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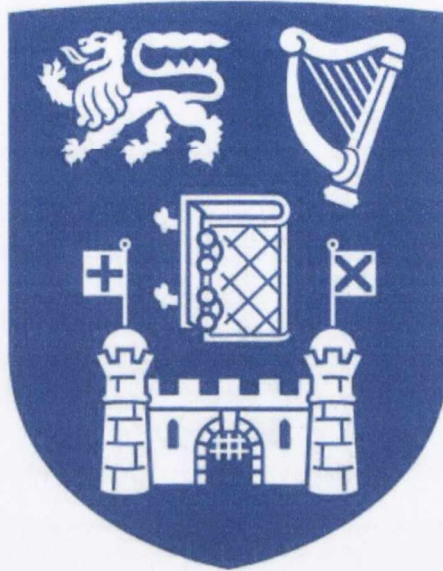
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2016

**Identification of novel immunomodulators in the
Vaccinia virus genome**

Mariann Landsberger

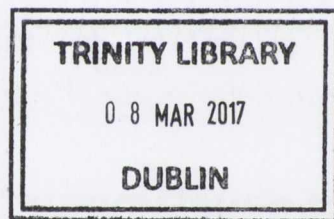


PHD
BIOCHEMISTRY
+ IMMUNOLOGY

Under the supervision of Prof. Andrew Bowie

Thesis submitted to the University of Dublin for the degree of Doctor of
Philosophy

School of Biochemistry and Immunology, Trinity Biomedical Sciences Institute, Trinity College Dublin,
Ireland



Thesis 11287

Declaration

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Abstract

A crucial early step for the initiation of an effective anti-viral response after sensing the presence of viruses is the activation of the transcription factors nuclear factor kappa-B (NFκB) and interferon regulatory factors (IRFs) by pattern recognition receptors (PRR). These transcription factors drive the expression of pro-inflammatory cytokines and type I interferons. Large double-stranded DNA poxviruses, such as *Vaccinia virus* (VACV), have evolved numerous strategies to evade these host signalling systems and elucidation of such strategies has identified key mechanisms of innate immunity. The aim of this project is to discover novel intracellular VACV immunomodulators and characterise the role of their host targets in innate immunity.

Seven novel poxviral NFκB and/or IRF inhibitors (M1, F17, A47, A57, B2, B12 and B13) were identified from a systematic reporter gene assay (RGA) screen for viral modulators of PRR signalling pathways, which included Toll-like-receptors, retinoic acid inducible gene 1-like-receptors, and the DNA sensor cyclic GMP-AMP synthase. Further RGA and functional analyses revealed that all seven proteins inhibited NFκB signalling activation by a broad variety of upstream PRR sensing pathways suggesting inhibition at points proximal to NFκB activation. In addition, F17, A47, A57, B2, B12 and B13 also inhibited IRFs, consistent with the ability of viral immunomodulators to target multiple host pathways. Stable-inducible cell lines expressing these novel inhibitors were used to identify their interaction partners by mass spectrometry analysis. The kinases vaccinia-related kinase 1 (VRK1), cyclin-dependent kinase 1 (CDK1) and the nucleosome remodeling and histone deacetylation (NuRD) complex were discovered to bind B12, F17, and A57, respectively. B12, F17, A57 and B2 emerged as the most promising novel VACV proteins, due to their significant inhibition of transcription factors, and to their putative host targets. B2 was the most potent PRR and pro-inflammatory cytokine signalling inhibitor discovered in this screen, however no interaction partners for this protein were identified. F17 is packaged into the nascent virion and can therefore exert its inhibition immediately after infection and prior to translation of other VACV immunomodulators. This protein was shown to be a potent cytosolic NFκB inhibitor, and delayed phosphorylation of p65. CDK1 was one of the identified host proteins that associated with F17. Based on this preliminary data we propose a role for CDK1 in innate immune signalling. B12, a nuclear pseudokinase, shares approximately 30 % amino acid sequence identity with VRK1, and was shown to bind VRK1. The nuclear serine/threonine kinase VRK1 has been reported in cell cycle regulation, yet no involvement in innate immunity has been discovered to date. B12 was the second strongest immunomodulator identified in this screen and we therefore propose that VRK1 plays a role in PRR and pro-inflammatory cytokine signalling. Several members of the NuRD complex were identified as interaction partners of A57, which may explain its ability to interfere with IRF and NFκB signalling proximal to these transcription factors, since this complex has been reported to repress expression of genes involved in innate immunity.

The present study revealed seven novel VACV immunomodulators, and thus implicated two cellular kinases and a chromatin remodeling complex as having roles in innate immunity due to their association with viral inhibitors. These results confirm the continuing usefulness of VACV as a model to investigate mechanisms in innate immunity.

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Abbreviations

5'pp	5'diphosphate
5'ppp	5'triphosphate
a.a.	Amino acid
AMP	Adenosine monophosphate
ANP32	Acidic leucine-rich nuclear phosphoprotein 32 family member
AP1	Activator protein 1
ATF	Activating transcription factor
ATP	Adenosine triphosphate
BAF	Barrier-to-autointegration factor
Bcl-2	B cell lymphoma 2
BLAST	Basic local alignment search tool
BSA	Bovine serum albumin
CARD	Caspase-recruitment domain
CARDIF	CARD adaptor inducing IFN β
CBP	CREB-binding protein
CDK	Cyclin-dependent kinase
CEV	Cell-associated enveloped virus
cGAMP	Cyclin GMP-AMP
cGAS	Cyclic GMP-AMP synthase
CHD4	Chromodomain-helicase-DNA-binding protein 4
ciAP1	Cellular inhibitor of apoptosis 1
CLR	C-type lectin-like receptor
CMLV	<i>Camelpox virus</i>
Cop	<i>Vaccinia virus strain Copenhagen</i>
COX-2	Cyclooxygenase-2
CPSF3	Cleavage and polyadenylation specificity factor 3
CPXV	<i>Cowpox virus</i>
CREB	Cyclic AMP response-element binding protein
CTL	Cytotoxic T lymphocyte
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DD	Death domain
DDX	DExD/H box polypeptide
DNA	Deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
dNTP	Deoxynucleotide
DOC1	Deleted in oral cancer 1
ds	Double-stranded
DTT	Dithiothreitol
EBV	<i>Epstein-Barr virus</i>
EDTA	Ethylenediaminetetraacetic acid
EEV	Extracellular enveloped virus
eIF2a	Eukaryotic initiation factor 2a

Abbreviations

ER	Endoplasmic reticulum
ERIS	Endoplasmic reticulum (ER) IFN stimulator
ERK	Extracellular signal-regulated kinase
EV	Enveloped virus
EV	Empty vector control
FADD	Fas-associated death domain protein
GATAD2B	Transcriptional repressor p66-beta
GMP	Guanosine monophosphate
GTP	Guanosine triphosphate
GUK1	Human guanylate kinase
h	Hour
HA	Hemagglutinin
HCMV	<i>Human cytomegalovirus</i>
HEK	Human Embryonic Kidney
HIV	Human immunodeficiency virus
HNRNPUL2	Heterogeneous nuclear ribonucleoprotein U-like protein 2
hpi	Hours post-infection
hps	Hours post-stimulation
hpt	Hours post-transfection
HSV	<i>Herpes simplex virus</i>
HTATSF1	HIV Tat-specific factor 1
IAV	<i>Influenza virus A</i>
ICAM-1	Intercellular adhesion molecule 1
IEV	Intracellular enveloped virus
IFI16	Gamma-interferon inducible protein 16
IFIT	Interferon-induced protein with tetratricopeptide repeats
IFN	Interferon
IFNAR	Interferon receptor
I κ B α	NF κ B inhibitor α
IKK	NF κ B inhibitor α kinase
IL	Interleukin
IL-1R	Interleukin-1 receptor
IL-1RAcP	Interleukin-1 receptor accessory protein
IMV	Intracellular mature virus
iNOS	Inducible nitric oxide synthase
IPS-1	Interferon-beta promoter stimulator 1
IRAK	Interleukin-1 receptor associated kinase
IRF	Interferon regulatory factor
ISG	Interferon-stimulated gene
ISGF3	Interferon-stimulated gene factor 3
ISRE	Interferon-stimulated response element
ITR	Inverted terminal repeat
JAK	Janus kinase
JIP1	c-Jun-amino-terminal-interacting protein 1
JNK	c-Jun N-terminal kinase

Abbreviations

kbp	Kilo base pairs
kDa	Kilo Dalton
KSHV	<i>Kaposi's sarcoma-associated herpesvirus</i>
LB	Luria-Bertani
LPG2	Laboratory of genetics and physiology 2
LPS	Lipopolysaccharide
LUBAC	Linear ubiquitin assembly complex
Lys	Lysine
m ⁷ Gcap	7-methylguanosine cap
MAL	MyD88-adaptor-like
MAPK	Mitogen-activated protein kinase
MAVS	Mitochondrial antiviral-signalling protein
MCP1	Monocyte chemotactic protein 1
MCV	<i>Molluscum contagiosum virus</i>
MDA-5	Melanoma differentiation-associated protein 5
Met	Methionine
MHC	Major histocompatibility complex
min	Minute
MIP2	Macrophage inflammatory protein 2
MITA	Mediator of IRF3 activation
MKK	Mitogen-activated protein kinase kinase
MPXV	<i>Monkeypox virus</i>
MPYS	Methionine-Proline-Tyrosine-Serine
MTA	Metastasis-associated protein MTA
MV	Mature virus
MVA	<i>Modified Ankara virus</i>
MyD88	Myeloid differentiation primary response 88
n.d.	Not determined
NAP1	NAK-associated protein 1
NEMO	NFκB essential modulator
NES	Nuclear export signal
NFκB	Nuclear factor kappa B
NK	Natural killer cell
NLR	Nod-like receptor
NLS	Nuclear localisation signal
ns	Not significant
NUCK1	Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1
NuRD complex	Nucleosome remodeling and deacetylase complex
OAS	2'-5' oligoadenylate synthetase
ORF	Open reading frame
PAMP	Pathogen-associated molecular pattern
PARP	Poly ADP ribose polymerase
PBS	Phosphate buffered saline
pc-Jun	c-Jun phosphorylated at Ser63

Abbreviations

pDC	Plasmacytoid dendritic cell
PFU	Plaque forming unit
PGE2	Prostaglandin-2
PKR	Protein kinase R
PMA	Phorbol 12-myristate 13-acetate
poly dA:dT	Poly(deoxyadenylic:deoxythymidylic) acid
poly I:C	Poly(inosinic:cytidylic) acid
PP2A	Protein phosphatase 2A
pp65	p65 phosphorylated at Ser536
PRR	Pattern recognition receptor
RALY	RNA-binding protein Raly
RANTES	Regulated on activation, normal T cell expressed and secreted
RD	Regulatory domain
RGA	Reporter gene assay
RHD	Rel homology domain
RIG-1	Retinoic acid-inducible gene 1
RING	Really interesting new gene
RIP1	Receptor-interacting protein 1
RIPLET	RING finger protein leading to RIG-I activation
RLR	RIG-like receptor
RNA	Ribonucleic acid
RNA pol III	RNA polymerase III
s	Second
SAMHD1	Sterile alpha motif and histidine-aspartate-domain-containing protein 1
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
Ser	Serine
SeV	<i>Sendai virus</i>
SH2	Src homology 2
shRNA	Small hairpin RNA
SINTBAD	Similar toNAP1 TBK1 adaptor
siRNA	Small interfering RNA
SIRT	NAD-dependent protein deacetylase sirtuin
ss	Single-stranded
STAT	Signal transducer and activator of transcription
STING	Stimulator of interferon genes
TAB	TAB1 binding protein
TAD	Transactivation domain
TAK1	Transforming growth factor β activated kinase 1
TANK	TRAF family member-associated NF-kappa-B activator
TBK1	TRAF family member-associated NFkB-activator-binding kinase 1
TBS	Tris-buffered saline
TGF β	Transforming growth factor β

Abbreviations

Th1	Type 1 T helper cell
Thr	Threonine
TIR	Toll-interleukin-resistance
TLR	Toll-like receptor
TMEM173	Transmembrane protein 173
TNFa	Tumour necrosis factor a
TNFR	TNFa receptor
TOP2	DNA topoisomerase 2
TRADD	TNFR1-associated death domain protein
TRAF	TNF receptor-associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR-domain-containing adapter-inducing IFN β
TRIM25	Tripartite motif-containing protein 25
TYK	Tyrosine kinase
Tyr	Tyrosine
und.	Undetected
VACV	<i>Vaccinia virus</i>
VARV	<i>Variola virus</i>
VHR	Vaccinia-H1 related kinase
VIPER	Viral Inhibitory Peptide of TLR4
VISA	Virus induced signalling adaptor
VRK	Vaccinia-related kinase
VSV	<i>Vesicular stomatitis virus</i>
VV-WR	<i>Vaccinia virus strain Western reserve</i>
WB	Western blot
β TrCP	β -transducin repeat-containing

Chapter 1 : Introduction

1.1. Poxviruses

1.1.1. Poxvirus taxonomy

The *Poxviridae* family comprises two sub-families: chordopoxviruses and entomopoxviruses infecting vertebrates and insects, respectively (Lefkowitz et al. 2006; Haller et al. 2014). Three genera have been described for entomopoxviruses (designated entomopoxviruses A-C). Chordopoxviruses are composed of the genera orthopoxviruses, parapoxviruses, avipoxviruses, capipoxviruses, leporipoxviruses, suipoxviruses, molluscipoxviruses, yatapoxviruses, cervidpoxviruses and crocodylipoxviruses. The two chordopoxviruses *Variola virus* (VARV), and *Molluscum contagiosum virus* (MCV), are the only obligate human poxviral pathogens, as no natural occurring animal host has yet been identified for either virus (McFadden 2005). Although humans are not the reservoir of orthopoxviruses, with exception of VARV, parapoxviruses, and yatapoxviruses, members of these genera have been reported to infect humans zoonotically (see 1.1.3).

1.1.2. Smallpox and vaccination

Variola virus is the causative agent of smallpox (Fenner et al. 1988), a febrile disease which presents with a characteristic rash, lesions, and a mortality rate ranging from 10 – 75 %. The earliest written records describing the disease have been found in China and dated back as far as the 4th century AD. Ever since, VARV has affected human populations worldwide until its eradication in 1980. During the early 20th century alone approximately 500 million people were killed by smallpox (Mahalingam et al. 2004). As a protection from the disease two practises became popular: variolation and vaccination (Fenner et al. 1988). Variolation was first described during the 10th century in China and consisted of intranasal or intradermal administration of pus or dried scab recovered from VARV-infected individuals. Despite lowering the case-fatality from 20 – 30 % to 0.5 – 2 %, this method was superseded by vaccination at the end of the 18th century. Edward Jenner coined the term vaccination in 1796, derived from ‘vacca’ the Latin word for cow, and was the first to document this practise (Riedel 2005; Sánchez-Sampedro et al. 2015). Vaccination consisted of intradermal administration of pus recovered from lesions of horses, cows, or individuals (e.g. dairymaids, horse handlers) suffering from what was presumably a *Cowpox virus* infection (CPXV, also an *Orthopoxvirus*). Adverse effects were mild compared to variolation, and generally limited to a scar at the site of inoculation. Thus, the first generation of smallpox vaccines were established (Jacobs et al. 2009; Verardi et al. 2012). It later became clear that members of the *Orthopoxvirus* genus conferred cross-protection to each other due to neutralising antibodies against viral membrane proteins (Fenner et al. 1988), which are very similar between strains. The vaccine virus strain, *Vaccinia virus* (VACV), was initially propagated in calf skin, however to limit contaminations, second generation vaccines were produced in tissue culture. Vaccination against smallpox for the general public was ceased after eradication of the disease, due to the occurrence of rare severe adverse effects, where the risk of VACV spreading systemically (e.g. generalised vaccinia, eczema vaccinatum, progressive vaccinia, postvaccinal encephalitis) became more probable than an encounter with smallpox due to the absence of an animal reservoir. To address adverse effects, third generation vaccines were developed, which were generated by the removal of non-essential genes from the viral genome through either passage in atypical hosts (e.g. *Modified Vaccinia Ankara* (MVA), (Antoine et al. 1998)) or through targeted deletion of

individual genes (e.g. NYVAC, (Tartaglia et al. 1992)). These virus strains are attenuated, and safer to use than their parental VACV strain, however they still bear risks particularly for use in immunocompromised individuals. For this reason there is an ongoing interest in characterising poxviruses, and in particular viral proteins that are able to modulate the immune system, the deletion of which may further reduce the adverse effects of these vaccines.

1.1.3. Zoonotic poxviruses

With waning cross-protection conferred by VACV, other orthopoxviruses may emerge as zoonoses (Shchelkunov 2013), some of which were documented already during the final stages of the smallpox eradication program e.g. *Monkeypox virus* (MPXV) (Ladnyj et al. 1972). Whilst the animal reservoir of MPXV is likely to be a rodent species (Essbauer et al. 2010), monkeys and humans can be infected and develop smallpox-like symptoms (McCollum & Damon 2014), which vary from mild to severe depending on the host species. Two distinct phylogenetic clades, i.e. Central African and West African, have been identified for MPXV, which differ in their virulence. Cases of zoonotic transmission have been repeatedly reported in the Democratic Republic of Congo (Rimoin et al. 2010), and its neighbouring countries (Damon et al. 2006), and in 2003 an outbreak caused by imported prairie dogs from Ghana occurred in the US, resulting in 72 infections (Reed et al. 2004).

Cowpox virus has played a crucial role in the eradication of smallpox (Essbauer et al. 2010). It causes a local, self-limiting disease in humans, characterised by pustular skin lesions at site of infection, which can develop into necrotic ulcers (Glatz et al. 2010; Favier et al. 2011). The animal reservoir of CPXV are rodents (Elsendoorn et al. 2011), with incidental hosts including cats, cows and zoo animals, which may facilitate transmission to humans. Recently, pet rats have been increasingly reported to transmit CPXV to humans (Elsendoorn et al. 2011).

Phylogenetic analyses have revealed a close evolutionary relationship between *Camelpox virus* (CMLV) and VARV (Gubser & Smith 2002). Similar to VARV, CMLV causes a systemic and often fatal disease in a single host (i.e. camels). During an outbreak in India in 2009, 3 human cases of CMLV were reported, however symptoms were limited to the primary site of infection (Bera et al. 2011).

The emergence of zoonoses involving VACV-like viruses such as *Buffalopox virus*, *Aracatuba virus* and *Cantagalo virus* has been documented in India and Brazil (Singh et al. 2012). In addition to cows, buffalos were used in India to produce the vaccine strain before cell culture systems were available. Furthermore, during eradication, VACV may have been spread to cows by vaccinated individuals handling cattle. No reservoir for VACV or VACV-like viruses has been found to date. Whilst infection of cattle and buffalos with VACV-like viruses causes severe disease in these animals, resulting in reduced milk production and death, to date immunocompetent humans solely present with lesions on hands and arms (Oliveira et al. 2013; Oliveira et al. 2014; Abrahão et al. 2015).

Other poxviruses with zoonotic potential are *Tanapox virus* of the *Yatapoxvirus* genus (Essbauer et al. 2010), and the *Parapoxvirus Orf virus*, which causes a mild cutaneous infection in humans following handling of contaminated goats (Kitchen et al. 2014; Turk et al. 2014; Keshan Zhang et al. 2014). Poxviruses, and in particular orthopoxviruses have the ability to infect humans, however human-to-human transmission of

these zoonotic viruses appears very restricted as only very few cases have been reported to date (Fine et al. 1988; Learned et al. 2005; Turk et al. 2014; Oliveira et al. 2014). Such low infection rates may be the result of existing cross-protection by the smallpox vaccine in a vaccinated population. However, to prevent the potential emergence of zoonotic poxviruses, a new generation of vaccines is under development. These vaccines are derived from a variety of poxviruses (e.g. ortho-, lepori-, and avipoxviruses), with the majority based on VACV (Jacobs et al. 2009), not only because it confers cross-protection, but also because of the extensive immunological and clinical knowledge of this virus gained during and since the smallpox eradication.

1.1.1. Vaccine vectors

Even prior to the eradication of smallpox, the scientific community recognised that VACV and other poxviruses could act as vaccine vectors in the vaccination strategies against other pathogens (Fenner et al. 1988). Recombinant VACV could be easily generated, as VACV was able to incorporate plasmid as well as genomic deoxyribonucleic acid (DNA) through homologous recombination (Evans et al. 1988). It was shown that the VACV genome supported the insertion of genes up to 25 kilo base pairs (kbp) (Smith & Moss 1983), and multiple genes encoding different proteins could be inserted (Perkus et al. 1985), which were expressed during vaccination and elicited an appropriate immune response against the expressed protein. Since then, numerous vaccines candidates have been developed for viral, bacterial and parasitic pathogens for use in animals and humans (Verardi et al. 2012; Sánchez-Sampedro et al. 2015). The rabies vaccine Raboral-VRG® is based on VACV, and has been used to successfully reduce the spread of this disease in wildlife in Europe and North America (Pastoret & Brochier 1996). Whilst no human poxviral vaccines have been licenced to date, numerous candidates are in preclinical and clinical trials (Verardi et al. 2012; Sánchez-Sampedro et al. 2015). MVA in particular has been intensively studied as a vaccine vector, and recently several successful phase one and two clinical trials have reported its potential for use against *Human immunodeficiency virus* (HIV) (Goepfert et al. 2014; Nilsson et al. 2014), *Influenza A virus* (IAV) (Kreijtz et al. 2014), tuberculosis (Tameris et al. 2014), and malaria (Hodgson et al. 2015).

1.1.2. Oncolytic virotherapy

The concept of treating cancer with viruses arose during the 1920's (Noguchi et al. 1963). In 1963, Noguchi and co-authors were able to successfully reduce skin cancers in 26 patients by directly injecting so-called "purified vaccine lymph", a suspension of live VACV, into the cancer. Whilst oncolysis was observed in the majority of treated patients, adverse effects such as fever also occurred. Vaccine vectors have since then been improved (Verardi et al. 2012; Sánchez-Sampedro et al. 2015). It was shown that non-essential genes of viruses could be deleted, which conferred a tropism for cancer cells (Guo et al. 2005; Guo et al. 2008). Accordingly, the objectives behind oncolytic virotherapy are the specific destruction of cancer cells by the virus and development of an immune response against infected cancer cells (Guo et al. 2008). Whilst numerous oncolytic poxviruses are currently in development, recently a VACV strain (Pexa-Vec) targeting primary liver cancer successfully completed phase 1 clinical trials and has entered phase 2 clinical trials

(Breitbach et al. 2015). A MVA strain (TroVACX) targeting metastatic colorectal cancer has also passed initial phase 1 and 2 trials (Rowe & Cen 2014).

