiquitin-UBAN domain interactions (**Figure 5.4.1**). Thus, as was the case for MC005, not only does this research help us understand how MCV is inhibiting inflammatory signalling, it also serves as a tool to confirm and elaborate models of IKK complex regulation.

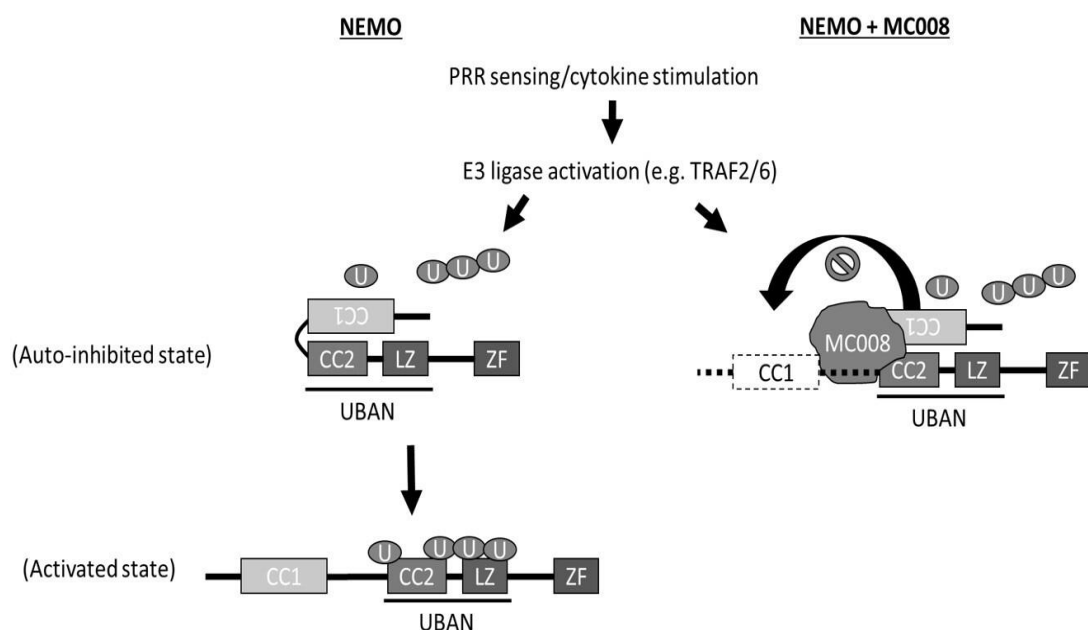


Figure 5.4.1 Model of MC008 inhibition of NF-κB signalling. PRR and cytokine signalling pathways drive signal transduction leading to the activation of E3 ligases such as TRAF proteins which generate ubiquitin chains. NEMO is locked in an auto-inhibited state by the CC1 domain until it is relieved by long ubiquitin chains binding to the UBAN domain. This binding event induces a conformational change, priming NEMO for downstream signalling. MC008 inhibits this ubiquitination event by binding to the CC1 and UBAN domains, thus preventing complex priming and downstream NF-κB signalling.

One perplexing aspect of the data is that MC008 inhibits both IKK α/β and p65 phosphorylation with only a minor delay in I κ B α degradation. In the canonical pathway, fully activated IKK β phosphorylates I κ B α on Serines 32 and 36 triggering its degradation (276). However, IKK β -mediated regulation of p65 by phosphorylation is more complex. During signalling, p65 is potentially phosphorylated on 11 known sites (277) controlling conformational changes, protein-protein interactions and stability. IKK β is associated with at least two of these, Serine 536 (278-282) and 468 (283). Whilst we did observe that MC008 inhibited Serine 536 phosphorylation, we did not detect Serine 468 phosphorylation in TNF α stimulation of HEK293Ts (data not shown). We propose that MC008 may inhibit IKK complex activation in a specific manner that targets regulation of p65 without a potent effect on I κ B α regulation. We are now investigating the precise nature of this difference which may give new insights into previously uncharacterised nuances of NEMO-induced IKK activation in NF- κ B regulation.

5.5 Conclusion

We describe a mechanism by which MC008 targets signalling within the known model of IKK complex regulation. Previously MC005, MC132, MC159 and MC160 have been described as inhibitors of NF- κ B signalling with MC008 adding to this class of MCV inhibitors. By specifically targeting NEMO in a variety of ways, viruses like MCV appear to have heavily invested in evolving the capacity to efficiently inhibit its activation, thus perturbing viral sensing-induced production of both type I interferon and proinflammatory cytokines and the activity of the latter within infected cells.