1.1.3. Genome organisation

The poxviral genome is composed of linear double-stranded (ds) DNA of 130 to 230 kb (Moss 2013). The two DNA strands are connected at their termini generating hairpin loops (Figure 1-1). Bioinformatic analyses have demonstrated that ninety viral proteins are conserved among chordopoxviruses, 49 of which are conserved within the *Poxviridae* family (Upton et al. 2003; Gubser et al. 2004). These genes have been mapped within the central 100 kb of the genome, which predominantly encodes proteins involved in ribonucleic acid (RNA) and DNA synthesis, protein processing, virion assembly and maturation. In comparison, the genomic termini are less well conserved as exemplified by a bioinformatical analysis that identified only twelve common proteins within the terminal 100 kb of the *Orthopoxvirus* genus. These proteins are termed “virulence factors” and intervene in host tropism and immune responses.

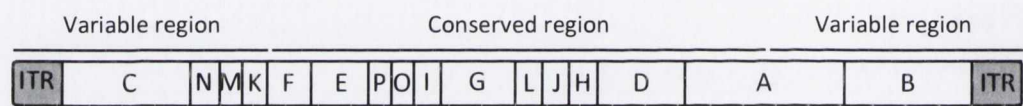


Figure 1-1 Schematic Hind III restriction map of VACV Copenhagen strain

VACV is a linear dsDNA molecule with covalently linked termini, which contain inverted terminal repeats (ITR). Variable regions and regions conserved amongst chordopoxviruses are indicated by bars. Hind III digestion fragments are designated by a letter (A-O). Adapted from (DeFilippes 1982).

By way of nomenclature, poxviral genes are annotated with reference to the VACV Copenhagen (Cop) strain given in the format X#(R/L), where X is a letter (A-O) designating the Hind III fragment (A-O), # is a number pertaining to the open reading frame (ORF), and R or L denoting a suffix indicating the direction of transcription. The latter is omitted when referring to proteins (DeFilippes 1982; Moss 2013).

1.1.4. Viral life cycle

VACV virions exist in two distinct infectious forms (Roberts & Smith 2008). The mature virus (MV, also known as intracellular mature virus (IMV)) contains a dumbbell-shaped core, which is flanked by aggregates of heterogeneous material called lateral bodies (Figure 1-2).



Figure 1-2 VACV particle
Cryo-electron micrograph of an isolated VACV mature virion. Scale bar: 200 nm (Cyrklaff et al. 2005)

The core and the lateral bodies are surrounded by a single membrane. MV is released upon cell lysis and represents the majority of VACV virions. The extracellular virus (EV, also known as extracellular enveloped virus (EEV)) has two membranes and is released by fusion with

the cell membrane. Although EV only represents 1 % of generated progeny, it is assumed to be crucial for cell-to-cell and *in vivo* spread (Figure 1-3).

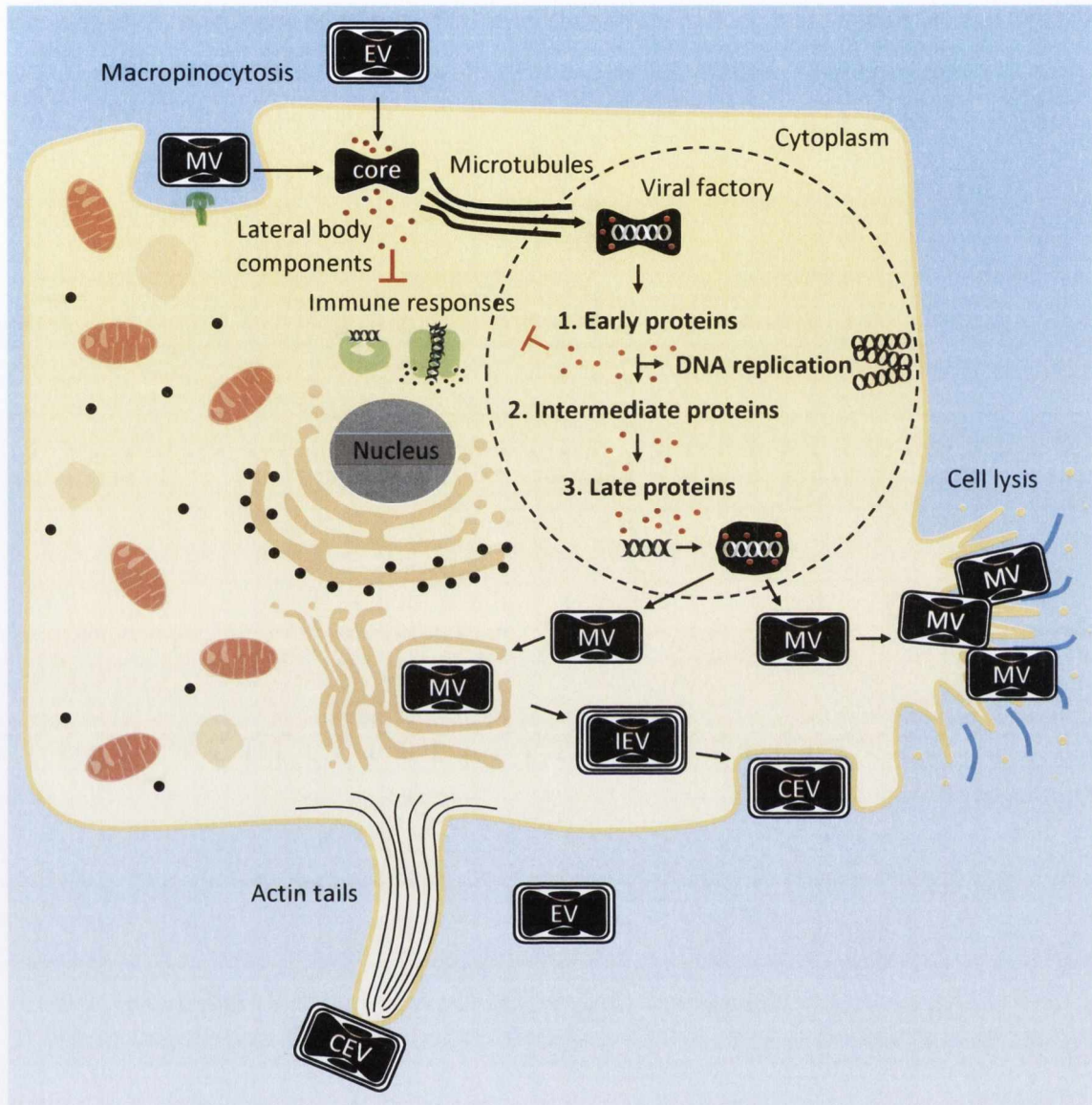


Figure 1-3 VACV life cycle

Schematic representation of VACV viral life cycle. Upon penetration of the host plasma membrane, viral membranes of MV and EV particles are removed and the viral core enters the cytoplasm. At perinuclear regions, viral factories are established, where transcription and translation of viral genes, genome replication and particle assembly take place. *De novo* formed MV are either released by cell lysis, or acquire additional membranes and are transported to the plasma membrane, where they become CEVs. CEVs use cellular projections to reach to non-infected cells.

Both EV and MV enter the cell by macropinocytosis (Mercer & Helenius 2008; Sandgren et al. 2010), however EV requires acidification of the vesicle to disrupt its outer membrane, and release MV (Schmidt et al. 2011), which in turn is able to fuse with endosomal membranes. The MV/EV core then enters the cytoplasm, and is transported to the perinuclear region via microtubules (Roberts & Smith 2008). The virus establishes viral factories, which are shielded from cellular organelles and are the site of genomic DNA replication, transcription, translation and virion assembly. The viral core is composed of structural proteins, the compacted genome and the early transcription machinery which includes a DNA-dependent RNA polymerase,

and enzymes able to generate mRNA that is capped, methylated and polyadenylated (Moss 2013). Partial uncoating of the core allows the transcription of early genes (Assarsson et al. 2008; Roberts & Smith 2008; Moss 2013), which are involved in host immune evasion, genomic DNA replication and regulation of intermediate-stage gene expression. Replicated DNA serves as the template for transcription of intermediate and late genes. The latter are implicated in virion assembly and also include transcriptional enzymes and factors required for transcription of early genes during the following infectious cycle. The sequential transcription of early, intermediate and late genes is regulated by consensus transcription start sites and termination sequences, which are recognised by stage-specific transcription machineries. Late genes rarely contain a termination sequence and, in addition, are transcribed from both DNA strands, thereby producing double-stranded RNA (Smith & Symons 1998). Approximately 10,000 genome copies are generated per infected cell (Joklik & Becker 1964), of which only 30 – 50 % are packaged into nascent virions. DNA replication occurs concomitantly with core disassembly at 1 to 2 hours post infection (hpi). To increase nucleotide levels, poxviruses encode enzymes involved in deoxynucleotide (dNTP) synthesis (e.g. thymidine kinase).

Genomic DNA is packaged into assembled brick-shaped IMV (Roberts & Smith 2008). These viral particles can be wrapped in double cellular membranes derived from endoplasmic membranes, or Golgi-cisternae generating intracellular enveloped virions (IEV). IEVs are transported to the plasma membrane via microtubules, where membrane fusion gives rise to cell-associated enveloped virus particles (CEV). Released CEVs are termed EVs. CEV trigger the formation of actin filaments which create cellular projections that transport individual viral particles to the surface of uninfected cells (Doceul et al. 2010).

1.2. Innate immunity

The immune system consists of an intrinsic as well as adaptive system of defence against various pathogens such as viruses, bacteria, fungi and multicellular parasites (Turvey & Broide 2010; Iwasaki & Medzhitov 2015). It is composed of three main arms with external barriers constituting the first line of defence: physical barriers such as the skin and epithelial cilia, and chemical barriers including skin pH and lysosomal enzymes. The second line of defence termed the “innate immune” system, mediates a rapid response to pathogens that have breached the first line of defence. The principal objective of the innate immune system is to distinguish between self and non-self, which is achieved through expression of a limited number of germline-encoded pattern recognition receptors (PRR) on hematopoietic and non-hematopoietic cells (i.e. fibroblasts, macrophages, dendritic cells (DC)). These receptors recognise pathogen-associated molecular patterns (PAMP) and danger-associated molecular patterns (DAMP). The third line of defence is called the “adaptive immune system”, the response of which to pathogens develops over a longer time-scale than the innate system. The adaptive immune system has the ability to develop a memory of the intruding pathogen, relying on a vast repertoire of lymphocytes expressing highly specific receptors (e.g. B and T lymphocytes). It consists of humoral (i.e. neutralising antibody production) and cell-mediated (CD4+ and CD8+ T lymphocytes, cytolytic T lymphocytes (CTL)) responses. The innate immune response plays a pivotal role in initial host protection, and furthermore, activation and instruction of the adaptive immune system (Iwasaki & Medzhitov 2015). The innate response is mounted within minutes upon detection of infection (Turvey & Broide 2010; Iwasaki & Medzhitov 2015). Infections are cleared by phagocytosis of extracellular pathogens, degranulation of antimicrobial cytotoxic molecules, which damage multicellular parasites, killing of virally or bacterial infected cells by natural killer cells (NK), and the production of small soluble mediators, i.e. cytokines and chemokines, in order to recruit additional immune cells from circulation and to activate the adaptive immune system. In addition to specialised immune cells, non-hematopoietic cells such as fibroblasts and epithelial cells have the ability to produce cytokines and chemokines to elicit a response to an invading pathogen.

1.2.1. Cytokine signalling pathways and function in immunity

Cytokines are small soluble proteins involved in auto-, para-, and endocrine cell signalling (Turvey & Broide 2010). Production and secretion of a subset of these small proteins is induced by recognition of PAMPs or DAMPs by sentinel cells such as epithelial cells, fibroblasts, DCs and macrophages. After binding to their cognate receptor, cytokines trigger activation of signalling cascades. This can lead to the establishment of a cell-intrinsic antimicrobial state, and initiation of the innate immune response by activating and recruiting immune cells such as neutrophils or NK cells from circulation to the site of infection. Following this, the adaptive immune system can be activated. Interferons (IFN), interleukins (IL), and tumour necrosis factor α (TNF α) are well known examples of PRR-induced cytokines and are described in further detail in the following section (Figure 1-4) (Turvey & Broide 2010).

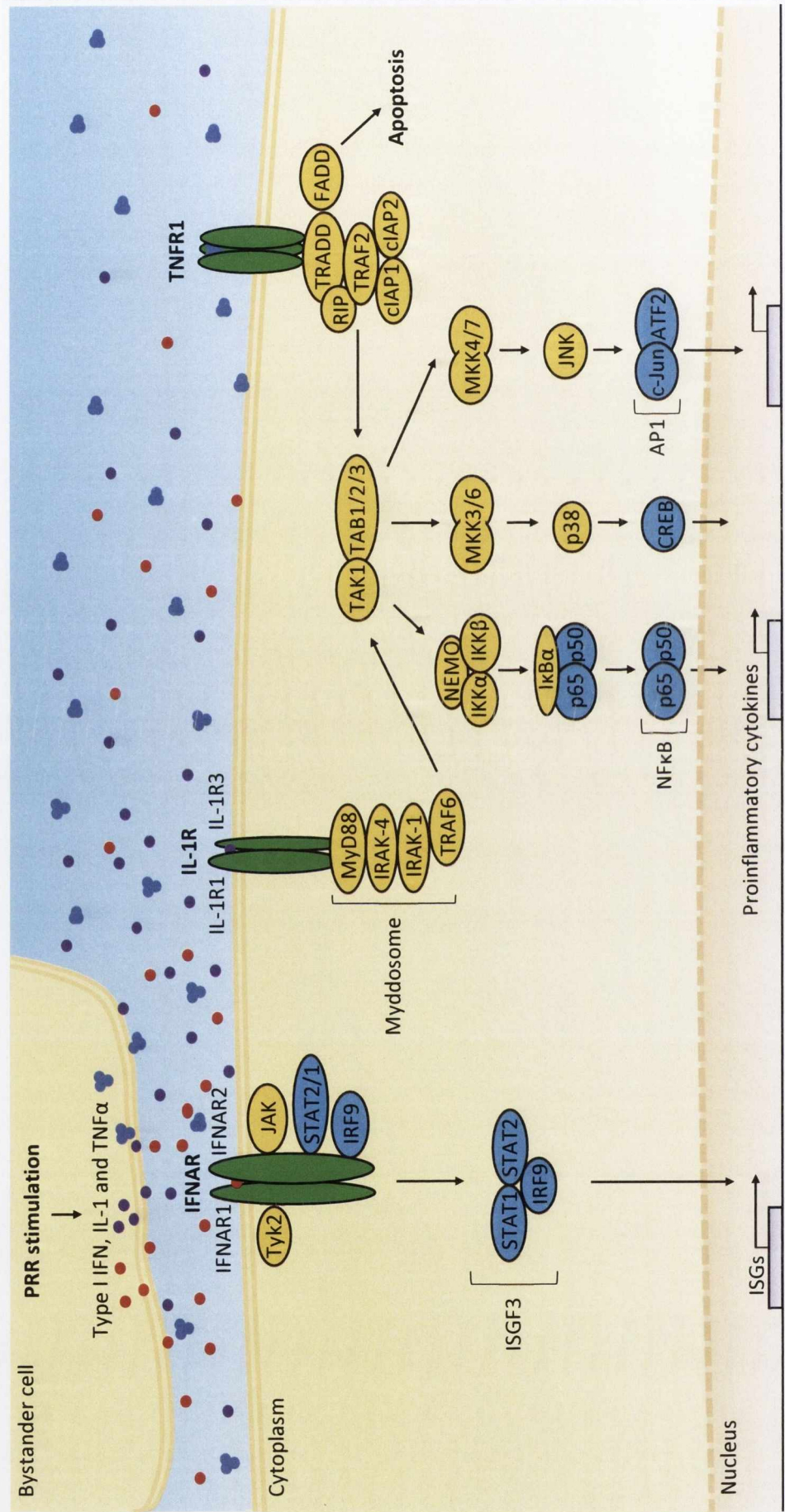


Figure 1-4 Overview of cytokine signalling pathways

Cytokines produced in response to pathogens (e.g. type I IFNs (red), IL-1 (purple) and TNFα (blue)) trigger oligomerisation of their cognate receptors and thereby activate a signalling cascade resulting in activation of transcription factors (e.g. STATs, IRFs, NFκB, and AP1). These transcription factors translocate to the nucleus, where they induce the expression of interferon-stimulated genes and pro-inflammatory cytokines.

1.2.1.1. Type I interferons

1.2.1.1.1. Introduction

Interferons were discovered as soluble factors secreted by cells that had been exposed to heat-inactivated IAV (Isaacs & Lindenmann 1957). These agents were found able to interfere with live virus challenges, thus termed 'interferons'. They are classified into three types according to their receptor and function (Schneider et al. 2014; McNab et al. 2015). In humans, type I IFNs are comprised of thirteen IFN α proteins, and a single form of IFN β , IFN ϵ , IFN κ and IFN ω . IFN γ is the only member of the type II IFN group, whereas type III IFNs contain four IFN λ proteins, which have similar functions to the type I IFNs. Type I IFNs signal through the heterodimeric IFN α receptor (IFNAR)1-IFNAR2 receptor complex (Uzé et al. 1990; Novick et al. 1994; Cohen et al. 1995), inducing the expression of IFN stimulated genes (ISG) (McNab et al. 2015). Both the innate and the adaptive immune response can be shaped by the activity of ISGs through activation of NK cells, enhancement of DC differentiation, increasing major histocompatibility complex (MHC) expression on the plasma membrane of DCs, promoting type 1 T helper cell (Th1) and CTL responses, and triggering antibody production by B cells.

1.2.1.1.2. Type I IFN signalling

Type I IFNs signal through the heterodimeric transmembrane receptor IFNAR (Uzé et al. 1990; Novick et al. 1994; Cohen et al. 1995), composed of IFNAR1 and IFNAR2 subunits (Figure 1-4). Under homeostatic conditions, tyrosine kinase 2 (Tyk2) is bound to the cytoplasmic domain of IFNAR1 (Abramovich et al. 1994), and Janus kinase 1 (JAK1) is bound to IFNAR2 (Novick et al. 1994). IFNAR2 is also associated with the transcription factor signal transducer and activator of transcription 2 (STAT2) (Li et al. 1997), and interacts weakly with STAT1. Furthermore, the transcription factor interferon regulatory factor 9 (IRF9) was reported to associate with STAT2 in absence of stimulation (Martinez-Moczygemba et al. 1997; Banninger & Reich 2004), which allows the STAT2-IRF9 complex to shuttle in between the cytoplasm and the nucleus due to a nuclear export signal (NES) present in STAT2 and a nuclear localisation signal (NLS) present in IRF9.

Upon ligand binding, IFNAR1 and IFNAR2 dimerise (Cohen et al. 1995), which juxtaposes the kinases JAK1 and Tyk2 leading to their transphosphorylation and activation (Müller et al. 1993). IFNAR1 is subsequently phosphorylated by Tyk2 at tyrosine (Tyr) 466 creating a docking site for STAT2 (Yan et al. 1996). STAT2 association with IFNAR1 is mediated by the STAT2 src homology 2 (SH2) domain (Fu et al. 1992). STAT1 and STAT2 activation is achieved through phosphorylation of STAT1 at Tyr701 by JAK1, and phosphorylation of STAT2 at Tyr690 by Tyk2 (Improta et al. 1994), leading to SH2-dependent STAT1-STAT2 heterodimerisation and dissociation from the receptor complex along with IRF9 (Kessler et al. 1990; Schindler et al. 1992). The *in situ* formed complex, interferon-stimulated gene factor 3 (ISGF3) (Banninger & Reich 2004), composed of STAT1, STAT2 and IRF9, is able to translocate to the nucleus due to an additional NLS provided by STAT1. In the nucleus, ISGF3 induces the expression of ISGs by binding to the IFN-stimulated response element (ISRE) present in the ISG promoter (Kessler et al. 1988).

1.2.1.1.3. Interferon stimulated genes

Interferon-stimulated genes have a wide gamut of functions in homeostasis and pathogenesis, with a particular role in viral infections as evidenced by knockout mouse infection models (Schoggins 2014). Currently, over 5000 ISGs have been reported (Rusinova et al. 2013), of which 3000 are induced by type I IFNs. Their roles range from upregulating the sensitivity of cells to infection (e.g. induction of PRRs, chemokine and cytokine receptors), direct antiviral effects, driving pro-apoptotic processes, but also directing a return to homeostasis (Schneider et al. 2014). The antiviral response mediated by ISGs targets cell entry, viral replication (e.g. sterile alpha motif and histidine-aspartate-domain-containing protein 1 (SAMHD1), oligoadenylate synthetase (OAS) and RNase L), translation of viral proteins (e.g. dsRNA-dependent protein kinase R (PKR) and interferon-induced proteins with tetratricopeptide repeats (IFIT)), and viral egress (e.g. tetherin). Several ISGs, including SAMHD1, OAS, PKR and IFIT, have been shown to intervene in VACV pathogenesis.

1.2.1.1.3.1. Sterile alpha motif and histidine-aspartate-domain-containing protein 1

SAMHD1 has been established as a host protein with triphosphate hydrolase activity which is able to restrict retroviral replication (Ballana & Esté 2015), but has also been reported to impair the life cycle of DNA viruses such as VACV and HSV-1 (Hollenbaugh et al. 2013). Upon binding of deoxynucleotides, SAMHD1 tetramerises, and subsequently converts dNTPs into deoxynucleosides and triphosphoate molecules, hereby limiting the amount of dNTPs available for viral and host replication. Whilst restriction of dNTP levels during viral infections is advantageous, it may have detrimental effects for normal cell processes (Ballana & Esté 2015). Consequently, it has been shown that SAMHD1 function can be modulated by cell cycle regulatory proteins such as the cyclin-dependent kinase (CDK) family (Cribier et al. 2013), which reduce SAMHD1 function in cycling cells.

1.2.1.1.3.2. Oligoadenylate synthase and RNaseL

The activity of OAS and RNaseL targets viral replication, too (Drappier & Michiels 2015). Interferon upregulates OAS levels, which in response to dsRNA stimulation polymerises adenosine triphosphate (ATP) into 2'-5' oligoadenylates. This second messenger is recognised by the endoribonuclease RNase L, which dimerises and cleaves single-stranded (ss) RNA, thereby limiting viral replication.

1.2.1.1.3.3. Proteinase kinase R and Interferon-induced proteins with tetratricopeptide repeats

Another strategy is the restriction of viral protein synthesis by preventing the assembly of the translation machinery (Munir & Berg 2013; Fensterl & Sen 2015). Both PKR and IFIT protein family members have been reported to reduce translation following direct recognition of viral RNA. Upon binding of dsRNA (Munir & Berg 2013), the inactive serine/threonine (Ser/Thr) kinase PKR homodimerises and phosphorylates the eukaryotic translation initiation factor 2 α (eIF2 α), which inhibits both viral and host mRNA translation. In contrast, IFIT1, (also called ISG56), recognises and sequesters 5'triphosphate RNA, thereby impairing translation (Pichlmair et al. 2011; Kumar et al. 2014; Fensterl & Sen 2015). In addition, IFIT1 binds methylated cap structures of viral mRNA, limiting their accessibility for eIF4 ϵ .

Interferons have well established roles in antiviral and anti-tumour responses, whilst pro-inflammatory cytokines such as interleukins and TNF α are best known for their ability to induce inflammation and fever.

1.2.1.2. Interleukin-1

1.2.1.2.1. Introduction

Interleukin-1, initially termed leukocytic pyrogen, was originally studied for its role in sterile fever induction (Wood 1958), and later for its involvement in lymphocyte activation (Rosenwasser et al. 1979). Since then its pleiotropic activities have been well established including regulating cortisol and insulin levels, reducing appetite, and promoting production of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and prostaglandin-2 (PGE2) (Dinarello 2015). These effects can result in the initiation of fever, hypotension and vasodilatation. Interleukin-1 also promotes neutrophil infiltration, expression of cytokines (e.g. IL-6), and drives the differentiation of DC, NK, T and B cells.

The IL-1 family comprises eleven members, of which IL-1 α and IL-1 β are the most studied (Dinarello 2009). In contrast to other interleukins, the members of the IL-1 family are not only able to influence immune responses by interacting with their cell surface receptor with subsequent induction of pro-inflammatory cytokines, but have also been reported to act directly as transcription factors in the nucleus, as exemplified by the non-processed form of IL-1 α (Werman et al. 2004).

Active IL-1 β is generated in a two-step process (Dinarello 2010). Stimulation of PRRs or the interleukin-1 receptor (IL-1R) initiates transcription and translation of IL-1 β mRNA, which leads to the production of biologically inert pro-peptide. Recognition of DAMPs and PAMPs leads to the assembly of a protein complex termed the “inflammasome”, which processes inactive pro-caspase-1 into active caspase-1. In turn, caspase-1 cleaves pro-IL-1 β into its mature active form, which is then secreted. Unprocessed IL-1 β may also be cleaved by the extracellular neutrophil- and mast cell-derived proteases such as proteinase-3, elastase, and chymase (Guma et al. 2009; Joosten et al. 2009). In contrast to IL-1 β , unprocessed IL-1 α is active, as demonstrated by its ability to act as a transcription factor (Werman et al. 2004), as well as to bind and to activate its receptor (Kim, Lee, et al. 2013). This cytokine is primarily released from necrotic cells inducing inflammation and is rarely secreted into circulation in response to PRR stimulation (Dinarello 2010).

Upon IL-1 β recognition, the IL-1 receptor complex assembles and recruits an oligomeric complex termed the “myddosome” (Motshwene et al. 2009; Lin et al. 2010). This complex is composed of the adaptor protein myeloid differentiation primary response 88 (MyD88) and members of the interleukin-1 receptor associated kinase (IRAK) family. Its recruitment results in activation of the transcription factor nuclear factor kappa B (NF κ B) (Figure 1-4).

1.2.1.2.2. IL-1 signalling

The IL-1R family is composed of ten members, two of which: IL-1R1 and the accessory protein IL-1R3 (also known as IL-1RAcP) (Sims et al. 1989; Greenfeder et al. 1995), are required for signal transduction elicited by IL-1 α and IL-1 β . The interleukin-1 receptors are transmembrane proteins (Boraschi & Tagliabue 2013), composed of an extracellular IL-1 binding domain containing three immunoglobulin G-like regions and an

intracellular Toll-interleukin-resistance (TIR) domain, which enables downstream signalling. The TIR domain is an evolutionary conserved cytoplasmic domain identified initially in the *Drosophila melanogaster* protein Toll, in human IL-1R, and in the antiviral resistance plant N protein (Rock et al. 1998). It was therefore termed “Toll-interleukin-resistance” domain. This domain was subsequently used to identify further TIR-domain containing homologues, such as the human Toll-like receptor family and numerous TIR-containing adaptors (see 1.2.2.1).

Upon IL-1 β binding by IL-1R1, the accessory protein IL-1R3 is recruited, forming the heterodimeric IL-1 receptor (Greenfeder et al. 1995; Boraschi & Tagliabue 2013), which stabilises the ligand-receptor complex. Receptor dimerization leads to approximation of the cytosolic TIR domains resulting in generation of a signalling surface for the TIR-domain containing adaptor protein MyD88 (Hultmark 1994; Muzio et al. 1997). MyD88 is composed of a C-terminal TIR domain, a short intermediate domain and an N-terminal interaction domain termed the “death domain” (DD) (Muzio et al. 1997; Wesche et al. 1997), which serves to recruit members of the IRAK family. Early reports demonstrated MyD88 association with IRAK-1, IRAK-2 and IRAK-4 (M Muzio et al. 1997; Wesche et al. 1997; Burns et al. 2003; Brikos et al. 2007). The role of IRAK-4 in IL-1 signalling as an adaptor linking MyD88 to downstream kinases has been well established, yet the respective roles of IRAK-1 and IRAK-2 are not entirely understood. Macrophages lacking both kinases are markedly impaired in cytokine production demonstrating their importance in IL-1 signalling (Kawagoe et al. 2008).

The IRAK family is composed of four members each characterised by an N-terminal DD and a proline-serine-threonine rich proST region, a central kinase domain, and C-terminal TNF receptor-associated factor 6 (TRAF6)-interaction motifs (Flannery & Bowie 2010). IRAK-4 lacks TRAF6-interaction motifs. Studies of crystallised death domains of adaptor complexes revealed TIR-induced helical oligomerisation of six MyD88 monomers (Motshwene et al. 2009; Lin et al. 2010), which recruit four IRAK-4 proteins to form the stable myddosome complex. Four IRAK-2 proteins are recruited to the MyD88-IRAK-4 oligomer (Lin et al. 2010), which are subsequently phosphorylated by IRAK-4. Early studies reported phosphorylation of key residues in IRAK-1 (i.e. Thr209 and Thr387) after recruitment to IRAK-4. This leads to a conformational change in IRAK-1, enabling the phosphorylation of the kinase domain activation loop. Fully activated IRAK-1 hyperphosphorylates itself within the proST region (Burns et al. 2003; Kollewe et al. 2004). Activated IRAK-1 recruits TRAF6 via its three TRAF6-interaction motifs (Ye et al. 2002; Kollewe et al. 2004).

The E3 ubiquitin ligase TRAF6 contains N-terminal really interesting new gene (RING), and zinc finger domains, and a C-terminal TRAF-C domain (Cao et al. 1996), which is required for interactions with other TRAF family members. TRAF6 recruits and activates a complex comprised of transforming growth factor β (TGF β) activated kinase-1 (TAK1), and TAK1 binding protein 1 (TAB1), TAB2 or TAB3 (Wang et al. 2001). Several studies reported the involvement of lysine 63 (Lys63)-linked polyubiquitin chains bound to TRAF6 in the activation of TRAF6 itself and TAK1 (Lamothe et al. 2007; Conze et al. 2008). These polyubiquitin chains can be generated by TRAF6 in association with the dimeric ubiquitin-conjugating complex Ubc13-Uev1A (Deng et al. 2000; Wang et al. 2001; Xia et al. 2009). Polyubiquitin chains are bound by the TAK1 scaffold proteins TAB2 and TAB3, as well as by the scaffold protein NF κ B essential modulator (NEMO) (Kanayama et al. 2004; Emmerich et al. 2013). NEMO is associated with the NF κ B inhibitor α (I κ B α) kinase (IKK) complex, and by binding polyubiquitin chains NEMO facilitates the activation of the IKK complex by TAK1 (Emmerich et al.

2013; Zhang, Clark, et al. 2014). A cell-free study demonstrated that unanchored polyubiquitin chains, generated by UbcH5C instead of Ubc13-Uev1A, were also bound by TAB2 and led to TAK1 activation, whereas TRAF6 bound ubiquitin chains were dispensable for TAK1 activation (Xia et al. 2009). Recently, other host protein complexes such as the linear ubiquitin assembly complex (LUBAC) were shown to generate methionine 1 (Met1)-linked polyubiquitin chains following TRAF6-mediated Lys63-polyubiquitin chain formation, and the occurrence of Lys63/Met1 hybrids was reported (Emmerich et al. 2013). It was demonstrated that TAB2/3 preferentially bound to Lys63-polyubiquitin chains, whereas NEMO favoured Met1-polyubiquitin chains.

Activated TAK1 phosphorylates key residues in the activation loops of its cytosolic targets IKK β and mitogen-activated protein kinase (MAPK) kinase 6 (MKK6) (Wang et al. 2001). Overexpression, as well as deletion of TAK1 revealed its crucial role in downstream activation to the MAPK p38, c-Jun N-terminal kinase (JNK), and transcription factors (Ninomiya-Tsuji et al. 1999; Wang et al. 2001; Sato et al. 2005; Shim et al. 2005). TAK1 activates the MAPK complexes MKK4-MKK7, which in turn recruits JNK (Dérjard et al. 1995; Tournier et al. 2001), leading to activation of the activator protein-1 (AP1). The AP1 transcription factor family are homo- and heterodimers composed of Jun (e.g. c-Jun), activating transcription factor (ATF), and musculoaponeurotic fibrosarcoma proteins (Meng & Xia 2011), which regulates various cellular processes including cell cycle and inflammation. Following activation of JNK, this kinase phosphorylates c-Jun (Dérjard et al. 1994), and ATF2 resulting in their activation (Gupta et al. 1995; van Dam et al. 1995), dimerization (Hai & Curran 1991), and nuclear translocation. TAK1 also activates the MAPK complex MKK3-MKK6 (Moriguchi et al. 1996; Wang et al. 2001), which phosphorylates p38, eliciting activation of cAMP response-element binding protein (CREB) (Deak et al. 1998).

In unstimulated cells, NF κ B, most commonly comprised of the subunits p65 (RelA) and p50, is sequestered in the cytosol by its inhibitor I κ B α in canonical signalling (Baeuerle & Baltimore 1988b). Upon activation, TAK1 phosphorylates the IKK complex (Takaesu et al. 2003), a higher order oligomer of 800 kilo Dalton (kDa) composed of IKK α , IKK β , and NEMO (Mercurio et al. 1997; Zandi et al. 1997; Rothwarf et al. 1998; Yamaoka et al. 1998). Recently, TAK1-mediated phosphorylation of IKK β has been shown to prime the kinase for autophosphorylation (Zhang, Clark, et al. 2014), which results in its activation. NF κ B-bound I κ B α is phosphorylated at Ser32 and Ser36 by the activated IKK complex creating a recognition site for the E3 ubiquitin ligase β -transducin repeat-containing (β TrCP) (Henkel et al. 1993; Traenckner et al. 1995; Spencer et al. 1999). Subsequently, β TrCP along with E1 and UBC5C polyubiquitinate phosphorylated NF κ B-bound I κ B α leading to its recognition and degradation by the 26S proteasome (Alkalay et al. 1995). The previously sequestered transcription factor NF κ B is released (Henkel et al. 1993), whereupon it translocates to the nucleus and binds to κ B binding sites in the promoter of NF κ B-stimulated genes (Sen & Baltimore 1986; Baeuerle & Baltimore 1988a; Kunsch et al. 1992).

The promoters of IFNs and of the pro-inflammatory cytokines IL-1 and TNF α contain κ B-binding sites, which result in the expression of these cytokines. In turn, secreted IL-1 and TNF α lead to further activation of the transcription factor NF κ B.

1.2.1.3. Tumour necrosis factor α

1.2.1.3.1. Introduction

The crucial role of TNF α in tumour necrosis was first demonstrated *in vivo* by Carswell and co-authors (Carswell et al. 1975). Tumours grafted superficially onto mice necrotised after intravenous injection of serum containing TNF α whilst healthy tissue was spared (Carswell et al. 1975). Since then the pleiotropic cytokine was shown to be involved in a large variety of biological functions including cell survival and death, cell differentiation and proliferation, and inflammation (Aggarwal et al. 2012; Waters et al. 2013). TNF α is an endogenous pyrogen eliciting fever and vasodilatation through production of pro-inflammatory mediators such as COX-2 and PGE2. It promotes secretion of cytokines (e.g. IL-6, IL-8, IL-18 and IFN γ -induced protein 10) and expression of adhesion molecules, which in turn recruit leukocytes to the site of infection. Furthermore, it was shown to regulate haematopoiesis, coagulation, and the activation of DCs and macrophages. The latter are the principal source of TNF α .

TNF α is a member of the TNF superfamily comprised of 19 ligands and 29 receptors (Waters et al. 2013), all of which mediate pro-inflammatory responses. The cytokine exists in a transmembrane and a soluble trimeric form, both able to interact with the tumour necrosis factor receptor (TNFR). Membrane-bound TNF α is cleaved by the protease TNF α -converting enzyme to generate the soluble active trimer (Black et al. 1997). Two distinct TNF α receptors, i.e. TNFR1 and TNFR2, have been described (Brockhaus et al. 1990). They are characterised by extracellular cysteine rich domains that form the binding pocket for TNF α . In addition, TNFR1 contains a cytosolic DD that serves to interact with adaptor proteins (Hsu et al. 1995). This domain is absent in TNFR2. Similar to the IL-1R family, ligand binding induces oligomerisation of the receptor and recruitment of adaptor proteins through specific interaction domains such as death domains ultimately leading to activation of TAK1, and subsequent activation of the IKK complex and MAP kinases (Figure 1-4).

1.2.1.3.2. TNF α signalling

Recognition of TNF α by TNFR1 induces recruitment of the adaptor TNFR1-associated death domain protein (TRADD) via DD homotypic interactions (Hsu et al. 1995). TRADD is involved in the initiation of two contrasting cellular processes (Hsu, Huang, et al. 1996; Hsu, Shu, et al. 1996). It can associate with Fas-associated death domain protein (FADD) to elicit apoptosis (Hsu, Huang, et al. 1996). It can also engage with TRAF2 and receptor-interacting protein 1 (RIP1) to activate NF κ B and MAP kinases resulting in cell survival and a pro-inflammatory response (Hsu, Shu, et al. 1996). Death domain-containing RIP1 is recruited by TRADD through its C-terminal DD, whereas TRAF2 interacts with the N-terminus of TRADD (Park et al. 2000). TRAF2 was shown to bind and recruit the E3 ubiquitin ligases cellular inhibitor of apoptosis 1 (cIAP1) and cIAP2 to the receptor complex, where they presumably activate RIP1 by polyubiquitination (Yin et al. 2009). RIP1 is a Ser/Thr kinase, yet it was shown that its kinase activity is not required for RIP1-dependent recruitment of TAK1 and of the IKKs to the TNFR1-TRADD-TRAF2-RIP1 complex (Devin et al. 2000; Lee et al. 2004; Ea et al. 2006). Similar to the IL-1R signalling pathway, TAK1 activation results in phosphorylation-initiated degradation of I κ B α (Zhang, Clark, et al. 2014), which releases the transcription factor NF κ B to translocate

into the nucleus and induce gene expression (Kunsch et al. 1992; Henkel et al. 1993). TAK1 also activates MAPK resulting in activation of AP1 and CREB (Deak et al. 1998).

1.2.2. Pattern recognition receptor signalling pathways

Since the prediction of PAMPs (Janeway 1989), a large variety of these unique invariable microbial molecules and molecular signatures from parasites, which trigger the innate immune response, have been discovered. These include viral PAMPs (e.g. viral nucleotides, capsid and glycoproteins) (Lester & Li 2014), bacterial PAMPs (e.g. bacterial nucleotides, flagellin and cell wall components such as peptidoglycan), and fungal PAMPs (e.g. carbohydrates like β -glucan) (Hansen et al. 2011; Plato et al. 2013).

The receptors recognising these PAMPs are commonly divided into five groups according to their receptor structure (Hansen et al. 2011): C-type lectin-like receptors (CLRs), which are primarily involved in fungal detection, virus-sensing retinoic acid-inducible gene 1 (RIG-I)-like receptors (RLRs), Toll-like receptors (TLRs), nucleotide-binding oligomerisation domain-like receptors (NLRs) and DNA sensors. TLRs, NLRs and DNA sensors recognise PAMPs of various origins. It is important to note, that DNA sensors do not share a common receptor structure as they are a diverse collection of receptors, grouped together based on their ability to sense cytosolic DNA (Dempsey & Bowie 2015). Examples of DNA sensors are DExD/H box polypeptide 41 (DDX41), absent in melanoma 2, IFN γ -inducible protein 16 (IFI16), RNA polymerase III (RNA pol III), DNA-dependent protein kinase (DNA-PK) and cyclic guanosine monophosphate (GMP) - adenosine monophosphate (AMP) synthase (cGAS). Alternatively, PRRs can be categorised according to their predominant site of ligand recognition (Iwasaki & Medzhitov 2015). Extracellular microbial patterns are sensed by the plasma membrane-associated receptors TLR1, TLR2, TLR4, TLR5, TLR6 and CLRs. PAMPs which enter the cell by endocytosis remain in endosomes for detection by TLR3, TLR7, TLR8 and TLR9. Plasma membrane and endosomal receptors are transmembrane proteins transducing external signals into the cell. In comparison, cytosolic PAMPs are recognised by NLRs, RLRs and DNA sensors, which are not localised to any specific cellular compartment. PRRs are expressed on all cell types of the immune system including monocytes, macrophages, DCs, neutrophils, T cells and B cells (Michallet et al. 2013). They are also expressed on non-haematopoietic cells such as epithelial and endothelial cells. In addition, PRR expression can be induced or enhanced in response to infection (Hansen et al. 2011).

PRRs are generally composed of a ligand binding domain and a signal transducing domain, which is capable of interacting (directly or indirectly) with adaptor proteins (Hansen et al. 2011). Initiation of PRR signalling cascades results in activation of MAPK and transcription factors (e.g. IRFs, NF κ B and AP-1), leading to secretion of type I IFNs and pro-inflammatory cytokines (e.g. TNF α and IL-1). These initial events shape the subsequent adaptive response (Iwasaki & Medzhitov 2015). For example, epithelial cells of the gastrointestinal tract express TLR2, TLR3, TLR4 and TLR5, which elicit secretion of chemokines that recruit leukocytes to the site of infection. Another example are CLR-expressing tissue resident DCs and macrophages that can give rise to Th1 responses upon fungal PAMP detection. In comparison, circulating plasmacytoid DCs (pDCs) secrete type I IFNs, initiate Th1 responses and antibody production through activation of TLR7 and TLR9 by viruses in the blood stream. Lymphocytes themselves also express a range of PRRs (e.g. TLRs, NLRs and

RLRs) (Michallet et al. 2013), which serve to facilitate cell proliferation, cell expansion and generation of optimal antibody responses.

1.2.2.1. Toll-like receptor family

1.2.2.1.1. Introduction

One of the first human TLRs was identified by searching for TIR domain homologues following the discovery of *Drosophila* Toll protein involvement in antifungal resistance in flies (Medzhitov et al. 1997). Shortly thereafter others were reported and now the TLR family comprises ten human transmembrane proteins (i.e. TLR1-10) (De Nardo 2015). The N-terminal extracellular region is composed of leucine-rich repeats that serve as ligand binding domains. A single transmembrane domain links the N-terminus to the TIR domain-containing C-terminus. TLRs are ubiquitously expressed (Zarembek & Godowski 2002), yet predominantly present in tissues exposed to the external environment as well as in tissues involved in generation of immune responses (e.g. spleen, lung, peripheral blood leukocytes), where they can be induced in response to invading pathogens.

Ligand-induced dimerisation of TLRs brings their intracellular TIR domains in close proximity (De Nardo 2015), which generates a signalling platform for signalling adaptors. Activation of TLR adaptors, TIR-domain-containing adapter-inducing IFN β (TRIF) and MyD88, elicits the signalling pathway activating the transcription factors IRF3, IRF7 and NF κ B (Figure 1-5).

1.2.2.1.2. TLR ligands

TLRs recognise a wide variety of ligands. They detect extracellular ligands through receptors situated at the plasma membrane, or endocytosed PAMPs via TLRs located on endosomal membrane. Studies of crystal structures of TLR ectodomains with their cognate ligands revealed distinct ligand-receptor interactions (Jin et al. 2007; Liu et al. 2008; Kang et al. 2009; Park et al. 2009; Yoon et al. 2012; Tanji et al. 2013; Ohto et al. 2015). However, all ligands crosslink receptor dimers, leading to conformational change in the receptor proteins, which brings the two TIR domains together.

Structural components such as viral capsid proteins, or bacterial cell wall components (e.g. peptidoglycan) are recognised by TLR1, TLR2, TLR4, TLR5, and TLR6. These structures are unique to pathogens, which allows a rapid self/non-self-discrimination by TLRs as demonstrated through examples in the following. TLR2 forms heterodimers with TLR1 and TLR6 and its ligands include viral glycoproteins (Bieback et al. 2002), and di- and triacylated lipoproteins (Takeuchi et al. 2001; Takeuchi et al. 2002). The fusion protein F of *Respiratory syncytial virus* can be recognised by TLR4 (Kurt-Jones et al. 2000), though it is known for recognition of bacterial lipopolysaccharides (LPS) facilitated by co-receptor myeloid differentiation factor (Park et al. 2009). TLR5 appears initially to form a heterodimer with bacterial flagellin (Yoon et al. 2012), and these heterodimers associate to activate the signalling cascade. The above described TLRs are generally located at the plasma membrane, with the exception of TLR2 and TLR4 (Kagan et al. 2008; Barbalat et al. 2009). The latter TLRs can be internalised through endocytosis resulting in distinct signalling pathways.

Viral and bacterial nucleic acids are recognised by TLRs (i.e. TLR3, TLR7, TLR8 and TLR9) situated at endosomal membranes. TLR3 senses viral and synthetic dsRNA (e.g. purified dsRNA from *Reovirus*, *West Nile Virus* and poly(inosinic:cytidylic) acid (poly(I:C))) (Alexopoulou et al. 2001), which is absent in eukaryotic cells under normal conditions. A minimum length of 40-50 bp is required to elicit TLR3 dimerisation (Leonard et al. 2008; Liu et al. 2008), which can form higher order oligomers with increasing dsRNA length. TLR7 and TLR8 recognise ssRNA molecules rich in guanine (G) and uracil (U), generated during the replication of IAV (Diebold et al. 2004), *Sendai virus* (SeV) and *Vesicular stomatitis virus* (VSV) (Lund et al. 2004). Despite the abundant presence of host ssRNA in cells, the presence of pathogenic ssRNA is specifically detected via the GU bias present in viral and bacterial ssRNA molecules (Heil et al. 2004). TLR9 is currently the only known TLR recognising DNA molecules. Recently, the crystal structure of the TLR9 ectodomain in association with CpG-DNA confirmed that it is actually the unmethylated CpG motifs that directly bind to the receptor (Ohto et al. 2015). In contrast to bacterial and viral DNA, genomic eukaryotic DNA generally contains methylated CpG-elements (Lund et al. 2003).

1.2.2.1.3. TLR signalling

Upon receptor dimerisation, four distinct TIR-containing adaptor molecules are involved in TLR signalling to activate the transcription factors IRF3 and NFκB: MyD88-adaptor-like protein (MAL), TRIF-related adaptor molecule (TRAM), TRIF and MyD88 (De Nardo 2015).