Further investigation of the MCV genome will likely reveal more inhibitors of innate immune signalling which will expand our understanding of this fascinating virus whilst

offering novel insights into innate signalling pathways and potentially new avenues of therapeutic intervention in human inflammatory disease.

Chapter 6 General Discussion

6.1 Summary

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MCV is a human-specific dermatotropic poxvirus, infection by which results the formation of benign papules on the surface of the skin that exhibit little to no signs of an inflammatory response (203). Like all poxviruses, MCV encodes an array of immunomodulatory genes that contribute to the evasion and subversion of host immune signalling mechanisms. The lab has previously published on MC132 and MC005, which inhibit NF- κ B activity by inducing p65 degradation, and by inhibiting conformational priming of NEMO, respectively (226, 227). This thesis describes another MCV protein MC008, providing novel insight into the mechanism of this proteins ability to inhibit inflammatory signalling.

The host laboratories initial MCV investigation began with a luciferase assay-based screen of the genome to identify novel inhibitors of inflammation, leading to the identification of MC008. This was followed by the analysis of MC008 immunoprecipitates using affinity purification mass spectrometry to identify potential MC008 binding partners, thus revealing an interaction with the E3 ligase, TRIM32. It was reported that TRIM32 plays a regulatory role in in the cGAS-STING response to DNA sensing, thus was the focus of our early investigation (73, 82). However, a series experiments aiming to decipher the mechanism by which MC008 disrupts TRIM32 positive regulation of this pathway were unsuccessful. During an experiment examining MC008 impact on the dynamic between TRIM32 and NEMO, it was noted that there was a reduction in baseline levels of NEMO ubiquitination. Therefore, we changed the focus of the investigation towards this region of the signalling pathway.

To identify where MC008 is exerting its inhibitory effects on inflammatory signalling, we performed a pathway screen using luciferase assays. In these assays, genes encoding proteins involved in canonical inflammatory signalling were overexpressed alongside MC008. We observed inhibition of NEMO, and the inhibitory effects of MC008 were largely bypassed downstream of this key regulatory protein. We also observed inhibition of cytokine induced inflammatory signalling and reduced proinflammatory cytokine production following PRR stimulation. These data indicated that MC008 targets a node common to all of the pathways investigated, most likely at the level IKK complex.

To further probe this, a series of co-immunoprecipitation-based analyses were performed to identify interactions between MC008 and proteins of the IKK complex/IKK proximal proteins. An interaction was observed between MC008 and NEMO. Additionally, co-localization was observed between MC008 and NEMO, providing further evidence that MC008 targets this protein. Following on from this, truncation experiments revealed an interaction between MC008 and multiple NEMO domains, with the CC1 domain appearing to be important due to reduced MC008 association when removed. Finally, we demonstrated that MC008 disrupts both mono-ubiquitination and poly-ubiquitination of NEMO, revealing for the first time, a mechanism of MC008 inhibition of inflammatory signalling.

6.2 Study Limitations

While this research contributes to knowledge on MCV immune evasion mechanisms and provides a foundation for the development of a novel therapeutic, there are limitations with this study. The lack of a cell culture model or animal model for efficient MCV replication hampers the ability to directly test the effect of MC008 on virulence (170). As such, knowledge surrounding MCV immune evasion strategies, to date, have been derived from

either expressing proteins independent of infection or by using a surrogate virus for infection models. Furthermore, technical difficulties with TRIM32 expression and uncertainty surrounding the reported role of TRIM32 in regulating DNA sensing obstructed our ability to implicate this protein in the system, despite the observing a consistent interaction with MC008.

6.3 Research Impact

Inflammation lies at the centre of human disease and is triggered via intracellular signalling pathways. This leads to a wide variety of autoimmune and autoinflammatory conditions, such as systemic lupus erythematosus, during which aberrant activation of signalling pathways play a significant pathological role (284). Innate immune signalling underlies and controls inflammation and, as such, understanding and manipulating the key elements of these pathways is crucial for the development of new anti-inflammatory therapies. Since viruses have specifically evolved to subvert the host immune system, allowing viral growth and replication, investigation of their mechanisms of immunomodulation offer an excellent tool for the identification of novel therapeutic targets. As viruses produce a vast array of inhibitory proteins like MC008 to block inflammation and antiviral responses, these may prove to be useful tools on which to base novel therapeutics to ameliorate human disease driven by aberrant innate responses. Furthermore, MC008 appears to inhibit early ubiquitination, thus interrupting later events in NEMO activation, thereby validating current models of IKK complex regulation.