The so-called 'sorting adaptors' MAL and TRAM are specifically required for TLR2 and TLR4 signalling pathways (Fitzgerald et al. 2001; Stack et al. 2014), directing TLR2/4 to different subcellular locations (i.e. plasma membrane or endosome) to initiate distinct signalling events. MAL is anchored to the plasma membrane (Kagan & Medzhitov 2006), and recruits the signalling adaptor MyD88 to TLR2 and TLR4 leading to NFκB activation. Alternatively, TLR2 and TLR4 can be internalised by endocytosis upon ligand recognition. TLR2 is associated to MAL and TRAM (Stack et al. 2014), which recruit MyD88 for IRF7 activation. In comparison, TRAM alone associates with TLR4 and recruits the signalling adaptor TRIF (Yamamoto et al. 2003; Kagan et al. 2008).

The signalling adaptor MyD88 is used by all TLRs, with the exception of TLR3 (De Nardo 2015). Its activation leads to the assembly of the myddosome (Lin et al. 2010), activation of the IKK complex through interaction with ubiquitin chains (Zhang, Clark, et al. 2014), and the subsequent release of the transcription factor NFκB from its cytosolic inhibitor (see 1.2.1.2.2) (Henkel et al. 1993).

The signalling adaptor TRIF is required for TLR3- and TLR4-driven activation of IRF3 and NFκB (Yamamoto et al. 2002). It interacts directly with TLR3, and via TRAM with TLR4 through homotypic interactions between the TLR and TRIF TIR domains. TRIF contains a C-terminal RIP homotypic interaction motif, and N-terminal TRAF2/6 binding domains, which serve to recruit RIP1 (Meylan et al. 2004), and TRAF6 for NFκB activation (Sato et al. 2003; Jiang et al. 2004). TRIF also recruits TRAF3 and the kinases TRAF family member-associated NFκB-activator-binding kinase 1 (TBK1) and IKKε (Sato et al. 2003; Tatematsu et al. 2010), which phosphorylate IRF3 (Katherine Fitzgerald et al. 2003; Sharma et al. 2003), and lead to its activation. Recently, it was reported that TRIF is phosphorylated by TBK1 creating a docking site for IRF3 to facilitate subsequent phosphorylation by TBK1 (Liu, Cai, et al. 2015). Activated IRF3 dimerises and translocates to the nucleus to

induce type I IFN expression (Lin et al. 1998; Yoneyama et al. 1998). In addition, TBK1 and IKK ϵ have been implicated in NF κ B signalling (Pomerantz & Baltimore 1999; Peters et al. 2000; Tojima et al. 2000), however there is controversy about their roles in activation of this transcription factor, since knockout mouse infection models have not supported their requirement in NF κ B pathway activation (Hemmi et al. 2004; McWhirter et al. 2004).

Plasmacytoid DCs produce large amounts of IFN α through activation of IRF7 (Izaguirre et al. 2003). In comparison to the IRF3 activation pathway which uses TRIF as a signalling adaptor, MyD88 is used as a signalling adaptor upon ligand recognition by TLR7 and TLR9 (Honda, Ohba, et al. 2005; Honda, Yanai, et al. 2005). Recently it was shown that MAL serves as a sorting adaptor at the endosomal membrane to recruit MyD88 to TLR9 (Bonham et al. 2014). Similar to the IL-1R signalling pathway the myddosome is assembled (Kawai et al. 2004). TRAF6 is recruited by MyD88 and in turn activates the kinases IRAK-1 and IKK α , which activate IRF7 through phosphorylation. Activated IRF7 dimerises and translocates to the nucleus where it induces type I IFNs (Sato et al. 1998).

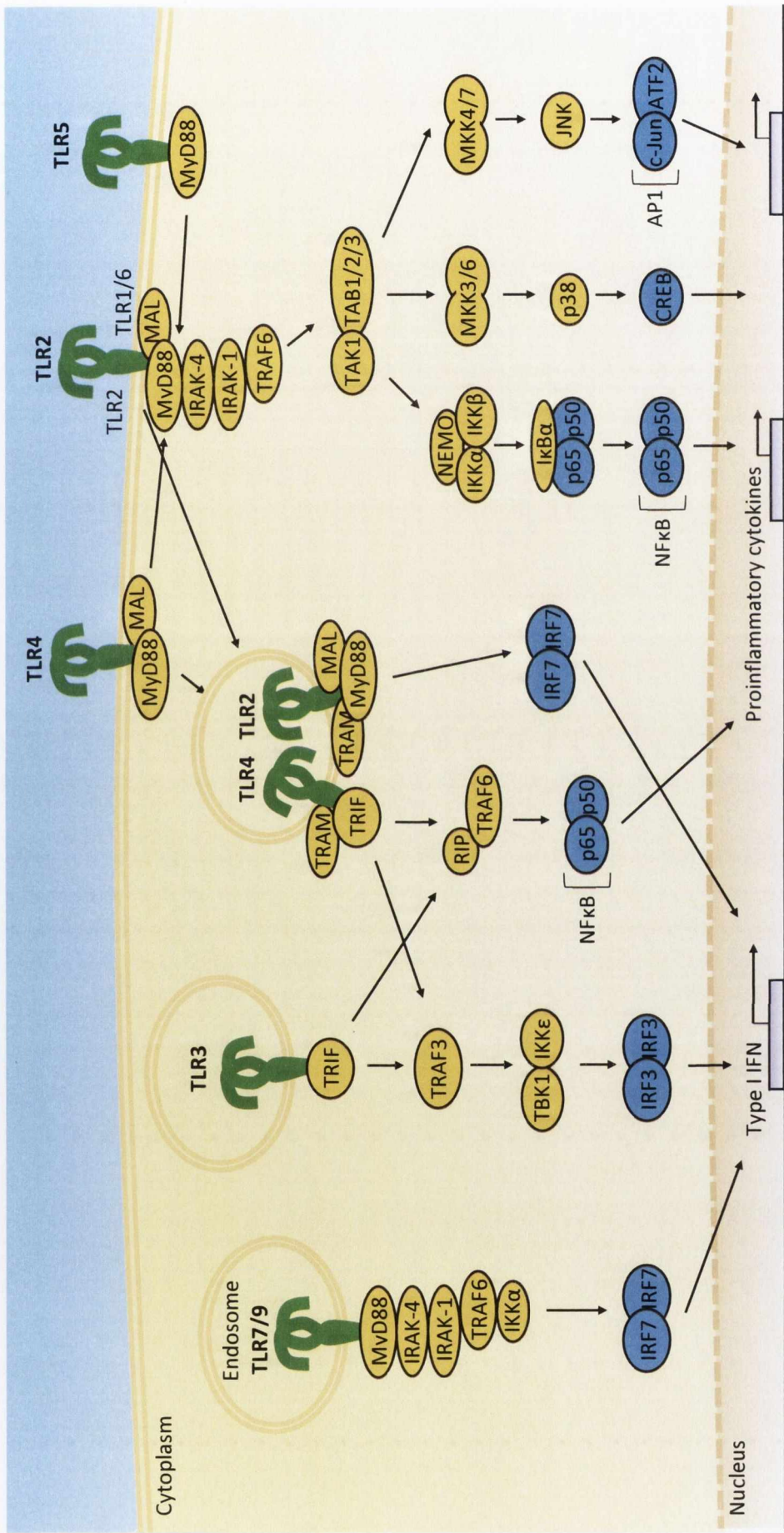


Figure 1-5 Overview of TLR signalling pathways

TLRs are membrane bound sensors recognising a broad variety of extracellular and endocytosed PAMPs such as bacterial LPS, fungal flagellin and viral or bacterial nucleotides. PAMP recognition leads to receptor dimerization and subsequent recruitment of adaptor molecules (MyD88, TRIF, MAL, TRAM). Adaptor proteins activate signalling cascades resulting in transcription factor phosphorylation by kinases. Activated transcription factors translocate to the nucleus, where they induce expression of type I IFNs and pro-inflammatory cytokines.

1.2.2.2. Retinoic acid-inducible gene 1-like receptor family

1.2.2.2.1. Introduction

Shortly after the discovery of the TLR signalling pathways it became clear that this host defence system was limited due to its recognition being restricted to extracellular and endosomal PAMPs. In 2004, Andrejeva and co-authors, and Yoneyama and co-authors identified the RNA-helicases melanoma differentiation-associated protein 5 (MDA-5), and RIG-I, which constitute the two principal RNA sensors of the RLR family (Andrejeva et al. 2004; Yoneyama et al. 2004). These proteins are ubiquitously expressed in immune specific as well as many other cells (Kang et al. 2002; Yoneyama et al. 2004), conferring a broad protection against intracellular pathogens. However, RLRs are not required for antiviral immune responses in pDCs (Kato et al. 2005), which therefore rely entirely on TLR and other cytosolic signalling pathways.

Upon ligand recognition, RLRs activate their common adaptor protein mitochondrial antiviral-signalling protein [MAVS, also referred to as interferon-beta promoter stimulator 1 (IPS-1), virus induced signalling adaptor (VISA), and caspase-recruitment domain (CARD) adaptor inducing IFN β (CARDIF)], which localises in the outer mitochondrial membrane (Kawai et al. 2005; Meylan et al. 2005; Seth et al. 2005; Xu et al. 2005). Following its activation, MAVS interacts with TRADD (Michallet et al. 2008), which in turn elicits signalling cascades resulting in the activation of IRF3 and IRF7, and of NF κ B (Figure 1-6).

1.2.2.2.2. RLR ligands

The three members of the RLR family, RIG-I, MDA-5 and laboratory of genetics and physiology 2 (LPG2) are characterised by a DDX RNA helicase domain (Kang et al. 2002; Yoneyama et al. 2004), and, with the exception of LPG2 (Cui et al. 2001), by two N-terminal CARD domains. In addition, RIG-I and LPG2 possess a C-terminal regulatory domain (RD). Viral detection occurs through RIG-I and MDA-5, whereas the role of LPG2 is less well understood. The sensors RIG-I and MDA-5 have non-redundant roles in the antiviral defence (Gitlin et al. 2006; Kato et al. 2006; Loo et al. 2008), as evidenced by their differential detection of distinct viruses. It was demonstrated that RIG-I selectively binds to shorter segments of nucleotides of either viral or synthetic origin (i.e. poly(I:C)) compared to MDA-5 (Kato et al. 2006; Kato et al. 2008). Studies attempting to define RIG-I ligands revealed the necessity of a blunt end bearing RNA sequence (Schmidt et al. 2009), occurring in dsRNA or in self-religated ssRNA, which takes the shape of a panhandle (Schlee et al. 2009). Furthermore, 5'di- or 5'triphosphate (5'pp or 5'ppp) ends are specifically recognised by RIG-I (Hornung et al. 2006; Pichlmair et al. 2006; Lu et al. 2010; Wang, Ludwig, et al. 2010; Luo et al. 2012; Goubau et al. 2014), whereas MDA-5 recognises the internal duplex structures of dsRNA (Wu, Peisley, et al. 2013). In addition, a report examining the recognition of self-RNA showed that 3'mono-phosphate dsRNA (Malathi et al. 2007), generated by the host antiviral defence enzyme RNaseL, was recognised by RIG-I and/or MDA-5. Crystal structures of the regulatory domain have provided structural evidence how aberrant RNA molecules are detected by RIG-I (Lu et al. 2010; Wang, Zhang, et al. 2010; Luo et al. 2012), however a recent study has shown that CARD domains are also able to regulate RNA affinity of RIG-I (Ramanathan et al. 2015). Whilst full-length RIG-I selectively binds to blunt-ended dsRNA, deletion of CARDs results in equal binding of blunt- and non-blunt-ended RNA molecules.

Double stranded or 5'pp- or 5'ppp-ends are absent in host RNA, which is either capped by a 7-methylguanosine (m⁷G cap) group, or phosphogroups are cleaved as part of the maturation process. Several viruses replicating in the cytoplasm mimic host RNA by capping their RNA and encoding 2'O-methyltransferases, which impedes detection of unmethylated caps by RIG-I and MDA-5 (Züst et al. 2011; Schuberth-Wagner et al. 2015; Devarkar et al. 2016). Two recent studies revealed how steric exclusion allows RIG-I to discriminate host and microbial RNA (Schuberth-Wagner et al. 2015; Devarkar et al. 2016). Whilst RNA bearing an unmethylated cap at its end was readily bound by RIG-I and activated type I IFN production, 2'O-methylation at the penultimate nucleotide of the RNA molecule sterically interfered with its association with the binding pocket of RIG-I.

Studies of IAV, SeV and *Reovirus* infected cells revealed that the "natural" RIG-I ligands are 5'pp- and 5'ppp-containing genomic RNA segments generated during viral replication, thus confirming how the RLR family distinguishes between self and non-self RNA (Baum et al. 2010; Rehwinkel et al. 2010; Goubau et al. 2014).

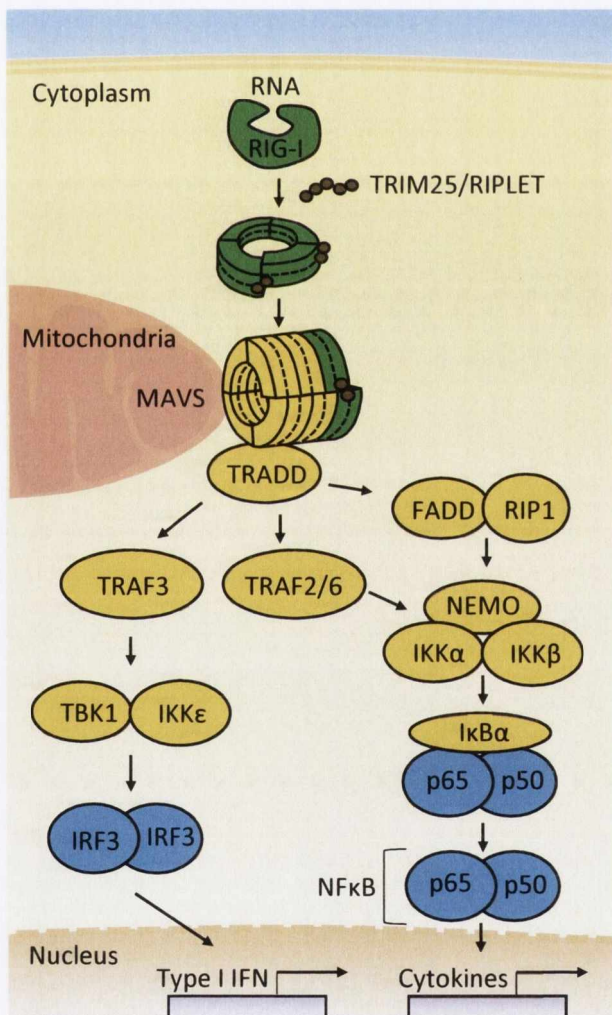


Figure 1-6 Overview of RLR signalling pathways

RLRs are cytosolic sensors of viral nucleotides, which recruit the adaptor molecule MAVS. MAVS activates signalling pathways leading to the phosphorylation of IRF3 and NFκB. Following activation, IRF3 and NFκB translocate to the nucleus, where they induce the expression of type I IFNs and pro-inflammatory cytokines.

1.2.2.2.3. RLR signalling pathway

In absence of viral nucleotides, RIG-I is held in an inactive conformation as the tandem CARDs are bound to the helicase domain (Saito et al. 2007; Kowalinski et al. 2011), masking the RNA binding domain as well as restricting the access of ubiquitin enzymes and free ubiquitin chains to bind to CARD. The 5'ppp group is recognised by the flexible regulatory domain (Lu et al. 2010; Wang, Ludwig, et al. 2010; Luo et al. 2012). Subsequently, viral RNA bound by the helicase domain, and ATP is hydrolysed (Luo et al. 2012), which induces a conformational change resulting in release of the two CARD domains, and in the dimerization of RIG-I (Cui et al. 2008; Zeng et al. 2010). The RIG-I dimers form tetramers, which can be further stabilised through Lys63-linked polyubiquitination of CARD domains at Lys172 by E3 ligase tripartite motif-containing protein 25 (TRIM25) (Gack et al. 2007), at C-terminal Lys849 and Lys851 by RING finger protein leading to RIG-I activation (RIPLET) (Oshiumi et al. 2009), or by non-covalent interactions of CARD with unanchored Lys63-linked polyubiquitin (Zeng

et al. 2010). Whilst both RIG-I and MDA-5 have been reported to form filaments on RNA molecules independent of ubiquitin chain binding (Peisley et al. 2013; Wu, Peisley, et al. 2013), Lys63-ubiquitin chains were shown to stabilise CARD interactions (Peisley et al. 2014). A minimum of four RIG-I molecules interact with MAVS via their CARD domains and nucleate the formation of MAVS filaments at the mitochondrial outer membrane (Hou et al. 2011; Wu et al. 2014). Next, TRADD associates with activated MAVS (Michallet et al. 2008), and in turn recruits either TRAF3 (Saha et al. 2006; Paz et al. 2011), TRAF2/6 (Xu et al. 2005; Liu et al. 2013), or the TRADDosome members FADD and RIP1. TRAF3 binds to the TRAF3 binding motif in MAVS (Michallet et al. 2008; Paz et al. 2011), and activates IRF3 by recruiting TBK1 and IKK ϵ (Michallet et al. 2008; Paz et al. 2011). Recently, TBK1 and IKK β -mediated IRF3 recruitment to MAVS has been described (Liu et al. 2013). Upon activation, these kinases phosphorylate MAVS at Ser442 creating a docking site for IRF3 (Liu, Cai, et al. 2015). Subsequently, TBK1 phosphorylates MAVS-bound IRF3, leading to its dimerization and dissociation from the adaptor complex. MAVS oligomerisation is required for IRF3 activation as a polymerisation defective MAVS mutant could not interact with IRF3 despite being phosphorylated at Ser442 (Liu, Cai, et al. 2015). NF κ B activation by the IKK complex can be achieved through IKK recruitment by either TRAF2/6 or by the TRADDosome (Xu et al. 2005; Michallet et al. 2008). Interestingly, stimulator of IFN genes (STING) was shown to interact with MAVS (Ishikawa & Barber 2008; Zhong et al. 2008), and to play a role in RLR-mediated IRF3 activation. However, recent reports on STING have demonstrated its pivotal role in orchestrating the signalling cascades leading to IRF and NF κ B activation in response to cytoplasmic DNA (Dempsey & Bowie 2015).

1.2.2.3. DNA sensors signalling through stimulator of interferon genes

1.2.2.3.1. Introduction

The immunostimulatory properties of foreign DNA, and UV-inactivated DNA viruses such as VACV were discovered over 50 years ago (Glasgow & Habel 1962; Rotem et al. 1963), however the underlying mechanisms have only begun to be elucidated recently (Dempsey & Bowie 2015). In these early studies, cells stimulated with calf thymus DNA (Rotem et al. 1963), or UV-inactivated VACV were shown to produce IFN, which in turn reduces replication of several DNA and RNA viruses (Glasgow & Habel 1962).

Almost 50 years later, the cytosolic adaptor STING (also known as mediator of IRF3 activation (MITA), Methionine-Proline-Tyrosine-Serine (MPYS), endoplasmic reticulum (ER) IFN stimulator (ERIS), and transmembrane protein 173 (TMEM173) was discovered, and reported to be involved in DNA-mediated immune responses (Ishikawa & Barber 2008; Jin et al. 2008; Zhong et al. 2008; Sun et al. 2009). STING is localised at the endoplasmic reticulum, and its crucial role as an adaptor protein to innate DNA sensors has been established over the past six years (Dempsey & Bowie 2015). Additionally, STING is able to directly sense bacterial cyclic di-nucleotides, which are conserved bacterial signalling molecules, and elicit type I IFN production (Burdette et al. 2011).

Hitherto, known nucleotide sensors were limited to endosomal TLR9, which senses unmethylated CpG-DNA (Lund et al. 2003), and members of the RLR family recognising various RNA species in the cytosol (Schlee et al. 2009; Schmidt et al. 2009). A STING-independent DNA sensor is RNA pol III. It transcribes adenine/thymine-

rich DNA (i.e. synthetic dsDNA molecule poly(deoxyadenylic:deoxythymidylic) acid (poly(dA:dT))) into 5'ppp dsRNA, which in turn activates the RIG-I signalling cascade. This mechanism appears to be involved in eliciting immune responses to *Adenovirus*, *Epstein-Barr virus* (EBV) and *Herpes simplex virus 1* (HSV-1) (Ablasser et al. 2009; Chiu et al. 2009).

In an attempt to identify DNA sensors, various proteins were reported to affect DNA-mediated immune responses, of which four have been described to signal through STING (i.e. cGAS, DDX41, DNA-PK, IFI16) (Dempsey & Bowie 2015). The cytosolic helicase DDX41 activates MAPK, IRF3 and NFκB in response to poly(dA:dT), HSV, *Adenovirus* and VACV. DDX41 has been shown to interact with both STING and TBK1 (Zhang et al. 2011). The catalytic subunit DNA-PK of the DNA damage response complex Ku70/Ku80/DNA-PK has been reported to activate IRF3, but not NFκB following MVA infection *in vitro* and *in vivo*. Further the subunit Ku70 associates with STING, possibly recruiting the adaptor to the receptor complex (Ferguson et al. 2012). IFI16 and cGAS have been reported to detect viral nucleic acids in several studies (Unterholzner et al. 2010; Gariano et al. 2012; Orzalli et al. 2012; Ablasser et al. 2013; Jakobsen et al. 2013; Sun et al. 2013; Ansari et al. 2015), hence they appear to be the predominant DNA sensors signalling through STING. IFI16 can directly associate with STING (Unterholzner et al. 2010; Ansari et al. 2015), whilst cGAS synthesises a second messenger molecule termed cyclic GMP-AMP (cGAMP) upon DNA binding, which binds STING leading to its activation (Figure 1-7) (Sun et al. 2013).

1.2.2.3.2. Gamma-interferon-inducible protein 16

IFI16 was identified as a DNA sensor through analysis of proteins binding to a 70 bp long dsDNA molecule derived from VACV (Unterholzner et al. 2010). In contrast to other nucleic acid sensors (e.g. TLR9, RIG-I), which were reported to recognise specific structures, co-crystallisation of the IFI16 DNA binding domain with B-form dsDNA revealed a sequence non-specific binding of dsDNA at the sugar-phosphate-backbone (Jin et al. 2012). It appeared that sufficiently long naked DNA is the ligand of IFI16, and IFI16 can assemble into filaments on DNA molecules of at least 60 bp (Morrone et al. 2014). However other forms of DNA have also been reported to activate IFI16 signalling (Jakobsen & Paludan 2014). An early study examining the DNA forms associated with IFI16 showed that IFI16 predominantly bound to cruciform and superhelical DNA (Brázda et al. 2012), and another study found IFI16 binding to the stem region of secondary structures generated by folding of pro-lentiviral ssDNA (Jakobsen et al. 2013). It remains unclear whether or how IFI16 distinguished between self and non-self DNA, as no pathogen-associated structures are recognised (Jin et al. 2012). The site of DNA recognition was suggested to determine the onset of an immune response, whereby cytoplasmic DNA was recognised as a DAMP (Paludan & Bowie 2013). In this model, the nucleus was assumed to be void of DNA sensors given the presence of host genomic DNA. However, IFI16 is a nuclear as well as cytoplasmic DNA sensor, therefore challenging this hypothesis. The observation of filament assembly on DNA suggests that IFI16 can distinguish self from non-self DNA based on the length of naked DNA. Self, nuclear DNA is normally found in complexes with host factors such as histones and would therefore provide a poor ligand for IFI16 activation (Dempsey & Bowie 2015).

Several DNA viruses and retroviruses, and *Listeria monocytogenes* have been reported to be recognised by IFI16 in the cytosol or in the nucleus such as HSV-1, *Human cytomegalovirus* (HCMV), EBV, *Kaposi's sarcoma-*

associated herpesvirus (KSHV) and HIV-1 (Unterholzner et al. 2010; Gariano et al. 2012; Horan et al. 2013; Jakobsen et al. 2013; Hansen et al. 2014; Ansari et al. 2015). The localisation of IFI16 is regulated via acetylation of its NLS by the acetyltransferase p300 (Li et al. 2012; Ansari et al. 2015), which prevents nuclear import, and by deacetylation via histone deacetylases, which allows sensing of HSV-1 in the nucleus.

Upon DNA-binding IFI16 associates with STING (Unterholzner et al. 2010; Ansari et al. 2015). STING, in turn, activates signalling pathways leading to activation of IRFs and NF κ B (Abe & Barber 2014; Liu, Cai, et al. 2015), the mechanism of which will be detailed in the description of cGAS signalling (see 1.2.2.3.3).

1.2.2.3.3. Cyclic GMP-AMP synthase

1.2.2.3.3.1. cGAS ligands

In search for DNA sensors activating STING, Wu and co-authors discovered by means of a cell complementation assays that the cyclic di-nucleotide cGAMP was generated by cells in response to DNA stimulation (Wu, Sun, et al. 2013). Subsequently, the same group identified cGAS as the enzyme (Sun et al. 2013), which generates cGAMP from ATP and GMP in response to DNA binding. Various dsDNA species have been reported to activate cGAS including interferon stimulatory DNA (dsDNA stretch of 45 bp), poly(dA:dT), a 50 bp stretch of GC-rich DNA, a 90 bp stretch of dsDNA derived from HSV-1, herring-testis-DNA and DNA from *Listeria monocytogenes* (Wu, Sun, et al. 2013; Abe & Barber 2014; Hansen et al. 2014). Crystal structures of cGAS with dsDNA have revealed a sequence unspecific binding of the sugar-phosphate backbone (Gao et al. 2013), and demonstrated how B-form DNA, yet not dsRNA, induces a conformational change in cGAS leading to activation of cGAS (Zhang, Wu, et al. 2014). Similar to the observations made for IFI16 (Jakobsen et al. 2013), a recent study demonstrated that cGAS binds to stem-loop structures found in HIV-1 ssDNA (Herzner et al. 2015). These secondary ssDNA structures contained additional unpaired guanosines in form of a Y-junction, which rendered the stem-loop particularly immunostimulatory (Herzner et al. 2015). It remains unclear how the distinction between self and non-self DNA is made. It is possible that cGAS, which is a predominantly cytosolic protein, recognises cytosolic DNA as a DAMP and thereby initiates an immune response. However, a recent study demonstrated that cGAS is present in the nucleus of certain cells types (Orzalli et al. 2015), where it interacts and stabilises the nuclear sensor IFI16.

1.2.2.3.3.2. cGAS signalling pathway

Binding of cytosolic DNA induces a conformational change in cGAS, leading to homodimerisation of cGAS in complex with two DNA molecules (Li et al. 2013; Zhang, Wu, et al. 2014), and rearrangement of the active site (Figure 1-7). The catalytic pocket opens to allow ATP and guanosine triphosphate (GTP) binding, resulting in the generation of the cyclic di-nucleotide cGAMP (Gao et al. 2013; Sun et al. 2013). STING then recognises cGAMP, leading to STING dimerisation and activation at the ER (Sun et al. 2009; Wu, Sun, et al. 2013).

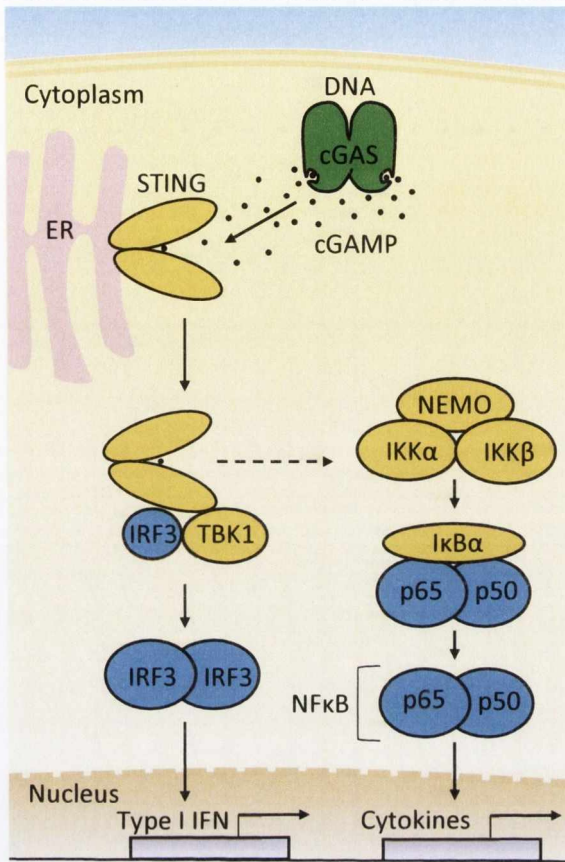


Figure 1-7 Overview of cGAS signalling pathway

The cyclic GMP-AMP synthase is a cytosolic DNA sensor, which synthesises the second messenger cGAMP upon DNA recognition. In turn, cGAMP activates STING leading to phosphorylation and nuclear translocation of IRF3 and NFκB. Activated IRF3 and NFκB induce expression of type I IFNs and pro-inflammatory cytokines.

In addition, cGAMP is able to diffuse into non-infected bystander cells via gap junctions to activate STING (Ablasser et al. 2013), and induce an immune response prior to production of type I IFNs or proinflammatory cytokines. Furthermore, two recent reports demonstrated that cGAMP was packaged into viral particles of MVA, HIV-1 and murine CMV infected cells (Bridgeman et al. 2015; Gentili et al. 2015), thereby eliciting type I IFN responses upon cell entry of these viruses. In a related manner, HSV-1 infection of Vero cells was shown to induce transport of STING to uninfected cells via exosomes (Kalamvoki et al. 2014).

Upon activation, STING traffics from the ER membrane via the Golgi apparatus to perinuclear membranes, where STING recruits TBK1 (Ishikawa et al. 2009; Saitoh et al. 2009). TBK1 subsequently phosphorylates STING at Ser366 creating a docking site for IRF3 (Liu, Cai, et al. 2015). IRF3 is phosphorylated by TBK1 (Tanaka & Chen 2012), which then dimerises and translocates to the nucleus to initiate type I IFN production. TRAF3 has been shown to associate

with STING when both proteins were ectopically expressed *in vitro* (Chen et al. 2008), which may suggest TRAF3's involvement in cGAS signalling. However, TRAF3 depletion in murine embryonic fibroblasts did not impair dsDNA-mediated activation of IRF3 (Abe & Barber 2014).

STING has been reported to activate NFκB in numerous studies, however the mechanism by which it activates NFκB is less clear compared to what is known for IRF3 (Ishikawa & Barber 2008; Sun et al. 2009; Wu, Sun, et al. 2013; Sun et al. 2013; Abe & Barber 2014). TBK1 and TRAF6 have been proposed to intervene in IKK complex recruitment, resulting in phosphorylation and nuclear translocation of the NFκB subunit p65 (Abe & Barber 2014).

1.2.3. Immune response to VACV

Poxviral infections cause a strong humoral and cell-mediated immune response (Moss 2011). VACV has a broad host tropism both *in vitro* and *in vivo* (McFadden 2005). Whilst it has been shown to successfully infect monocytes (Sánchez-Puig et al. 2004), B lymphocytes and NK cells, its replication in DCs and macrophages is abortive, and severely impaired in T lymphocytes (Moss 2011). Early during infection, sentinel cells, including non-hematopoietic (e.g. fibroblast) and specialised innate immune cells (e.g. DC, macrophages, NK cells) are

activated by VACV (see 1.2.3.1), and induce the expression of interferons (Barbalat et al. 2009; Martinez et al. 2010a; Martinez et al. 2010b), and cytokines (Zhu et al. 2007). Furthermore, virus-infected cells are lysed by NK cells (Martinez et al. 2010a; Sutherland et al. 2011). In addition, the complement system is able to bind MVs (Isaacs et al. 1992), and has been shown to be required for neutralisation of EVs (Benhnia et al. 2009). Then, the adaptive response is mounted which consists of neutralising antibodies as well as CD4+ and CD8+ T cell responses. Whilst the latter has been reported to have a minor role in clearing the virus from infected mice in presence of a potent humoral response (Xu et al. 2004), CD8+ T cell-mediated cytolytic effects are protective and limit pathogenesis in the absence of a sufficient antibody response. Neutralising antibodies are produced against membrane-associated proteins present in MV and EV membranes (Moss 2011), and can be detected at approximately two weeks postvaccination in humans. Studies examining the half-life of adaptive immune responses against VACV in humans demonstrated that CD4+ and CD8+ T cell responses were still detectable at 75 years postvaccination (Hammarlund et al. 2003), however their efficacy declined progressively at 7 years postvaccination. In contrast, neutralising antibodies were stable for a lifetime (up to 88 years postvaccination) and did not lose efficacy (Hammarlund et al. 2003; Taub et al. 2008).

1.2.3.1. Detection of VACV by innate immune signalling

VACV generates a variety of PAMPs (e.g. DNA, RNA, and proteins) throughout its life cycle, which are recognised by different PRRs. Viral challenges in cGAS knockout mice demonstrated the crucial role of this DNA sensor in eliciting antiviral responses to VACV (Schoggins et al. 2014). The role of TLR9 as an orthopoxviral DNA sensor is less evident, an initial study reported TLR9-dependent IFN α secretion in response to MVA in certain DC subpopulations (Samuelsson et al. 2008). However, three subsequent studies demonstrated that TLR9 is not involved in VACV sensing (Barbalat et al. 2009; Martinez et al. 2010b; Cao et al. 2012).

VACV generates dsRNA molecules throughout the entire transcription process (Duesberg & Colby 1969; Willis et al. 2011), the detection of which have been reported for several PRRs. These PRRs include: RIG-I, MDA-5 (Pichlmair et al. 2009; Myskiw et al. 2011), TLR3, TLR7 (Cao et al. 2012), TLR8 (Martinez et al. 2010b), and the ISGs OAS and PKR (Rice et al. 1984; Willis et al. 2011). Although the adaptor protein TRIF has been described as protective in VACV infection in the context of TLR4 signalling, TLR3-mediated activation of TRIF has a detrimental effect on the host upon viral challenge mediating an excessive inflammatory response for reasons that are not entirely clear (Hutchens, Luker, Sottile, et al. 2008).

TLR2 and TLR4 have been reported to be involved in VACV detection (Zhu et al. 2007; Hutchens, Luker, Sonstein, et al. 2008), though their ligands have not yet been identified. However, contention about the requirement of TLR2 is ongoing, whilst its involvement has been demonstrated in several studies examining its role in DCs (Zhu et al. 2007), monocytes (Barbalat et al. 2009), NK cells (Martinez et al. 2010a), and CD8+ cells (Quigley et al. 2009), others did solely support the necessity of MyD88 (Zhao et al. 2009; Sutherland et al. 2011; Davies et al. 2014), but not TLR2.

1.3. Viral evasion

Viruses are obligate intracellular parasites; they rely on host cells to complete their replication and life cycle, at the expense of their host. To counteract the constant threat posed by viruses (and other pathogens), hosts have evolved complex and multi-faceted immune systems. Viruses have, in turn, adapted to overcome these defence systems (Daugherty & Malik 2012), developing mechanisms to negate, modulate and evade immune responses (Figure 1-8).

1.3.1. Evolution of poxviral evasion strategies

The majority of known immunomodulators are encoded by genes located at the termini of the VACV genome (Gubser et al. 2004). Despite having no effect on viral growth *in vitro* (Smith et al. 2013), these proteins contribute significantly to the virulence of the virus *in vivo* (Smith et al. 2013). Due to the low error rate of their polymerase, poxviruses cannot solely rely on random mutations occurring during replication to generate a large diversity of inhibitory proteins and rapidly adapt to novel immune responses. Instead, a process termed the “gene accordion” has been recently identified (Elde et al. 2012). In this process, the copy number of individual genes expands rapidly, giving the virus the ability to overcome a certain immune response quantitatively. Subsequently, individual copies accumulate mutations, leading to diversification of the initial gene. Copy numbers are then reduced, preserving only genes harbouring beneficial mutations (Elde et al. 2012). Another process contributing to host evasion is termed “microbial mimicry” (Elde & Malik 2009), whereby pathogens mimic host genes in order to modulate host processes. Intriguingly, bioinformatic analyses have revealed that poxviral proteins are more similar to eukaryotic proteins than to viral, prokaryotic or archaeobacterial proteins (Lefkowitz et al. 2006). Acquisition of host proteins and subsequent gene expansion and diversification can be exemplified by the poxviral B-cell lymphoma 2 (Bcl-2) fold-like protein family (i.e. C16, C6, C1, N1, N2, K7, F1, A46, A49, A52 and B14) (Neidel et al. 2015). The host protein Bcl-2 is involved in apoptosis, whilst in VACV this gene has evolved to encode at least eleven distinct viral Bcl-2 fold-like proteins targeting different processes in PRR signalling and cell death.

1.3.2. Viral evasion and immunomodulation mechanisms

Strategies of viral evasion and immune modulation can be roughly divided into three types. Prevention of detection, impairment of signalling following pathogen detection, and inhibition of the antiviral response itself (Alcami & Koszinowski 2000).

To interfere with recognition by the immune system, pathogens have evolved to mimic their hosts (Elde & Malik 2009), and reduce and/or vary patterns and structures, which identify them as non-self. Viruses, in particular RNA viruses and retroviruses use antigenic variability to avoid detection by neutralising antibodies and cell-mediated adaptive immune responses (Alcami & Koszinowski 2000). VACV is the only virus which uses two morphologically different infectious forms (i.e. MV, EV) (Bidgood & Mercer 2015). It was shown that distinct antigens are recognised by the adaptive immune system, and thereby EV generation has the effect of diverting immune responses away from MV. Both MV and EV membranes contain host proteins, which prevent the activation of the complement system (Vanderplasschen et al. 1998). Enveloped viruses such as

VACV, VSV and *Ebolavirus* have been shown to enter host cells by mimicking membranes of apoptotic cells (Amara & Mercer 2015), which display phosphatidylserines on the extracellular side of their plasma membrane. This strategy does not only facilitate uptake of viral particles, but may also provide viruses with a less alert environment, since uptake of apoptotic cells has been linked to downregulation of innate immune signalling (Rothlin et al. 2007; Bhattacharyya et al. 2013).

In the cytoplasm two complementary mechanisms have been discovered. VACV and other viruses are able to synthesise methylated, capped and polyadenylated mRNA (Moss 2013), which mimics host mRNA to the extent that it cannot be recognised by cytosolic receptors such as IFIT proteins (Daffis et al. 2010). Examination of avian and human IAV strains revealed that IAV has gradually adapted to its human host by the removal of CpG dinucleotides from its genome (Greenbaum et al. 2008). Alternatively, viral nucleotides can be sequestered to interfere with recognition, as has been shown for the poxviral protein E3 (Chang et al. 1992).

Cells have evolved elaborate systems to detect viral infections. A key signalling network is the PRR signalling pathways. Viruses have been shown to impair these pathways by physically preventing interaction of signalling molecules, modifying host proteins (i.e. posttranslational modifications), providing decoy targets for host proteins, and by degradation of signalling components. The intracellular strategies of immune evasion and modulation of PRR and pro-inflammatory cytokine signalling by VACV are detailed in the following section.

Upon recognition of a pathogen a signalling cascade is activated which leads to the production of interferons, ISGs, cytokines and chemokines, the activity of which is also targeted by viruses. To this end, viruses secrete so-called virokines and viroceptors, which mimic host cytokines and receptors (Alcami et al. 1998). In addition, viral proteins can pose as targets for intracellular ISGs, for example VACV K3 competes with eIF2 α for phosphorylation by PKR (Dar & Sicheri 2002).

1.3.3. Immunomodulators of pattern recognition pathway signalling

The importance of cGAS as sensor of VACV DNA has been demonstrated *in vitro* and *in vivo* (Schoggins et al. 2014), and therefore the existence of poxviral antagonists of cGAS-STING signalling is anticipated. Despite a predicted DNA binding domain in E3 (Valentine & Smith 2010), this protein was shown to be unable to bind DNA. C16 has been shown to impair dsDNA recognition by DNA-PK by interacting with the subunits K70 and Ku80 (Peters et al. 2013). The amount of dsRNA generated during replication is limited by the organisation of the VACV genome, which can be divided into four sections. Generally, genes belonging to a section are transcribed in the same direction. Early transcribed genes are located at the termini and are transcribed towards their respective ends (Smith & Symons 1998; Lefkowitz et al. 2006), thereby avoiding the generation of dsRNA. Three additional strategies are used by VACV to avoid dsRNA detection (Chang et al. 1992), one of which is the sequestration of dsRNA by E3. VACV mRNA is methylated and capped, which prevents detection by IFIT proteins (Daffis et al. 2010). In addition, it has recently been shown that the VACV decapping enzymes D9 and D10 remove the cap structure from viral RNA to render it sensitive to degradation by host

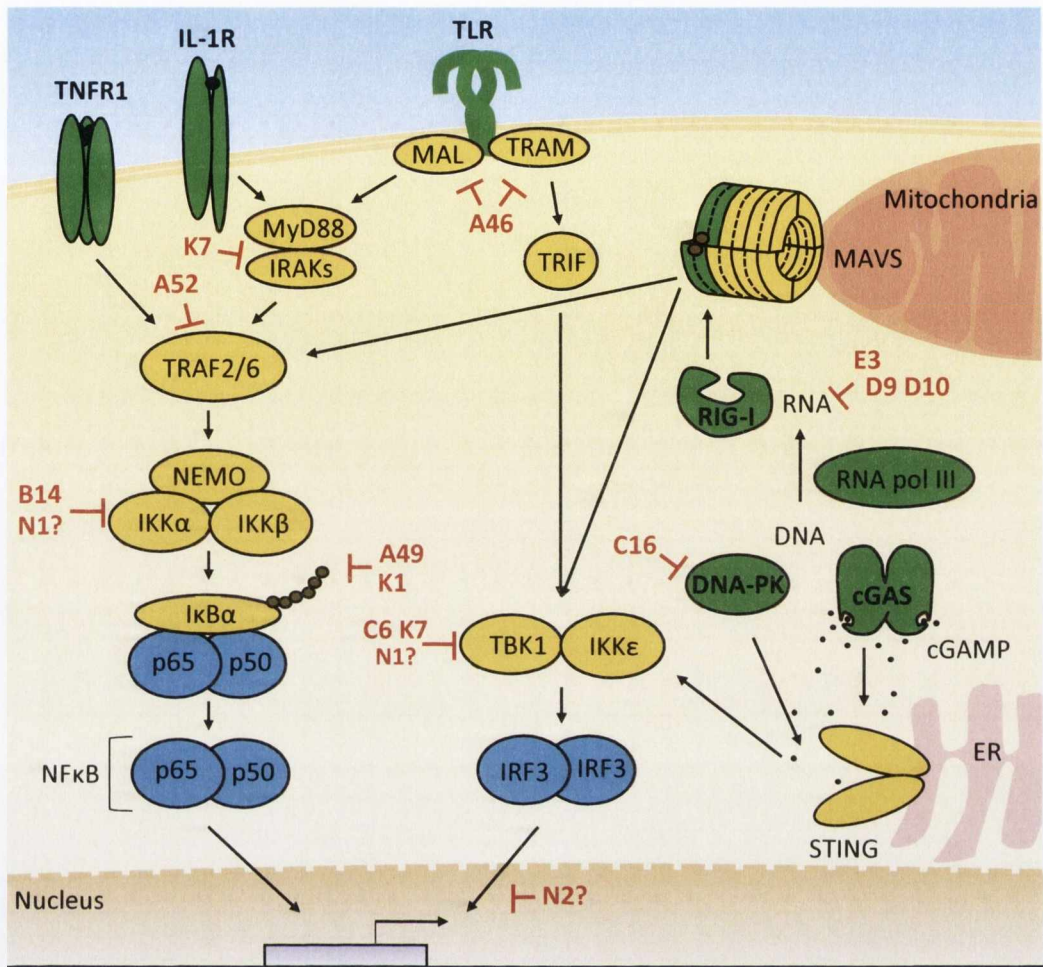


Figure 1-8 Overview of VACV intracellular evasion strategies

VACV has evolved numerous strategies to evade innate immune signalling. E3, D9, D10 and C16 act proximal to the receptor and prevent detection through sequestration or targeted degradation of viral nucleic acids, and through interference with the DNA sensor DNA-PK. Inhibitors of activated signalling cascades include A46, K7 and A52, which target adaptor and downstream signalling molecules of PRR signalling pathways. B14, C6 and K7 act proximal to transcription factors and prevent phosphorylation of IRFs and NFκB by activated kinase complexes. Nuclear translocation of NFκB is also inhibited by A49 and K1. In the nucleus, N2 is able to impair induction of gene expression mediated by IRF3.

5′to3′exoribonuclease 1 (Burgess & Mohr 2015; Liu, Katsafanas, et al. 2015). Both mechanisms inhibit activation of IRF3 and NFκB through dsRNA detection by RLRs, RNA pol III, OAS, and PKR. An early study examining 2′-5′ oligoadenylates produced by OAS in response to interferon during VACV infection discovered synthesis of numerous aberrant oligomers, which were unable to stimulate RNaseL activity (Rice et al. 1985). Whilst none of these non-stimulatory oligomers had an inhibitory function as observed in HSV-1 infected cells (Cayley et al. 1984), they may represent another strategy to impair OAS signalling. A46 is an IL-1R- and TLR-specific inhibitor. Prior to the characterisation of A46 (and A52) (Bowie et al. 2000), TLRs were only reported to sense bacterial and fungal PAMPs. A46, was one of the first viral proteins shown to impair TLR signalling, thus indicating the now established role of TLRs in antiviral immunity. A46 disrupts interaction of TRAM and MAL with its receptor TLR4 (Stack & Bowie 2012), thereby impairing IRF3 and NFκB activation.