Protein-based therapeutics represent a major leap in medicine with over 100 proteins currently approved for clinical use. Recombinant proteins such as insulin-like growth factor (285) and antibodies such as rituximab (286) are among many examples of highly effective protein therapeutics. However, the majority of these therapeutics exert their effects on

extracellular targets emphasising the challenges of designing effective protein therapeutics with targets not exposed to the extracellular environment. The cell membrane presents as a major obstacle for therapies aiming to target intracellular mechanisms. As a result, there has been a recent surge in studies focusing on alternative drug delivery systems (287-289). Cell-penetrating peptides (CPPs) are short peptides containing a motif such as poly-arginine that enables either passive or active transport across biological membranes. Currently there are upwards of 25 CPP-conjugated drugs in clinical trials that have the potential to treat a variety of diseases including those involving inflammation (289, 290). There has been some success in the intracellular delivery of a DNA virus-derived peptides using CPPs, one example being viral inhibitor peptide of TLR4 (VIPER), an 11-amino acid peptide derived from the VACV protein A46, which potently inhibits LPS-activated NF- κ B with systemic efficacy in murine LPS-induced sepsis models (291). VIPER inhibits NF- κ B by masking binding sites on MAL and TRAM, thus preventing downstream signalling. This highlights the potential therapeutic value of reducing the anti-inflammatory activity of viral immunomodulators with intracellular targets to CPP-tagged peptides, particularly those derived from viruses adapted to human infection. Through further peptide screening of emerging viral immunomodulators targeting key pathways in human disease, more effective therapeutics for treating human disease may be derived by allowing virus evolution to select concise points of inhibition.

6.4 Future directions

In this body of work, we describe a mechanism by which MC008 targets inflammatory signalling within the known model of IKK complex regulation. However, a number of questions remain to be answered. As our investigation into the effect of MC008 on TRIM32-driven signalling were hindered by technical difficulties, sequencing of the plasmid would be an important next step to ensure mutations were not responsible for the

observed inconsistencies. Additionally, by using an appropriate siRNA to knock-down TRIM32 or a CRISPR/Cas9 knock-out cell line, further insights into the role for MC008 in the pathway and how it perturbs events further downstream may be revealed.

Our finding that MC008 inhibits both mono- and poly-ubiquitination of NEMO can also be built upon. Future work on this should include a screen using linkage-specific ubiquitin antibodies or ubiquitin lysine mutants to characterise the type of poly-ubiquitin chains inhibited. We also observed an interesting effect whereby MC008 inhibits both IKK α/β and p65 phosphorylation with only a minor delay in I κ B α degradation. Probing with a broader range of site-specific phospho-p65 antibodies in the context of MC008 expression may provide new insights into NEMO-induced IKK activation in NF- κ B regulation.

Currently the host lab is investigating an MC008-based CPP which has demonstrated promising therapeutic potential during *in vitro* studies. Upon receiving ethical approval for murine studies, this CPP will be tested in disease-relevant *in vivo* models, such as an imiquimod-induced mouse model of psoriasis, to determine CPP efficacy.

Chapter 7 Bibliography & Appendix

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7.2 Appendix

Dynamic Assay for Profiling Anti-SARS-CoV-2 Antibodies and Their ACE2/Spike RBD Neutralization Capacity

This chapter contains extracts originally published in Viruses. Phelan T, Dunne J, Conlon N, Cheallagh CN, Abbott WM, Faba-Rodriguez R, et al. Dynamic Assay for Profiling Anti-SARS-CoV-2 Antibodies and Their ACE2/Spike RBD Neutralization Capacity. Viruses. 2021;13(7):1371.

7.2.1 Overview

During the course of my investigation into the MCV protein MC008, the coronavirus disease 2019 (COVID-19) pandemic caused significant disruption to this PhD research, including closures of many higher education research facilities around the world. For many months, lab-based higher education research in Ireland was restricted to essential activities including COVID-19 research, thus presenting an opportunity for scientists to change focus and work together towards a common goal. I was able to obtain a 6-month ‘off-books’ period to conduct COVID-19 research. Although not related to the MC008 investigation, I have included a brief overview of my work undertaken during that period for the interest of readers.

Serological assays provide a valuable tool in tackling the pandemic and investigating vaccine responses. At the time of writing, available assays did not allow determination of neutralization capacity within the assay protocol. Furthermore, commercial serology assays had a high buy-in cost that was inaccessible for many research groups.

7.2.2 Results

We replicated the assay published by Amanat et al. (292), with minor modifications. These modifications include not employing serum serial dilutions to measure antibody reactivity, the use of IgG1 as a marker for IgG humoral responses against SARS-CoV-2 and the integration of an in vitro ACE2 binding assay into the protocol. In total, we tested 103 pre-COVID-19 samples and 91 samples from patients with confirmed SARS-CoV-2 infection.

Optical density (OD) values representative of immunoglobulin levels in the cohorts were obtained for three relevant isotypes (**Figure A1.1A–F**): IgG1, IgM and IgA. A cut-off value for each isotype was derived from the mean value of the pre-COVID-19 samples, along with the addition of three standard deviations. Samples exceeding the cut-off were considered positive. We obtained a sensitivity and specificity of 88.5% (95%CI 79.5–93.81) and 99.1% (95%CI 94.8–99.95) respectively, for combined IgG1 and IgM results. As evident in the longitudinal samples (**Figure A1.1G–J**), the stage of infection plays an important role in the detection of anti-spike antibody isotypes in COVID-19 patients. This is also consistent with the kinetics of antibody responses during SARS-CoV-2 infection (293).