VACV encodes numerous inhibitors acting on downstream signalling events. Viral proteins targeting activation and action of IRF3 include; K7 (DDX3, an IKKε-interacting protein) (Schröder et al. 2008), N1

(presumably TBK1), C6 (scaffold proteins TRAF family member-associated NF-kappa-B activator (TANK), NAK-associated protein 1 (NAP1) and Similar to NAP1 TBK1 adaptor (SINTBAD), scaffold for TBK1/IKK ϵ) (Unterholzner et al. 2011), and N2 (Ferguson et al. 2013). Characterisation of host proteins targeted by K7 led to the discovery of an innate function for DDX3 (Schröder et al. 2008), an RNA helicase, which interacts with IKK ϵ to initiate IRF3 activation.

Along with A46 and E3, ten NF κ B inhibitors have been reported. K7 and A52 associate with IRAK-2 and TRAF6 preventing NF κ B activation (Harte et al. 2003; Schröder et al. 2008). The discovery of A52 as a viral inhibitor of IRAK-2 led to a detailed investigation of IRAK-2 in PRR signalling. Hitherto, IRAK-2 was assumed to play a minor role in signal transduction downstream of MyDD88, acting redundantly in the absence of IRAK-1. However, Keating and co-authors provided evidence that it has a crucial role in TLR signalling (Keating et al. 2007). No precise mechanism has been reported for C4 inhibition of NF κ B activation (Ember et al. 2012), but it appears to act downstream of TRAF2 and TRAF6. Phosphorylation and activation of IKK β is impaired by B14 (Chen et al. 2008), which directly binds to IKK β . A49 mimics I κ B α (Mansur et al. 2013), and binds to β -TrCP, thereby preventing ubiquitination and degradation of phosphorylated I κ B α . K1 has also been reported to impair I κ B α degradation (Shisler & Jin 2004). NF κ B activation is inhibited by N1 via an unknown mechanism. This inhibition was initially assumed to be mediated by association with the IKK complex (DiPerna et al. 2004), however these results could not be reproduced in a subsequent study (Chen et al. 2008). Extracellular-signal regulated kinases (ERK) 2 mediated NF κ B activation is inhibited by M2 (Gedey et al. 2006), which prevents phosphorylation of ERK2.

Despite the large number of existing viral inhibitors of the NF κ B signalling pathway, a recent study has suggested the existence of additional immunomodulators (Sumner et al. 2014). The VACV Copenhagen strain vv811 lacks C4, N1, M2, K1, K7, B14, and presumably A46, and A52 due to deletions (Δ C23L-F4L, Δ B13R-B29R), truncation (A46), and point mutations (A52) (Fagan-Garcia & Barry 2011), yet is still able to prevent IL-1- and TNF α -driven nuclear translocation of p65. Additional deletion of A49 (Sumner et al. 2014), generating the virus vv811 Δ A49, abolished the ability of the parental vv811 strain to prevent NF κ B translocation to the nucleus (Sumner et al. 2014). In contrast, activation of NF κ B-reporter genes mediated by IL-1 and TNF α was still inhibited by more than 50 %. The virus vv811 Δ A49 was also still able to reduce production of certain cytokines (regulated on activation, normal T cell expressed and secreted (RANTES, also called CCL5) and monocyte chemotactic protein 1 (MCP1, also called CCL2)). These results suggest that additional NF κ B inhibitors are encoded in the VACV genome.

1.4. Aims

The VACV genome codes for the production a large repertoire of proteins involved in immune modulation and evasion. Elucidation of these inhibitory strategies have been used in the past to identify key mechanisms in innate immunity. Recently, the cytosolic DNA sensor cGAS has been identified as a novel PRR signalling through the adaptor STING to activate IRF3 and NFkB (Sun et al. 2013). Various members of the *Herpesviridae*, the *Flaviviridae* and the *Coronaviridae* families have been reported to antagonise cGAS and/or STING function, supporting a role for these host proteins in innate immune signalling against viruses (Ma & Damania 2016): The herpesviruses KSHV, *Murid herpesvirus 68*, *Rhesus monkey rhadinovirus* and EBV encode proteins, that have been shown to directly bind to cGAS and to DNA (Wu et al. 2015); KSHV protein vIRF1 was shown to prevent STING phosphorylation and subsequent TBK1 recruitment (Ma et al. 2015), *Dengue virus* protein NS2B/3 is able to bind and cleave STING (Aguirre et al. 2012; Yu et al. 2012), and *SARS coronavirus* protein PLpro can prevents STING dimerization and polyubiquitination (Sun et al. 2012). However, no poxviral immunomodulators of the cGAS-STING signalling pathway have been reported to date.

A recent study, performed in human cell lines (Pichlmair et al. 2012), has revealed that in comparison to the average human proteins, targets of viral proteins are more well connected to other host proteins, take central positions in affected networks and are more likely to participate in more than one cellular pathway. In the past, studies of these viral proteins have discovered not only viral immune evasion strategies, but also key mechanisms of innate immunity. *Vaccinia virus*, the model poxvirus used in this study, is a well-known example of a virus whose genome encodes a large number and broad variety of immunomodulators.

The aim of this project is to identify and characterise novel cGAS-STING signalling pathway inhibitors encoded by the VACV genome, achieved by completing the following objectives:

1. Identification of potential VACV inhibitors of IRF3 and NFkB activation and delineation of host targets through use of reporter gene assays (RGA).
2. Characterisation of identified VACV proteins and identification of host interaction partners through the generation of stable-inducible cell lines.
3. Validation and characterisation of host targets.

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2.1. Materials

All general reagents were purchased from Sigma-Aldrich, whereas plastics came from Cruinn and Fischer Scientific, unless otherwise stated.

2.1.1. Buffers

Table 2-1 Buffers and their composition

Name	Composition
1x Phosphate-buffered saline (PBS)	145 mM NaCl, 3.9 mM NaH ₂ PO ₄ , 2.27 mM Na ₂ HPO ₄ , per 1 l H ₂ O
1x Tris-buffered saline (TBS)	25 mM Tris, 150 mM NaCl, 2 mM KCl, per 1 l H ₂ O, pH 7.4
Resolving Gel Buffer	1.5 M Tris, pH 8.8
Stacking Gel Buffer	0.5 M Tris, pH 6.8
3x Western blot (WB) sample buffer	10 % (v/v) Glycerol, 10 % (w/v) sodium dodecyl sulphate (SDS), 0.1 % (w/v) Bromophenol Blue, 62.5 mM Trizma Base, 50 mM dithiothreitol (DTT, Fisher Scientific, CAT: 10592945), pH 6.8
10x WB Running buffer	0.25 M Trizma Base, 1.9 M Glycine, 35 mM SDS, per 1 l H ₂ O
10x WB transfer buffer	0.25 M Trizma Base, 1.9 M Glycine, per 1 l water
Washing buffer (WB)	1x PBS, 0.1 % (v/v) Tween-20
Luciferase mix	1.1.1.20 mM Tricine, 1.07 mM (MgCO ₃) ₄ Mg(OH) ₂ •5 H ₂ O, 2.67 mM MgSO ₄ , 0.1 M Ethylenediaminetetraacetic acid (EDTA), 33.3 mM DTT (Fisher Scientific, CAT: 10592945), 270 M coenzyme A (Sigma-Aldrich, CAT: A2181), 470 mM Luciferin (Biosynth, CAT: 2591-17-5), 530 mM ATP (Sigma-Aldrich, CAT: A6419)
Isotonic buffer (VACV DNA isolation)	10 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, pH 8.0
Hypotonic buffer (VACV DNA isolation)	10 mM Tris-HCl, pH 9.0
Sucrose buffer (VACV DNA isolation)	10 mM Tris-HCl, pH 9.0, 36 % (w/v) sucrose
Crystal violet (plaque assay)	15 % (v/v) ethanol, 0.1 % (w/v) crystal violet
1x TBA (agarose gel)	40 mM Tris, 20 mM acetic acid, and 1 mM EDTA pH 8.0
IP lysis buffer	50 mM Tris pH 7.5, 100 mM NaCl, 1 mM EDTA pH 8.0, 10 % glycerol, 0.5 % NP-40
Paraformaldehyde (confocal microscopy)	1x PBS, 3% (w/v) Bovine serum albumin (BSA), 4 % (v/v) paraformaldehyde
Blocking and lysis buffer (confocal microscopy)	1x PBS, 3% (w/v) BSA, 0.5 % TritonX1000
Wash buffer (IL-8 ELISA)	1x PBS, 0.05 % (v/v) Tween-20
Block buffer (IL-8 ELISA)	1x PBS, 1% (w/v) BSA
Reagent diluent (IL-8 ELISA)	1x PBS, 0.1 % (w/v) BSA, 0.05 % (v/v) Tween-20
Stop solution (IL-8 ELISA)	1 M H ₂ SO ₄

2.1.2. Cloning

Table 2-2 Reagents and kits used for generation of expression constructs

Reagent/kit	Source
Low viscosity carboxymethylcellulose	VWR international (CAT: 276494N)
High Pure RNA isolation kit	Roche (CAT: 11828665001)
Primer	MWG Biotech AG or Integrated DNA Technology
M-MLV Reverse transcriptase (incl. buffers)	Promega (MyBio, CAT: M1705)
Deoxyribonucleotides	New England Biolabs (CAT: N0446S)
RNAse OUT	ThermoFisher Scientific (Biosciences, CAT: 10777-019)
KOD DNA polymerase (incl. buffers, dNTPs)	Novagen (Merck Millipore, CAT: 71085)
2-log DNA ladder (incl. gel loading dye)	New England Biolabs (CAT: N3200S)
Agarose GelExtract Mini Kit	5 prime (CAT: 2300510)
Restriction enzymes (incl. buffers)	New England Biolabs
T4 DNA ligase (incl. buffer)	New England Biolabs (Brennan and Co., CAT: M0202S)
Novablue Single competent cells	Novagen (Merck Millipore CAT: 70181)
Luria-Bertani (LB) agar, Miller and broth, Miller	Fischer Scientific (CAT: 10081163 and 10113293)
QIAprep Spin Miniprep Kit	Qiagen (CAT: 27106)
EndoFree Plasmid Maxi kit	Qiagen (CAT: 12362)
DNA Sequencing	Source Bioscience LifeScience

Table 2-3 List of cloning and sequencing primers

Primer name	Primer sequence	Description of use
pCMV forward	GAGCTCGTTTAGTGAACCGTC	pCMV forward sequencing primer supplied by Source Bioscience LifeScience
CMVHARP	TTTCACACAGGAAACAGC	pCMV-HA reverse sequencing primer
F-WR014	GTGAATTCGTATGTCTTATGCTTGCCCAATTCTTAGTA	Sequencing pCMV-HA VV-WR 014
ORF7FP1	CAGATTAGACGTGCTGGTGAATA	Sequencing pCMV-HA C9L
ORF7RP1	TCTATGCGTGAGTTGGATGTG	Sequencing pCMV-HA C9L
F-WR020	GTGAATTCGTATGTCTTCGATTAGATTTATTGCATGTC	Amplification pCMV-HA C8L
R-WR020	GTGGTACCTCAGTTATGTATGGATTTAGCACGTTTG	Amplification pCMV-HA C8L
ORF15RP	CCCACTTCAACAGCCATCTTA	Sequencing pCMV-HA M1L
ORF19RP2	AGTTTCAGAAATGTCCTCCATTAC	Sequencing pCMV-HA F3L
ORF20FP	GCTAGCCACCGAGGATAAA	Sequencing pCMV-HA F5L
ORF24FP	TAGACCAGCTGACTGTTTCTTG	Sequencing pCMV-HA F11L
ORF24RP	ACCTTGGCTGTCTCATTCAATA	Sequencing pCMV-HA F11L
ORF29FP1	GGATATCTGCACGAGGACAAG	Sequencing pCMV-HA E2L
ORF29RP1	TCCAGAGCTCGCTCTATCTT	Sequencing pCMV-HA E2L
ORF34FP	ATGGGATACCTTTGAGCCTAAC	Sequencing pCMV-HA A55R
ORF34RP1	CCACTGCGAAACTACACCTAT	Sequencing pCMV-HA A55R

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Primer name	Primer sequence	Description
F-WR182	GTGGTACCCTAATTAGTGTAAATACATTCTAATACGGTC	Amplification and sequencing pCMV-HA A57R
R-WR182	GTGAATTCGTATGGAACGAGAAGGTGTTGATTACATT	Amplification and sequencing pCMV-HA A57R
ORF36RP	CTTAGTCCATGGCAAGATACCTC	Sequencing pCMV-HA B1R
ORF39FP1	TCGAACATACGAGAAAGTGGATAA	Sequencing pCMV-HA B4R
ORF39FP2	CCGAAGACAGTTACGGTTGTA	Sequencing pCMV-HA B4R
F-WR188	GTGAATTCGTATGTCTTCTTCAGTGGATGTTGATATCT AC	Sequencing pCMV-HA B6R
F-WR193	GTGAATTCGTATGGATACAGATGTTACAAATGTAG AAGATATC	Sequencing pCMV-HA B11R
R-WR193	GTGGTACCCTATATTCCTACATCGTGACAGATACAT	Sequencing pCMV-HA B11R
F-WR194	GTGAATTCGTATGGAATCCTCAAGTATTGTTTGATAAC	Sequencing pCMV-HA B12R
ORF43RP	TCCAGAGGTTCAATTCCTTCAG	Sequencing pCMV-HA B12R
ORF44FP	TCTATGTACGGCAAGGCATTTA	Sequencing pCMV-HA B13R
ORF44RP	CGATAAACGTAGCTTCCAGAGAG	Sequencing pCMV-HA B13R
ORF46FP	CGAGCCAGTAATCTTACCATGT	Sequencing pCMV-HA B16R
ORF46RP	AACACGTCTCTTATCGGTCATATAG	Sequencing pCMV-HA B16R
ORF47FP	CCAGAATACTATAGCGCGGAAA	Sequencing pCMV-HA B17R
ORF47RP	TGCTATGATTAACCTCCACGATAC	Sequencing pCMV-HA B17R
UFPVVORF	CCGGTAACATGTACCCATACGATG	Amplification pMEP4-HA VV-WR forward
SRP15	GCGGCTCGAGTAAAAATAATCATCGAATAC	Amplification pMEP4-HA VV-WR M1L rev
SRP28	CGCGCTCGAGTTAGTTCAAAGTCTCGACGC	Amplification pMEP4-HA VV-WR F17R rev
SRP31	CCGGCTCGAGTTATCCCATTCCAATCACCTT	Amplification pMEP4-HA VV-WR A47L rev
SRP35	GCCGCTCGAGCTAATTAGTGTAAATACAT	Amplification pMEP4-HA VV-WR A57R rev
SRP37	GGCGCTCGAGTTACCTCGATGGTGCCGCCGA	Amplification pMEP4-HA VV-WR B2R rev
SRP43	GCGGCTCGAGTCAATCTTGATAAACAGTTT	Amplification pMEP4-HA VV-WR B12R rev
SRP44	GGCGCTCGAGTTAACAATTAGTTGTCGGAGA	Amplification pMEP4-HA VV-WR B13R rev
SRP50	CGGGCTCGAGTTAGTAGATGGGTAGTATATT	Amplification pMEP4-HA VV-WR 204 rev
VRK1FP	GGG GGTACC ATGCCTCGTGTAAAGCAGC	Amplification hVRK1 forward
VRK1flagRP	CCCCCTCGAGTCACTTGTCGTCGTCGTCCTTGTAGTCCTTCTGGACTCTCTTCTGG	Amplification hVRK1 rev, flag-tagged
FLAGB12FP	GGGGGTACCATGGACTACAAAGACGATGACGACAAGATGGAATCCTTCAAGTATTGT	Amplification pCEP4 FLAG VV-WR B12 forward (incl. FLAG-tag)
(FLAG)B12RP	CCCCCTCGAGTCAATCTTGATAAACAG	Amplification pCEP4 FLAG VV-WR B12 rev

Table 2-4 Restriction enzymes

Restriction enzyme	conditions	Description of use
EcoRI	Buffer 2, 37 °C	pCMV-HA VV-WR cloning
KpnI	Buffer 2, 37 °C	pCMV-HA VV-WR and pMEP-HA VV-WR cloning
KpnI	Buffer 1, 37 °C	pCEP-Flag (UT, myc) VRK1 cloning
Sall	Buffer 3, 37 °C	pCMV-HA VV-WR cloning
XhoI	Buffer 3, 37 °C	pCMV-HA VV-WR and pMEP-HA VV-WR cloning
XhoI	Buffer 1, 37 °C	pCEP-Flag (UT, myc) VRK1 cloning

2.1.3. Cell culture

Table 2-5 Cell lines and respective media conditions

Cell line	Description	Medium
HEK 293T	Human Embryonic Kidney 293 cells, expressing SV40 large T-antigen	DMEM (Gibco, Biosciences, CAT: 61965-059), 10 % (v/v) FCS (Labtech, CAT: FCS-SA/500), Penicillin (100 U/ml) and Streptomycin (100 µg/ml, Pen-Strep Mixture, Invitrogen, CAT: P4333)
STING HEK 293T	HEK 293T cells stably expressing STING (Gift from Lei Jin (Albany Medical College, USA))	DMEM, 10 % v/v FCS, Penicillin (100 U/ml) and Streptomycin (100 µg/ml)
pMEP4 VV-WR HEK 293T	HEK 293T cells stably transfected with CdCl ₂ -inducible pMEP4-HA constructs	DMEM, 10 % v/v FCS, Penicillin (100 U/ml) and Streptomycin (100 µg/ml), Hygromycin (300 µg/ml, HygroGold, Invivogen, CAT: ant-hg-1)
HEK-blue™ IFNα/β cells	Human embryonic Kidney 293 cells, stably expressing STAT2 and IRF9 (Gift from Prof. O'Neill (Trinity College Dublin, Ireland), Invivogen, CAT: hkb-ifnab)	Assay medium: DMEM, 10 % v/v FCS, Penicillin (100 U/ml) and Streptomycin (100 µg/ml), and Normocin (100 µg/ml, Invivogen, CAT: ant-nr); Growth medium: Assay medium supplemented with Zeocin (100 µg/ml, Invivogen, CAT: ant-zn) and Blastidicin (30 µg/ml, Invivogen, CAT: ant-bl)
BS-C-1 cells	African green monkey kidney epithelial cells	DMEM, 10 % v/v FCS, Penicillin (100 U/ml) and Streptomycin (100 µg/ml)
TK143	Human embryoblast cell line, stably expressing plasmid containing the native HSV-1 thymidine kinase	MEM (Gibco, Biosciences, CAT: 61965-059), 10 % (v/v) FCS, Penicillin (100 U/ml) and Streptomycin (100 µg/ml), L-glutamine (1.8 mM, Sigma-Aldrich, CAT: G7513)
A549	Human lung epithelial cells	DMEM, 10 % v/v FCS, Penicillin (100 U/ml) and Streptomycin (100 µg/ml)

Table 2-6 Viruses

Name	Description	Source
<i>Vaccinia virus</i> WR	Strain Western Reserve (Baroudy et al. 1982; Baroudy & Moss 1982; Baroudy n.d.; Esposito et al. n.d.; Esposito et al. 2003)	Gift from G.Smith (University of Cambridge, United Kingdom)
VSV-AV3-GFP	Attenuated Recombinant <i>Vesicular stomatitis virus</i> , Indiana serotype, contains a transgene encoding GFP (Boritz et al. 1999)	Gift from A.Pichlmair (Max-Planck Institute for Biochemistry, Germany)
<i>Sendai virus</i>	Cantell strain, mixed virus stock composed mostly of copyback defective-interfering genomes, used as interferon inducer (Strahle et al. 2006)	LGC Standards (CAT: ATCC-VR-907)

2.1.4. Transfections and plasmids

Table 2-7 Transfection and reporter gene assay reagents

Reagent	Description of use	Source
GeneJuice®	Transfection reagent for expression constructs	Novagen (Merck Millipore, CAT: 70967)
Lipofectamine®2000	Transfection reagent for ligands	Invitrogen (Biosciences, CAT: 11668019)
Passive lysis buffer	Reporter gene assay lysis buffer	Promega (MyBio, CAT: E1941)
Colenterazine	Substrate for Renilla luciferase	Biotium Incorporated (CAT: 10111-1)
Luciferin	Substrate for Firefly luciferase	Biosynth (CAT: 2591-17-5)

Table 2-8 List of reporter and expression plasmids

Plasmid	Description	Source
pCMV-HA constructs	CMV immediate early promoter, N-terminal hemagglutinin (HA) epitope tag, for VV-WR expression plasmids	Clontech (CAT: 635690)
pMEP4	Expression vector for stable cell line development, CdCl ₂ -inducible promoter, Hygromycin selection	Invitrogen (ThermoFisher Scientific)
pCEP4	Expression vector for stable cell line development, CMV promoter, Hygromycin selection	Invitrogen (ThermoFisher Scientific, CAT: V044-50)
pPK-IFN β -luc	Firefly luciferase reporter under IFN β promoter control	Gift from T. Taniguchi (University of Tokyo, Japan)
pPK-ISRE-luc	Firefly luciferase reporter under ISRE element control, (Katherine Fitzgerald et al. 2003)	Gift from K. Fitzgerald (University of Massachusetts Medical School, USA)
pPK-NF- κ B-luc	Firefly luciferase reporter under NF- κ B element control, (Bowie et al. 2000)	Gift from R. Hofmeister (University of Regensburg, Germany)
pAP-1-luc	Firefly luciferase reporter under AP1 promoter control	Gift from P. Moynagh (Maynooth University, Ireland), Stratagene (Agilent Technologies, CAT: 219074)
pPG13LUC	Firefly luciferase reporter under p53 element control, polyomavirus early promoter, 13 copies of a synthetic consensus p53-binding site, (Bates et al. 2005)	Gift from F.V. Fuller-Pace (University of Dundee, United Kingdom)
pRL-TK	Renilla luciferase reporter under HSV-TK promoter control	Promega (CAT: E2241)
pFR-luc	Firefly luciferase reporter under the control of a promoter with five tandem repeats of the yeast GAL4 binding sites	Stratagene (Agilent Technologies, CAT: 219050)
pcDNA-Flag constructs	IKK- ε , IKK- α , IKK- β , TBK1 Flag expression plasmids, (Peters et al. 2000)	Gift from L. O'Neill & K. Fitzgerald (Trinity College Dublin, Ireland)
pcDNA-FLAG STING	Human STING FLAG expression plasmid, (Ishikawa & Barber 2008)	M. Baran (Bowie group)
pCDNA3-flag-cGAS, MAVS	cGAS, MAVS Flag expression plasmid, (Xu Zhang et al. 2013; Seth et al. 2005)	Gift from J. Chen (University of Texas Southwestern Medical Center, USA)

Plasmid	Description	Source
pCMV2-flag-TRIF	TRIF flag expression plasmid, (Stack et al. 2005)	Gift from S. Sato (University of Tokyo, Japan)
pRK5-FLAG-TRAF2, TRAF 6	TRAF2, TRAF6 expression plasmid, (Stack et al. 2005; Harte et al. 2003)	Gift from D. Goeddel (Tularik Inc., San Francisco, USA)
pFA2-Elk1	Fusion protein with Elk1 activation domain sequence fused to DNA binding domain of yeast Gal4	Stratagene (Agilent Technologies, CAT: 219005)
p21v-HA-ras	RAS expression plasmid, , constitutively active, (Izquierdo et al. 1993)	Gift from D. Cantrell (University of Dundee, United Kingdom)
P65-HA	P65 HA expression construct, (Brady et al. 2015)	Gift of G.Brady (Trinity College Dublin, Ireland)
pCMV-flag constructs	IRF3 5D, IRF7 and IRF7 4D Flag expression plasmids, (Katherine Fitzgerald et al. 2003)	Gift from J. Hiscott (Istituto Pasteur-Fondazione Cenci Bolognetti, Italy)
Gal4-IRF3 and Gal4-IRF7	Fusion protein with IRF3 or IRF7 activation domain sequence fused to DNA binding domain of yeast Gal4, (Katherine a Fitzgerald et al. 2003)	Gift from K. Fitzgerald University of Massachusetts Medical School, USA)
pCDNA3-p53	Untagged p53 expression plasmid, (Bates et al. 2005)	Gift from F.V. Fuller-Pace (University of Dundee, United Kingdom)

Table 2-9 Cell stimulants

Stimulant	Description	Source
hTNF α	TNF-receptor ligand	Peprotech (CAT: 300-01A)
hIL-1 α	IL-1 receptor ligand	National cancer institute (Frederick, MD)
hIL-1 β	IL-1 receptor ligand	Peprotech (CAT: 200-01B)
3'3'-cGAMP	cGAS ligand	Invivogen (CAT: tlr-cga)
hIFN α_{2a}	Type I IFN receptor ligand	PBL Assay Science (CAT: 11100-1)
Phorbol 12-myristate 13-acetate (PMA)	NF κ B and MAPK inducer	Invivogen (CAT: tlr-pma)

Table 2-10 List of siRNA target sequences

All small interfering RNA (siRNA) were purchased from Qiagen.

Name	Target sequence	Description
siVRK1-01	AAGCTAAGCTTAAGAATTCTG	hVRK1 siRNA
siVRK1-02	AACAAGGAACCTGGTGTGAA	hVRK1 siRNA
Negative Control siRNA	AATTCTCCGAACGTGTCACGT	Negative control siRNA (Qiagen, CAT: 1027310)

2.1.5. Western Blot

Table 2-11 Western blot reagents (excluding antibodies)

Reagent	Description	Source
Protogel	30 %, SDS PAGE gel for electrophoresis	National Diagnostics (Biosciences, CAT: EC890)
Protein ladder	Molecular weight marker for SDS PAGE gel (250 kDa)	ThermoFisher Scientific (Fisher Scientific, CAT: 11852124)
Amersham Protran 0.45 nitrocellulose	Transfer membrane	GE Healthcare (Fischer Scientific, CAT: 15209804)
Immun-Blot® PVDF Membrane 0.2 µm	Transfer membrane	Thermo Fisher Scientific (Fisher Scientific, CAT: 10078559)
Benzonase® Nuclease, Purity > 90 %	Sample buffer	Novagen (Merck Millipore, CAT: 70746)

Table 2-12 Composition of electrophoresis gels

1.5 mm	Stacking gel (4)	Resolving gel (2)			
		10 %	12 %	15 %	18 %
Water	5.5 ml	7.9 ml	6.6 ml	4.6 ml	2.8 ml
A-B mix 30 %	1.3 ml	6.7 ml	8 ml	10 ml	12 ml
TRIS (0.5 M pH 6.8)	1 ml	-	-	-	-
TRIS (1.5 M pH 8.8)	-	5 ml	5 ml	5 ml	5 ml
SDS 10 %	80 µl	200 µl	200 µl	200 µl	200 µl
APS	80 µl	200 µl	200 µl	200 µl	200 µl
TEMED	10 µl	20 µl	20 µl	20 µl	20 µl

Table 2-13 Antibodies

Name	Description	IB	Confocal	Source
Anti-HA	Mouse monoclonal IgG1, epitope: YPYDVPDYA, 1 mg/ml, clone:16B12	1/1000-1/2000	1/500	BioLegend (Biosciences, CAT: 901514 or MMS-101R)
Anti-FLAG	Mouse monoclonal, epitope: DYKDDDDK, 2-5 mg/ml	1/2000	-	Sigma-Aldrich (CAT: F3165)
Anti-β-actin	Mouse monoclonal, epitope localised at N-terminus: DDDIAALVIDNGSGK, 2 mg/ml	1/10000	-	Sigma-Aldrich (CAT: A5316)
Anti-IκB	Mouse monoclonal	1/1000	-	Gift from R. Hay (Dundee, UK)
Anti-p65	Rabbit polyclonal IgG, epitope localised at C-terminus, 200 µg/ml	1/1000	1/500	Santa Cruz (CAT: sc-372)
Anti-pp65	Rabbit monoclonal, phosphorylation at Ser536	1/1000	-	Cell signalling (CAT: 3033)
Anti-VRK1	Mouse monoclonal IgG, against recombinant human VRK1, 200 µg/ml	1/1000	1/500	Santa Cruz (CAT: sc-101554)
Anti-pc-Jun	Rabbit polyclonal, phosphorylation at Ser63	1/1000	-	Cell Signalling (CAT: 9261)
Anti-GFP	Rabbit monoclonal, GFP, YFP and CFP	1/1000	-	Cell Signalling (CAT: 2956S)

Name	Description	IB	Confocal	Source
Anti-mouse-IR	Secondary antibody, goat, IRDye 680 conjugated, 1 mg/ml	1/10000	-	Li-Cor (CAT: 92668070)
Anti-rabbit-IR	Secondary antibody, goat, IRDye 800CW conjugated, 1 mg/ml	1/10000	-	Li-Cor (CAT: 92632211)
Anti-HA Alexa Fluor 594	Directly labeled with Alexa Fluor 594 fluorescent dye, polyclonal goat, epitope: CYPYDVPDYASL, 1 mg/ml	-	1/500	ThermoFisher Scientific (Biosciences, CAT: A-21288)
Anti-Flag Alexa Fluor 488	Directly labeled with Alexa Fluor 488 fluorescent dye, polyclonal rabbit, epitope: DYKDDDDK	-	1/500	Cell signalling (CAT: 5407S)
Anti-M Alexa Fluor 488	Anti-mouse, conjugated with Alexa Fluor 488 fluorescent dye, polyclonal goat, 2 mg/ml	-	1/1000	ThermoFisher Scientific (Biosciences, CAT: A11029)
Anti-R Alexa Fluor 647	Anti-rabbit conjugated with Alexa Fluor 647 fluorescent dye, polyclonal goat, 2 mg/ml	-	1/1000	ThermoFisher Scientific (Biosciences, CAT: A21245)

2.1.6. Other reagents

Table 2-14 Reagents used for immunoprecipitation, Type I IFN bioassay, ELISA and confocal microscopy (excluding antibodies)

Reagent	Description	Source
Anti-flag M2 affinity gel	Flag beads	Sigma-Aldrich (CAT: A2220)
Flag peptide	Elution of flag beads	Sigma-Aldrich (CAT: F3290)
MOWIOL-4-88	Mounting solution	Calbiochem (Merk Millipore, CAT: 475904)
DAPI (4',6-Diamidino-2-Phenylindole, Dilactate)	DNA stain to visualise nuclei	Invitrogen (ThermoFisher Scientific, CAT: D3571)
Quanti-Blue™	SEAP substrate for IFN type I bioassay	Invivogen (CAT: rep-qb1)
Human CXCL8/IL-8 DuoSet ELISA	DuoSet ELISA Development kit for detection of IL-8	R & D Systems (CAT: DY208)
BD OptEIA™ ELISA TMB substrate reagent set	ELISA substrate reagent	BD Biosciences (CAT: 555214)

2.2. Methods

2.2.1. Cloning

2.2.1.1. VACV DNA isolation

A monolayer of TK-143 cells (T-175 flask, Table 2-5) was infected with VV-WR (Table 2-6, at 0.1 plaque forming units (PFU)/cell in 5 ml MEM, 1.8 mM L-glutamine, 5 % (v/v) FCS, pen-strep), and incubated for 90 minutes (min) with rocking. Subsequently, the viral inoculum was replaced by fresh medium (Table 2-5, 2.5 % (v/v) FCS only). Cells were cultured until cytopathogenic effects were observed (2 days), then scraped into medium, and centrifuged at 800 g for 5 min at 4 °C. The cell pellet was washed with ice-cold isotonic buffer (Table 2-1) and again pelleted by centrifugation at 800 g for 5 min at 4 °C, before resuspension and incubation in 1 ml hypotonic buffer (Table 2-1) for 15 min at 4 °C. After lysing the cells by 25 strokes in a dounce homogeniser, cells were centrifuged at 2000 g for 5 min at 4 °C to pellet nuclei. Supernatants were transferred to new tubes, and the pellet was resuspended in hypotonic buffer and subsequently lysed using a dounce homogeniser. After centrifugation, supernatants were pooled, and sonicated for 1 min. Supernatants were layered onto a 36 % sucrose solution (Table 2-1) in an AH629 36 ml ultracentrifuge tube, and hypotonic solution was used to fill volume up to 32 ml. Virus cores in supernatants were sucrose purified by ultracentrifugation at 32775 g for 80 min at 4°C. The virus pellet was resuspended in 1 ml hypotonic buffer, vortexed, sonicated for 15 seconds (s), left on ice for several minutes, vortexed, then sonicated for another 15 s and stored at – 80 °C. For every 200 µl sucrose-purified virus, 3.75 µl β-mercaptoethanol, 50 µl 20 % sarcosyl salt solution, and 12.5 µl proteinase K (10 mg/ml) was added and incubated for 30 min on ice. The virus solution was incubated overnight at 55 °C, after addition of 350 µl 54 % sucrose solution. One volume of virus solution was added per volume of phenol (pH 8.0)-chloroform-isoamyl alcohol solution, mixed by pipetting and then centrifuged for 10 min at 9300 g and 4 °C. The upper aqueous phase, containing viral DNA, was transferred to a new tube and phenol-chloroform-isoamyl alcohol extraction was repeated. One volume of chloroform was added to the aqueous phase, mixed by pipetting and centrifuged for 10 min at 9300 g and 4 °C. Chloroform extraction was repeated on the aqueous phase. Viral DNA was then precipitated for several hours (h) at – 80 °C in 0.1 volume sodium acetate pH 7.0 and 3 volumes absolute ethanol. This precipitation was then centrifuged for 15 min at 16000 g and 4 °C, washed with 300 µl 70 % (v/v) ethanol, centrifuged for 5 min at 16000 g and 4 °C, and resuspended in 500 µl nuclease free water.

2.2.1.2. Generation of HEK 293T genomic DNA

HEK 293T cells were scraped in 1x PBS from 10 cm dishes in which they were grown, and RNA was extracted from them using High Pure RNA extraction kit (Table 2-2). Extracted RNA was reverse transcribed for 1 h at 37 °C in a total volume of 500 µl containing 100 µl M-MLV Buffer 5x, 100 µl diluted dNTPs, 25 µl random primers, 12.5 µl M-MLV reverse transcriptase, 12.5 µl RNase OUT, 5 µl RNA (1 µg/5 µl) (Table 2-2), and water.

2.2.1.3. PCR amplification

For plasmid construction, DNA fragments were amplified from either HEK 293T cDNA, VV-WR genomic DNA, or VV-WR expression vectors by polymerase chain reaction (PCR) using KOD DNA polymerase (Table 2-2). In a total volume of 50 μ l, 1 μ g DNA template was mixed with 5 μ l KOD Buffer 10 x, 5 μ l 2 mM dNTPs (final concentration 0.2 mM), 3 μ l 25 mM MgSO₄ (final concentration 1.5 mM), 1 μ l 10 μ M forward primer (final concentration 0.2 μ M), 1 μ l 10 μ M reverse primer (final concentration 0.2 μ M), 0.4 μ l 2.5 U/ μ l KOD DNA polymerase (final concentration 0.02 U/ μ l) and nuclease free water. PCR cycling conditions are illustrated in Figure 2-1. Table 2-3 gives an overview of primers used.

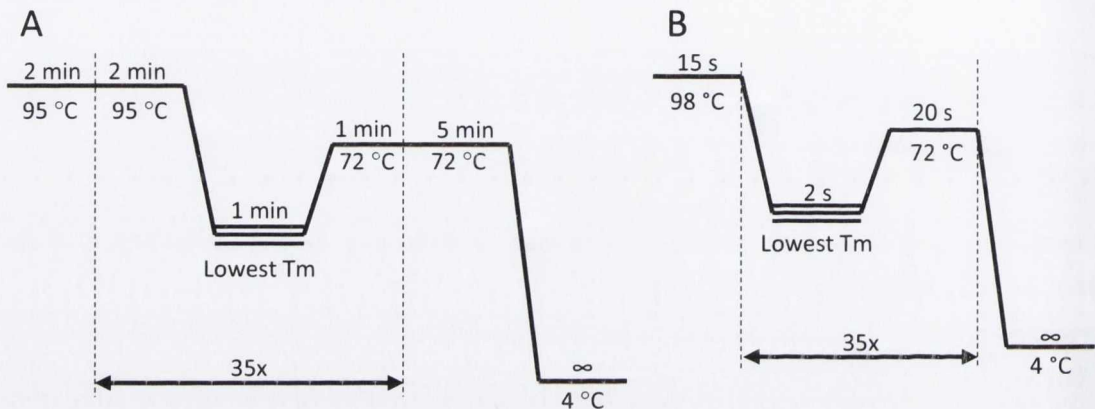


Figure 2-1 Thermal Cycling conditions

For amplification of nucleotide sequences originating from (A) VV-WR expression constructs or (B) viral or cellular genomic DNA. Heating to 95 – 98 °C ensures DNA denaturation, followed by a primer annealing step at lowest T_m of the desired primer pair (temperature dependent on the primer length and sequence). DNA synthesis was carried out at 72 °C and 1 min/kb was set up for the elongation step. Steps in between broken lines were repeated 35 times.

2.2.1.4. Restriction reaction

Large-scale restriction reactions performed on plasmids and amplicons were used to prepare vectors and inserts for plasmid construction. A reaction mixture of 20 or 50 μ l contained 1-5 μ g DNA, 0.5 to 1 μ l restriction enzyme, 2 or 5 μ l 10 x restriction enzyme buffer and nuclease-free water. Reactions were carried out overnight at 37 °C. Table 2-4 provides an overview of restriction enzymes used and correspondent conditions.

2.2.1.5. Agarose gel electrophoresis

Agarose gel electrophoresis was used for analytical purposes such as verification of plasmid linearisation, integrity of nucleic acids or correct fragment amplification, and to purify digested fragments for plasmid construction. Agarose gels were prepared thus: 1 % agarose powder, 1x TAE (Table 2-1) and 1 μ g/ml ethidium bromide. The Agarose GelExtract Mini kit (Table 2-2) was used to PCR purify amplicons and gel-purify digested vectors and inserts. To gel-purify digestion products, the entire reaction mix was run at 100 V in an agarose gel. Bands were visualised under low intensity UV light and excised using a sterile scalpel. DNA was purified according to manufacturer’s instructions and eluted in 25-50 μ l elution buffer.

2.2.1.6. T4 DNA ligase

Constructs were ligated in a final volume of 20 μ l by mixing 50 ng vector and 10-50 ng insert DNA with 2 μ l 10 x T4 DNA ligase buffer, 1 μ l T4 DNA ligase (Table 2-2) and water. Reactions were carried out at room temperature for 10 min.

2.2.1.7. Transformation

Novablue single competent cells (Table 2-2) were used for transformations. Twenty-five microliters of competent cells were mixed with 5 μ l of ligation reactions (or library plasmids if repreping it) for 30 min on ice. Cells were heat-shocked at 42 °C for 30 s and returned on ice for 5 min. Then, 100 μ l of LB (Table 2-2) medium were added and, cells were spread on LB-agar plates (Table 2-2) containing either 0.1 g/l ampicillin or 0.03 g/l kanamycin. Bacteria colonies were allowed to grow overnight at 37 °C.

2.2.1.8. Small and large scale preparations

Single colonies from transformation were transferred to tubes containing 5 ml LB with either 0.1 g/l ampicillin or 0.03 g/l kanamycin, and incubated overnight at 37 °C and 220 revolutions per minute (rpm). One millilitre of bacterial culture was used to extract plasmid DNA using the QIAprep Spin miniprep kit (Table 2-2), which was eluted in 50 μ l elution buffer.

For large-scale preparations, a small-scale bacterial culture was set up as described in the previous section. After 8 h of incubation, the bacterial culture was added to 200 ml LB liquid medium containing either 0.1 g/l ampicillin or 0.03 g/l kanamycin and incubated as described previously. Plasmid DNA was purified using the EndoFree Plasmid Maxi kit (Table 2-2), following protocols provided by the manufacturer.

2.2.1.9. Sequencing

Sequencing of expression plasmids was processed by Source Bioscience LifeScience sequencing service (Table 2-2). For sequencing reactions, 5 μ l of 100 ng/ μ l plasmid DNA and 5 μ l of 3.2 pmol/ μ l primer were provided. Sequencing results were analysed with Geneious version 6.1.5. (<http://www.geneious.com>, Kearse et al. 2012). A list of sequencing primers is given in Table 2-3.

2.2.2. Cell culture

2.2.2.1. Resuscitation of frozen cell lines

Cells, taken from liquid nitrogen stocks, were thawed at room temperature and resuspended in 25 ml growth medium (Table 2-5) in a T-175 flask. Cells were cultured at 37 °C with 5 % CO₂ until reaching 90 - 100 % confluency, when they were split. Cells from the second passage onwards were used for experiments.

2.2.2.2. Cell culturing

Cells were maintained in appropriate growth media as indicated in Table 2-5 at 37 °C and 5 % CO₂. Cells were split every 48 h at a 1:4 dilution, and at a dilution of 1:8 over the weekend. To do so, medium was removed,

cells were washed with approximately 10 ml of 1x PBS, and subsequently cells were incubated at 37 °C for 5 min with 5 ml of trypsin. When detachment of cells became visible, 10 ml of prewarmed medium was added and cells were transferred to a 50 ml falcon. Cells were centrifuged for 5 min at 200 g, and the cell pellet was resuspended in 8 ml prewarmed medium. A fraction of this cell suspension was added to 20 ml of prewarmed medium in a new T-175 flask according to desired dilution.

2.2.2.3. Freezing cell lines

Ninety to one hundred percent confluent cells were harvested as described in the preceding section. The cell pellet was resuspended in 10 % (v/v) DMSO containing FCS, and aliquoted into cryotubes. The cell suspension was frozen at – 80 °C and then transferred to liquid nitrogen storage.

2.2.2.4. Plaque assay

To determine PFU of VV-WR stock, virus was serially diluted in DMEM containing 2.5 % (v/v) FCS and Pen-Strep mix. Serial virus dilutions were used to infect duplicate wells of a 6-well plate containing confluent monolayers of BS-C-1 cells. After a 90 min incubation at 37 °C, with rocking, the viral inoculum was replaced by fresh medium containing 1.5 % CMC (Table 2-1). After 72 h, medium was removed, cells were washed with 1x PBS and stained for 2 h with 1 ml crystal violet (Table 2-1) at room temperature. Plates were washed with water, and then left to dry. Viral titres (expressed in PFU/ml) were determined based on number of plaques and dilution factor.

2.2.2.5. Transfections and stimulations

To transfect cells with reporter and/or expression constructs (Table 2-8), cells grown overnight in 96-well plates, 24-well plates, 6-well plates, or 10 cm dishes were transfected with 230 ng, 500 ng, 3000 ng and 8000 ng DNA using 0.56 µl, 2 µl, 6 µl, and 15 µl Genejuice® (Table 2-7) in 25 µl, 100 µl, 100 µl, and 235 µl serum free medium (SFM), respectively. After 5 min incubation at room temperature, the GeneJuice®-SFM mix was added to the DNA, incubated for 20 min, and then added in a dropwise manner to cells.

To stimulate cells grown in 96-well plates with 3'3'-cGAMP (Table 2-9), a mixture of 0.5 µl Lipofectamine®2000 (Table 2-7) and 25 µl SFM incubated for 5 min at room temperature, was added to 5 µg 3'3'-cGAMP. The mixture was incubated 20 min at room temperature and then added to cells.

Stimulations with TNFα, IL-1, IFNα_{2a}, SeV and PMA (Table 2-9) were carried out by directly adding these ligands to cells in the absence of any transfection reagent.

All experiments were performed over the course of 24 h, except for 24 h stimulations, which were preceded by a 24 h transfection. Furthermore, pMEP4-HA expression plasmid transfected HEK 293T cells (see 2.2.4) or siRNA transfected HEK 293T cells (see 2.2.3) were stimulated with TNFα over the course of 120 min.

2.2.3. siRNA protocols

HEK 293T and A549 cells were transfected with 100 nM siRNA (Table 2-10) in Lipofectamine®2000 (2 µl/well of 6-well plate, 0.25 µl/well of 96-well plate) and SFM (100 µl/well of 6-well plate, 8.5 µl/well of 96-well plate) following the transfection protocol described in the previous section, and then cell were seeded at a concentration of 1.2×10^6 cells/ml.

2.2.4. Generation of pMEP HEK 293T cell lines

HEK 293T cells were seeded at 1×10^6 cells in a 10 cm dish, and transfected 24 h later with 8 ng pMEP VV-WR expression construct (Table 2-8) as described in the previous section (2.2.2.5). Cells were selected 24 hours post transfection (hpt) in 300 µg/ml Hygromycin containing medium (Table 2-5), which was replaced every three days. After reaching confluency, cells were transferred to T75 and T175 flasks whilst maintaining them in a medium containing 300 µg/µl Hygromycin. Expression was tested by seeding cells at 1.2×10^6 cells/well in a 6-well plate and inducing these cells with 1 µM CdCl₂ solution. Cells were harvested 48 hours post induction (hpi) and analysed by Western blot (see 2.2.13).

2.2.5. VSV-GFP replication assay

A549 cells were transfected with siRNA, and seeded at 1×10^5 cells/ml in 6-well plates. Seventy-two hours later, viral dilutions of VSV-GFP (Table 2-6) were used to infect duplicate wells of a 6-well plate containing confluent monolayers A549 cells. After 90 min incubation at 37°C, with rocking, viral inoculum was replaced with fresh medium. GFP levels were analysed by microscopy and western blot (see 2.2.13) at 24 hpi.