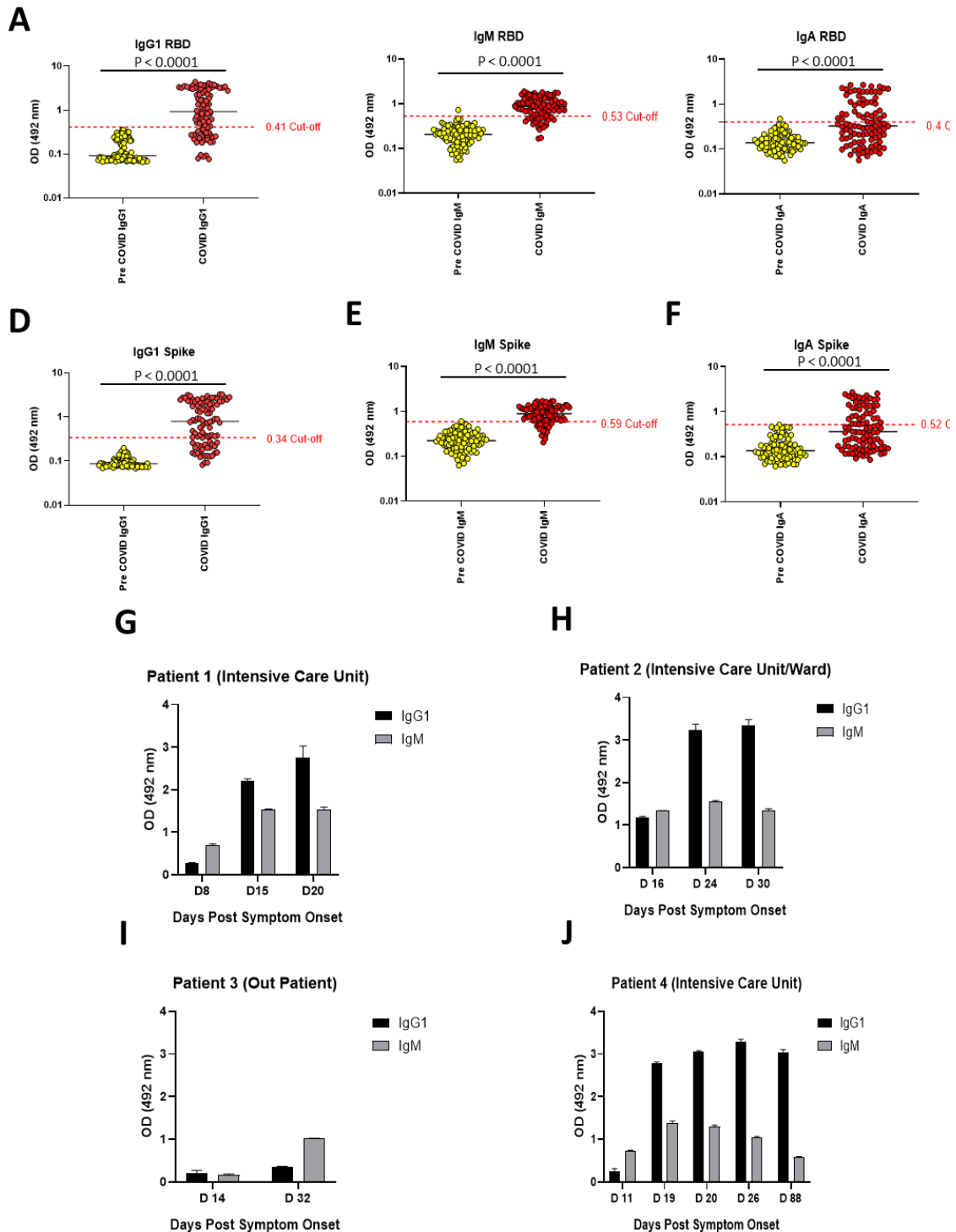


Figure A.1.1 OD values representative of antibody reactivity in sera from pre-COVID-19 and confirmed SARS-CoV-2 patients. (A–C) Results of samples tested against RBD. (D–F) Results of samples tested against full-length spike protein. Statistical analysis was performed using Mann–Whitney U tests in GraphPad Prism. The samples represented here were taken >7 days post symptom onset and include 103 pre-COVID-19 and 91 SARS-CoV-2 positive samples. Horizontal lines represent mean values and

samples were performed in triplicate. (G–J) Examples of results from longitudinal samples.

We analysed samples that were tested by commercial SARS-CoV-2 antibody serology assays (Roche Elecsys Anti-SARS-CoV-2 and Abbott SARS-CoV-2 IgG anti-N protein assays) and compared our results for IgG1 and IgM (**Figure A1.2**). Detection of either isotype was considered a ‘positive’ result for the sample. We demonstrated that the Krammer serology assay is comparable to the widely used and approved Roche and Abbott N-protein assays in terms of both sensitivity and specificity with concordances of 94.2% and 91.7% respectively.

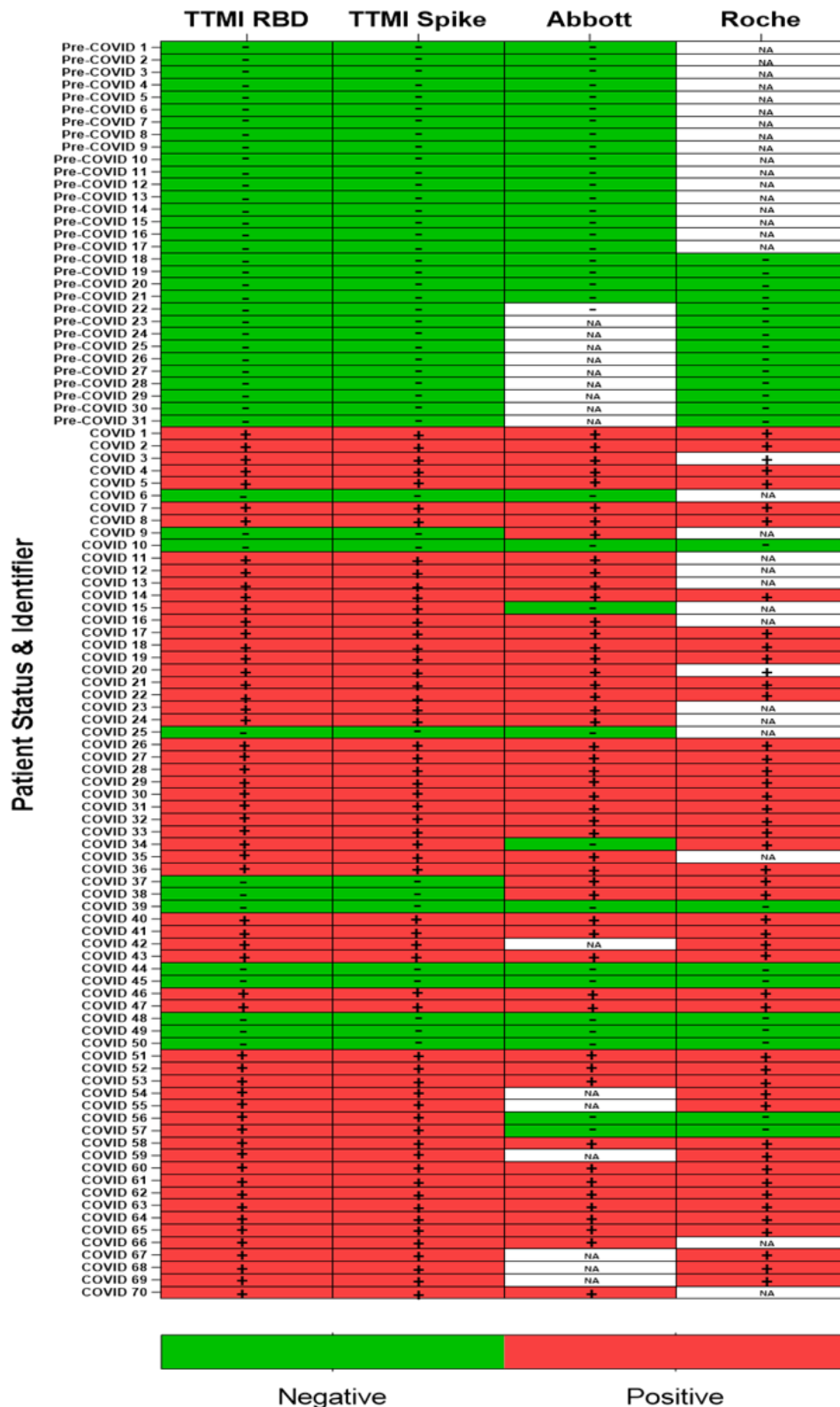


Figure A1.2 Comparison between the SARS-CoV-2 anti-spike serology assay and commercially available assays. Samples tested with the RBD and spike proteins are shown as independent assays and are comprised of IgG1 and IgM patient data. These

were compared with the same samples tested by the Roche and/or the Abbott assays where N=69 and N=84, respectively. Samples indicative of seropositivity are shown as “+” and negative as “-“. The tiles containing “NA” indicate samples in which no data is available for that assay.

In order to investigate the neutralization capacity in selected patient samples, we integrated an in vitro ACE2 binding assay into the serology protocol. We performed the in vitro ACE2 binding assay using patient serum samples, which we categorized into four different immunoglobulin profiles by serology assay screening; (High IgG, High IgM), (High IgG, low IgM), (Low IgG, High IgM), and (low IgG, low IgM). Individual patient samples from each category were distinguished as high and low for each isotype based on the ratio to the cut-off value and the samples were taken 21–88 days post symptom onset (n = 3). A sample was considered to have a high reactivity for IgM if it was $\geq 3\times$ the cut-off value or a high reactivity for IgG if it was $\geq 7\times$ the cut-off value. Samples for both isotypes were considered to have a low reactivity if they were positive but $< 2\times$ the cut-off value. These values are based on the maximum and minimum antibody reactivity for each isotype obtained during the prior serology analysis. In the patients considered to have high IgG1 and high IgM antibody reactivity, a 1 in 50 dilution of serum results in 80% reduction in the signal indicating potent neutralization (**Figure A1.3A**). While the High IgG1, Low IgM samples also possessed potent ACE2-binding neutralization capacity, particularly at lower dilutions, these were not as effective as the former (**Figure A1.3B**). Patients with low IgG and high IgM, along with low IgG and low IgM profiles, exhibited poor ACE2-binding neutralization capacity in comparison with the first two categories (**Figure A1.3C-D**). These patterns, as expected, were similar in two additional patient samples from these immunoglobulin profile categories.

To correlate the activity in the ACE2-RBD binding assay we employed a pseudovirus neutralization assay derived from the previously published method (294), to test those sera. This system involves measuring the infection of stable ACE2-expressing HEK293T cells (Invivogen) with pseudovirus containing the SARS-CoV-2 spike protein, which expresses firefly luciferase on successful infection. Neutralizing capacity of post-infection sera can therefore be estimated through a decrease in luciferase activity when compared to control sera. Similar profiles were generated using this pseudovirus system shown in red, when compared to the in vitro ACE2 binding assay results shown in blue (**Figure A1.3A–E**). This indicates a consistent effect and a reasonable correlation between the in vitro ACE2 binding assay and the cell-based pseudovirus infection assay, which was expected given the pseudovirus system, while more complex, is also primarily based on the spike-ACE2 interaction, though in a pseudoviral cell infection model.

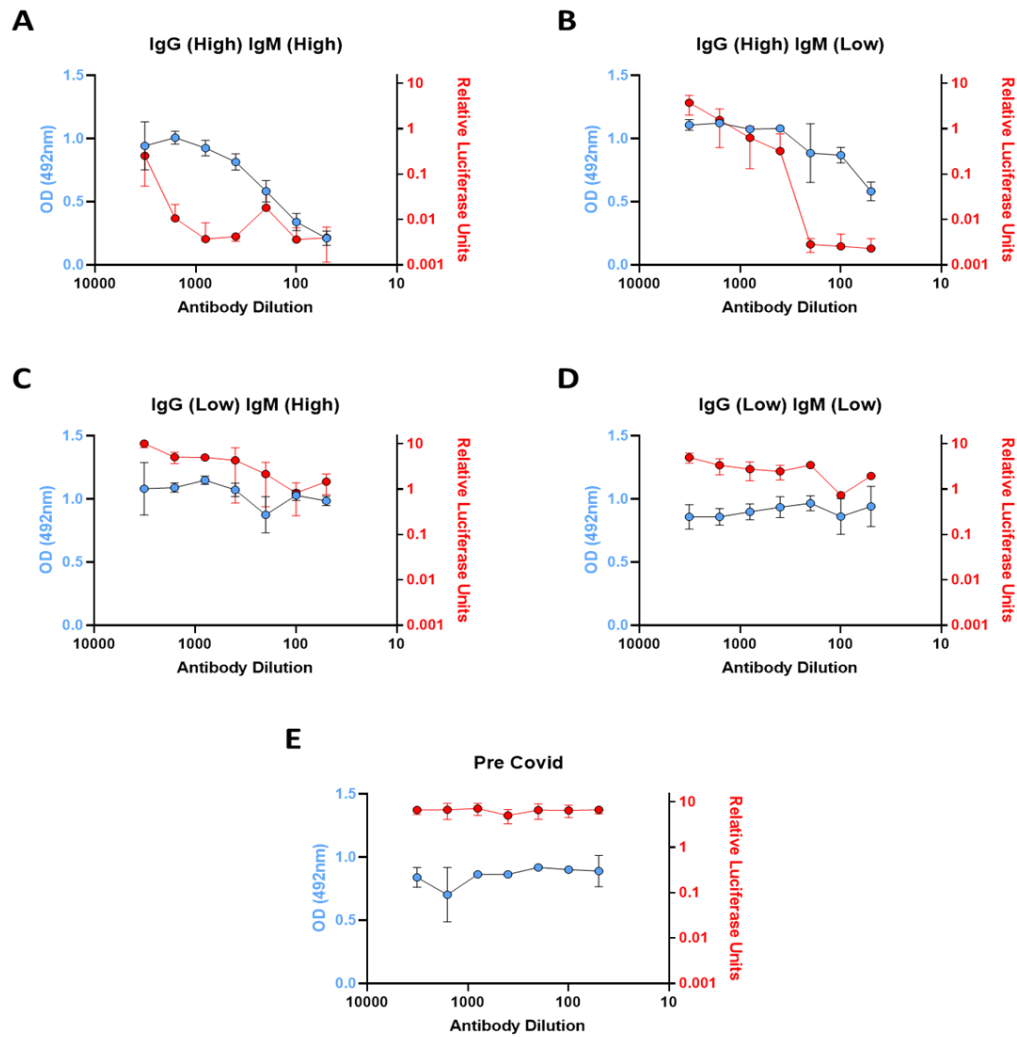


Figure A1.3 Antibody spike RBD neutralisation capacity in patient sera with different immunoglobulin profiles. (A-E) Demonstrates the spike RBD neutralisation capacity of patients with various immunoglobulin profiles using serially diluted serum. The blue points represent the data generated using the in vitro ACE2 binding assay and the red points represent data generated from the same sample using the pseudovirus assay. Data are the mean $\pm$ the standard deviation of triplicate samples from a representative experiment (N=3).

7.2.3 Conclusion

Here we have replicated a versatile and cost-effective alternative ELISA-based serology assay to those commercially available. Using this assay, we have also integrated an element of antibody neutralization into the protocol which can determine the neutralizing capacity of antibodies that block the ACE2-spike interaction in sera from patients with different immunoglobulin profiles. While antibody responses represent only one arm of the immune response against SARS-CoV-2, these tools provide an affordable and versatile option for future research.