2.2.6. Reporter gene assay

All reporter gene assays were performed in 96-well plates containing $2-3 \times 10^4$ HEK 293T cells/well, or 2×10^4 STING HEK 293T cells/well (Table 2-5). Cells were transfected with 60 ng/well reporter plasmid (pPK-IFNβ-luc, pPK-ISRE-luc, pPK-NF-κB-luc, pFA-CMV); 20 ng/well pRL-TK reporter gene as transfection efficiency control, for trans-activation assays; 3 ng/well IRF3-, IRF7 - Gal4 fusion protein or pFA2-Elk1, and a mixture of expression plasmids at desired concentrations (Table 2-8, Table 2-15). The total amount of transfected DNA for each experiment was kept constant at 230 ng by addition of pCMV-HA empty vector. Twenty-four hours post transfection, cells were either stimulated with ligands, or harvested. To harvest cells 24 hpt or 24 hours post stimulation (hps), medium was removed, and cells were lysed in 50 µl/well passive lysis buffer (Table 2-7) for 20 min at room temperature and 250 rpm. Samples were divided in two, and to 20 µl of cell lysate, either 40 µl of luciferase mix (Table 2-1) or 40 µl 1 µg/ml colentraine (Table 2-7) in PBS were added. Reporter gene activity was measured spectrophotometrically using the Luminoskan machine (Thermo) and Ascent Software v2.6. The reporter activity was normalized to activity of the renilla transfection efficiency control.

Table 2-15 Amounts of innate immune signalling component expression constructs used to activate reporter genes

Reporter gene	Activating signalling component expression construct
pFA2-Elk-1	25-50 ng RAS
pPK-IFN β -luc/ pPK-ISRE-luc	5 ng cGAS and 10 ng STING
	10 ng TRIF
	50 ng TBK1
pPK-ISRE-luc	50 ng MAVS
	20 ng IRF3 5D
	25 ng IRF7
	10 ng IRF7 4D
Gal4-IRF3/7	50 ng TBK1
	50 ng IKK ϵ
pPK-NF- κ B-luc	5 ng cGAS and 10 ng STING
	25 ng MAVS
	10 ng TRIF
	50 ng TRAF2
	50 ng TRAF6
	50 ng IKK α
	50 ng IKK β
	50 ng IKK ϵ
pCDNA3-p53	2.5 ng -10 ng p65
	10 ng p53

2.2.7. ELISA

HEK 293T cells containing CdCl₂ inducible pMEP4-HA expression constructs were seeded in 96-well plates at a concentration of 1.2 x 10⁶ cells/well in presence or absence of 1 μ m CdCl₂. Forty-eight hours later, cells were stimulated with 5 – 20 ng/ml IL-1 for 12 h – 24 h (Table 2-9). IL-8 in supernatants was detected using the Human CXCL8/IL-8 DuoSet ELISA kit (Table 2-14), following manufacturer’s instructions. ELISAs were performed in 96-well plates and reagent volumes (capture antibody, blocking buffer, samples, detection antibody, Streptavidine solution and substrate solution, Table 2-1) were set to 50 μ l. The reaction was stopped after 5 – 10 min by adding 25 μ l stop solution (Table 2-1), and the plate was read using a Multiskan™ FC Microplate Photometer (ThermoFisher Scientific) at absorbance of 450 nm and 540 nm. Data was analysed using Thermo Scientific™ SkanIt™ Software 2.5.1.

2.2.8. Type I IFN bioassay

HEK 293T cells containing CdCl₂ inducible pMEP4-HA expression constructs were seeded in 96-well plates as described above. HEK 293T cells were transfected with siRNA as described above. Forty-eight hours later, cells were transfected with 5 ng TRIF or 25 ng MAVS expression construct (Table 2-8), and supernatants were

harvested 24 hpt. HEK-Blue™ IFN α / β cells (Table 2-5) were seeded in a 96-well plate at a concentration of 3×10^5 cells/ml onto 50 μ l of supernatants in test medium and incubated for 24 h. To determine type I IFN levels, cells were seeded onto 50 μ l of serial dilutions of human recombinant IFN α_{2a} protein (Table 2-9). Then supernatants were harvested from HEK-Blue™ cells, and in a 96-well plate, 50 μ l of supernatant were incubated with 150 μ l QUANTI-blue™ (Table 2-14) at 37°C for 5-20 min. Plate was read using a Multiskan™ FC Microplate Photometer (ThermoFisher Scientific) at absorbance of 450 nm and 540 nm. Data was analysed using Thermo Scientific™ SkanIt™ Software 2.5.1.

2.2.9. TNF α stimulations

HEK 293T cells containing CdCl₂ inducible pMEP4-HA expression constructs were seeded in 6-well plates as described above (see 2.2.4). Alternatively, HEK 293T cells were transfected with siRNA as described above. At forty-eight hours post induction or transfection, cells were stimulated with 50 ng/ml TNF α (Table 2-9) at 0, 5, 15, 30, 60 and 120 min. Cells were then harvested and protein content was analysed by Western blot (see 2.2.13).

2.2.10. Mass spectrometry

Two T175 flasks of pMEP4-HA VV-WR HEK 293T cells were induced with 1 μ M CdCl₂ solution when cells reached 80 % confluency. To harvest cells 24 hpi, cells were washed with ice cold PBS (Table 2-1), scraped into 10 ml of PBS, pooled, and centrifuged for 5 min at 200 g and 4 °C. The cell pellet was washed with ice-cold PBS, excess supernatant was removed until 1 ml remained, into which the cells were resuspended. The cell suspension was transferred to an eppendorf, centrifuged for 5 min at 850 g and 4 °C. The supernatant was removed and the cell pellet was then snapfrozen in liquid nitrogen. Before centrifugation, 30 μ l of cell suspension were taken to test successful induction of plasmid expression in samples by Western blot (see 2.2.13). Snapfrozen cell pellets were analysed by immunoprecipitation and mass spectrometry by our collaborators Dr. Andreas Pichlmair and Dr. Darya Haas (Max Planck Institute of Biochemistry, Germany).

2.2.11. Confocal microscopy

HEK 293T cells seeded at 0.5×10^5 cells/well on glass coverslips in 24-well plates were grown overnight and were then transfected with HA- or flag-tagged expression plasmids (Table 2-8). Cells were washed 24 hpt with ice cold PBS and fixed for 20 min on ice in 500 μ l paraformaldehyde solution (Table 2-1). To evaluate nuclear p65 translocation, cells were stimulated for 20 min with 50 ng/ml TNF α (Table 2-9) prior to fixation. After a further washing step, cells were incubated for 20 min at room temperature and 100 rpm in blocking and lysis buffer (Table 2-1). Cells were then washed in PBS, and incubated at room temperature and 100 rpm shaking in darkness, either for 2-3 h in primary antibody solution (anti-HA antibody or anti-p65 (Table 2-13)) in washing buffer (Table 2-1) containing 3 % (w/v) BSA, or for 4-5 h in fluorescently labelled antibody solution (anti-HA alexa or anti-flag alexa (Table 2-13) in washing buffer containing 3 % (w/v) BSA). Next, cells previously incubated in primary antibody solution, were washed in PBS, and incubated in fluorescently labelled secondary antibody solution (anti-M alexa 488 antibody or anti-R alexa 647 antibody (Table 2-13) in washing

buffer containing 3 % (w/v) BSA) for 1-2h at room temperature and 100 rpm in darkness. After three PBS washes, cell were mounted with MOWIOL 4-88 (Table 2-14) containing 1 µg/ml DAPI (Table 2-14). Images were taken with the Olympus FV1000 scanning confocal microscope or the confocal laser-scanning microscope Leica TCS SP8. Data were analysed using Olympus FV10-ASW 2.0 Viewer Software and LAS Leica Application Suite 2.6.3. build 8173 Software. Confocal images were generated using Imaris 7.6.3. Software. To determine nuclear translocation of p65, cells were counted using ImageJ 1.50e software. Cell number was determined in reference to DAPI stain. Nuclear p65 levels were measured in all cells and a threshold was set by quantifying the mean levels of nuclear p65 levels in unstimulated cells.

2.2.12. Immunoprecipitations

HEK 293T cells seeded at 1.5×10^6 cells in 10 cm dishes were grown overnight and were transfected with 8 µg expression constructs the next day. Twenty-four hours post transfection, cells were washed in ice cold PBS and lysed in 400 µl lysis buffer (Table 2-1) containing 1 mM phenylmethanesulfonylfluoride, 1 mM sodium vanadate, 1 ng/ml Leupeptin and 0.5 % (v/v) aprotinin for 15 min on ice. Cell lysates were centrifuged for 15 min at 16000 g and 4 °C to pellet cell debris, and supernatants were used for coimmunoprecipitations. One hundred microliters of supernatant were used for verification of protein expression in lysates by western blot analysis. Before immunoprecipitations were performed, anti-flag M2 beads (Table 2-14) were washed three times by resuspension in 1 ml lysis buffer and 10 s centrifugation at 370 g and 4 °C. For immunoprecipitation, a solution of 10 µl anti-flag M2 beads and 10 µl lysis buffer was incubated rolling overnight at 4 °C with 400 µl cell lysates. Next, beads were washed three times as previously described, and incubated rotating for 30 min at 4 °C in 80 µl flag peptide solution (0.15 µg/µl Flag peptide in 1x TBS (Table 2-1, Table 2-14)). Beads were centrifuged for 10 s at 16000 g and 4 °C, supernatants were transferred to new tubes, and 10 µl sample buffer containing DTT was added to supernatants. Protein contents of cell lysates and immunoprecipitations were analyses by western blot (see 2.2.13).

2.2.13. Western blot

Cells grown in 6-well plates (e.g. for expression verification, TNFα stimulation) were harvested in 70 – 100 µl sample buffer (Table 2-1) containing 50 mM DTT and 1 µl/ml benzonase (Table 2-11). The 30 µl sample taken from mass spectrometry analysis (see 2.2.10) was lysed in 100 µl sample buffer containing 50 mM DTT and 1 µl/ml benzonase (Table 2-11). To verify protein expression in immunoprecipitation experiments (see 2.2.12) 80-100 µl samples were taken from lysates and were mixed with 20 µl sample buffer containing 50 µl/ml DTT. To separate proteins, 10 - 18 % acrylamide resolving gels and 5 % acrylamide stacking gels (Table 2-12) were cast. Samples in sample buffer were then heated for 5 min at 99 °C, and 5 – 30 µl were loaded into wells. The electrophoresis was carried out at 100 V in 1x running buffer (Table 2-1). Proteins were transferred in 1x transfer buffer (Table 2-1) to a nitrocellulose or PVDF membrane (Table 2-11) by either, semi-dry blotting at 100 mA/membrane for 1 h using the BIOMETRA system, or wet blotting at 100 W for 90 min using the Bio-Rad system. After a blocking step in washing solution containing 3 % (w/v) BSA for 1 h at RT, the membrane was incubated overnight at 4 °C with desired primary antibody solution (Table 2-13). The membrane was

washed for 5 min three times in washing solution and incubated for 1 h at RT with secondary antibody solution (anti-mouse or anti-rabbit, Table 2-13). The membrane was washed three times with washing solution. Protein bands were detected by fluorescence using the Li-Cor Odyssey scanner. Data was analysed using Odyssey 3.0.16 Software.

2.2.14. Statistical analysis

Graphs were generated following transfer of data into GraphPad Prism v.5.02 software. Statistical analysis was performed using two-sample, two-tailed student *t* tests on at least three independent experiments (biological replicates) with triplicate determination (technical replicates).

2.2.15. Bioinformatical analysis

Nucleotide and amino acid sequence alignments were generated using Geneious 6.1.5. Software.

Chapter 3 : Results I – screen of *Vaccinia virus* proteins for innate immune signalling inhibitors

3.1. Introduction

The search for DNA sensors signalling via STING has led to the discovery of several candidates such as IFI16, and most recently cGAS (see 1.2.2.3.3,(Dempsey & Bowie 2015)). Whilst both have been shown to activate IRF3 through the STING-TBK1 axis (Tanaka & Chen 2012; Liu, Cai, et al. 2015), the mechanism by which this pathway activates NFκB is less clear and requires further investigation (Ishikawa & Barber 2008; Abe & Barber 2014). A growing body of evidence suggests that cGAS, IFI16 and STING are important in the antiviral response to HIV (Jakobsen et al. 2013; Monroe et al. 2014), and play an aberrant role in chronic autoimmune diseases such as systemic lupus erythematosus, systemic fibrosis, rheumatoid arthritis, Aicardi-Goutières syndrome, and STING-associated vasculopathy with onset in infancy (Gall et al. 2012; Connolly & Bowie 2014; Jeremiah et al. 2014; Liu et al. 2014; Gao et al. 2015; Gray et al. 2015). Knockout mouse infection models confirmed the role of cGAS and STING in protection against viral pathogens such as HSV-1 and VACV (Ishikawa et al. 2009; Li et al. 2013; Schoggins et al. 2014). Studies in cells treated with siRNA or small hairpin RNA (shRNA) against IFI16 have indicated similar functions for IFI16 (Conrady et al. 2012; Gariano et al. 2012; Lo Cigno et al. 2015). Various members of the *Herpesviridae*, the *Flaviviridae* and the *Coronaviridae* families have been reported to antagonise cGAS, IFI16 and/or STING function, supporting a role for these host proteins in innate immune signalling against viruses (Ma & Damania 2016).

Poxviruses encode a large number of proteins involved in immune evasion and subversion, and study of these immunomodulators has led to a better understanding of not only the virus, but also the innate immune signalling they subvert (see 1.3, (Smith et al. 2013)). For example, study of *Vaccinia virus* protein K7 led to the discovery of DDX3, and the involvement of this cellular helicase in IRF3 activation by TBK1 (Schröder et al. 2008). Yet, to date no poxviral inhibitors of IFI16, cGAS or STING have been reported. We therefore decided to screen intracellular VACV proteins for their ability to inhibit cGAS- mediated interferon production.

3.2. Results

3.2.1. Selection process

The genome of *Vaccinia virus* strain *Western Reserve* (VV-WR) contains 223 genes according to NCBI (AY243312), of which 42 open reading frames (ORF) were previously selected by former group member Dr. Unterholzner for investigation of their immune inhibitory potential (Unterholzner et al. 2011). The remaining 181 were excluded through several criteria based on assessing probability of immunomodulatory function: Location within the central genomic region, as this area mainly contains conserved well-characterised house-keeping genes less likely to possess immunomodulatory traits (Lefkowitz et al. 2006); published secreted proteins, as the objective was to discover novel inhibitors of intracellular PRR pathways; and a size limitation was set to a minimum molecular weight of 8 kDa to ensure detection by standard molecular biology techniques. Proteins with well characterised, unrelated functions and duplicates of repeated ORFs with nearly 100 % sequence identity were also excluded. A literature search was carried out on the 42 remaining VV-WR candidates as well as a protein based basic local alignment search tool (BLAST) search for identification of homologies and similarities to other microbial or eukaryotic proteins. The results are summarised in Table 3-1 and will be described in further detail for proteins of interest (i.e. A47, F10, B1, B12, F17, B13) in the remainder of this chapter (**Error! Reference source not found.**).

3.2.2. Summary of background search

The result of this present analysis of the 42 previously selected ORFs was the identification of:

- 8 completely uncharacterised proteins (VV-WR 003, F6, F7, F14, F15, B11, B17, C14);
- 17 viral candidates with predicted motifs, with no established functions (C20, C19, VV-WR 014, VV-WR 015, C9, C8, C5, C1, M1, K5, A57, B2, B6, B10, VV-WR 203, VV-WR 204, VV-WR 208);
- 11 poorly or partially characterised proteins, which still lack a defined mechanism of action (C7, F3, F5, F16, F17, A47, A51, A55, B3, B4, B12);
- 5 VV-WR ORFs encoding proteins involved in other viral or cellular processes (F10, F11, E2, B1, B13),
- 1 established inhibitor of the IRF3 signalling cascade (N2)

After completion of the reporter-based screens, a study of VACV protein N2 revealed its ability to block type I IFN expression in the nucleus after phosphorylation and nuclear translocation of IRF3 (Ferguson et al. 2013). Also, a VACV translome study was published, which detailed the timing and abundance of VACV gene expression. This study reported that the following proteins were not detected during infection: VV-WR 003, C20, VV-WR 014, VV-WR 015, B3, and VV-WR 204 (Yang et al. 2015).

3.2.2.1. A57 – predicted guanylate kinase-like domain

Guanylate kinases catalyse the ATP-dependent phosphorylation of GMP (Fitzgibbon et al. 1996; Zhang et al. 2015). In the context of the recent discovery of the DNA sensor cGAS, which synthesises the secondary messenger 2'3'-cGAMP in response to DNA (Sun et al. 2013), we postulated that this protein may play a role in deregulating nucleotide levels to interfere with signal transduction. A57 shares 45 % amino acid (a.a.) sequence identity with the human guanylate kinase (GUK1, Uniprot ID: Q16774), although it lacks this protein's N-terminal domain (data not shown). The N-terminal region of GUK contains an ATP-binding site as well as four of the eight residues required for GMP binding (Zhang et al. 2015). Despite lacking the ATP-binding site, A57 still harbours the remaining four residues required for GMP binding. It appears improbable that A57 acts as a kinase able to modulate nucleotide levels, however it may have the ability to sequester GMP in the cytosol or interact directly with a guanylate kinase.

3.2.2.2. Serine/Threonine kinases

Vaccinia virus encodes three proteins with predicted Ser/Thr kinase motifs, namely F10, B1 and B12 (Traktman et al. 1989; Howard & Smith 1989; Lin & Broyles 1994). Whilst viral and cellular substrates have been identified for F10 and B1 (Banham et al. 1993; Betakova et al. 1999; Brown et al. 2000; Doglio et al. 2002; Szajner et al. 2004b; Nichols et al. 2006; Santos et al. 2006), it remains unclear whether B12 can function as a kinase (Howard & Smith 1989; Banham & Smith 1993).

Viral proteins required for virion membrane formation are phosphorylated by late expressed F10, which is packaged into nascent virions (Betakova et al. 1999; Derrien et al. 1999; Doglio et al. 2002; Szajner et al. 2004b). B1 is an early expressed essential kinase, which is packaged into virions and has multiple roles (Banham & Smith 1992; Rempel & Traktman 1992; Lin et al. 1992). It is required for viral DNA replication (Rempel & Traktman 1992), transcription and virion formation (Kovacs et al. 2001; Jamin et al. 2015), as well as for antagonism of viral DNA sequestration by the host protein barrier-to-autointegration factor (BAF) (Nichols et al. 2006), for activation of the c-Jun signalling pathway (Santos et al. 2006), and for impairment of p53 activity (Santos et al. 2004). B1 and B12 share an approximately 40 % (B12: 30 %) a.a. sequence identity with the human protein vaccinia-related kinase 1 (VRK1), which was named after B1 (Nezu et al. 1997). Although B1 phosphorylates targets of VRK1 (e.g. BAF, c-Jun and p53), no direct interaction between the two proteins has been reported (Lopez-Borges & Lazo 2000; Sevilla, Santos, Barcia, et al. 2004; Nichols et al. 2006). Furthermore, VRK1 is predominantly a nuclear kinase whereas B1 is a cytoplasmic protein localised to viral factories (Banham & Smith 1992; Lopez-Borges & Lazo 2000). It appears unlikely that B12 functions as an active kinase, as it lacks a recognisable ATP-binding site and key residues identified in other kinases (Howard & Smith 1989; Banham & Smith 1993). Furthermore, it has been shown that, in contrast to F10 and B1, B12 was unable to phosphorylate casein and histones *in vitro* (Banham & Smith 1993). Deletion mutant viruses lacking B12 did not display any disadvantage compared to wild type *Vaccinia virus in vitro* or *in vivo* (Banham & Smith 1993; Tschärke et al. 2002), however a functional complementation by its viral homologue B1 cannot be excluded.

3.2.2.3. Proteins packaged into virions

The VACV proteins F10, B1 and F17 are packaged into newly generated virions, which may be explained by their role in virion assembly and/or in cell entry (Zhang & Moss 1991; Lin et al. 1992; Banham & Smith 1992; Derrien et al. 1999). Once the core enters the cell, innate immune responses need to be immediately downregulated to allow for transcription of viral genes and DNA replication (see 1.1.7). *Vaccinia virus* protein F17 (also called F18 or VP11) is expressed late during infection and appears to be required for virion formation (Wittek et al. 1984; Zhang & Moss 1991). It constitutes the majority (approximately 70 %) of the lateral body mass within the nascent viral particle (Schmidt et al. 2013), which could indicate a role very early in infection before onset of early gene transcription. F17 is localised in the cytoplasm at viral factories and appears to form oligomers (Kao et al. 1981; Reckmann et al. 1997). Phosphorylation appears to regulate F17 involvement in viral protein processing prior to virion assembly and it was shown *in vitro* that, in contrast to F10, cellular kinases such as ERK, JNK and CDK1 can phosphorylate F17 (Wickramasekera & Traktman 2010). This process is reversed by the VACV dual-specificity phosphatase H1 (Liu et al. 1995). F17 has been reported to bind superhelical DNA (Kao et al. 1981), and actin (Reckmann et al. 1997), yet these associations may be due to the basic nature of F17, as no functional consequences of these activities have been demonstrated.

3.2.2.4. B13 – caspase inhibitor

The serine protease inhibitor B13, (also known as SPI-2), shares a 92 % a.a. homology with the *Cowpox virus* protein CrmA (Smith et al. 1989). B13 is an early expressed non-essential gene, which prevents Poly (adenosine diphosphate ribose) polymerase (PARP)-cleavage, pro-IL-1 β processing, and caspase-3 and -7 activity (Kettle et al. 1995; Kettle et al. 1997; Veyer et al. 2014). Despite its established anti-apoptotic effect (Dobbelstein & Shenk 1996; Kettle et al. 1997; Li & Beg 2000; Veyer et al. 2014), no other function related to innate immune signalling has been reported. Poxvirus proteins often have more than one function: For example, the potential apoptosis inhibitors N1 and C7 have been shown to also prevent NF κ B, and IRF activity, respectively (DiPerna et al. 2004; Nájera et al. 2006; Cooray et al. 2007; Meng et al. 2012). No differences *in vitro* or in intranasally infected mice were observed when comparing deletion mutant viruses lacking B13 to wild type VACV (Kettle et al. 1995). However, intradermally infected mice displayed larger lesions at the site of infection if B13 was deleted from the virus genome (Tscharke et al. 2002).

The present protein BLAST search and literature analysis revealed new aspects of the selected VV-WR ORFs, which may help infer a mechanism of inhibition for these viral proteins. The data on N2, VV-WR 003, C20, VV-WR 014, VV-WR 015, B3, and VV-WR 204 were published after commencement of the current project (Ferguson et al. 2013; Yang et al. 2015), and these proteins were thus excluded at a later point in time. In order to rapidly identify putative inhibitors of cGAS-STING signalling pathways, RGAs were used to screen selected viral proteins for their inhibitory potential.

3.2.3. Assembly of VACV protein expression constructs

VV-WR expression constructs were previously generated by Dr. Unterholzner by amplifying individual viral ORFs from VV-WR genomic DNA and inserting amplicons into the pCMV-HA expression vector. Constructs were freshly prepped for the present study, verified for encoding correct VV-WR sequences and five expression constructs had to be generated *de novo*. Insert bands of the expected size were obtained after double digestion of constructs, and PCR amplified ORFs were verified by sequencing (Figure 3-2A, data not shown). After repeated unsuccessful attempts at amplification, C7L and VV-WR 208 were excluded from the study, which reduced the number of viral candidates to 40. Sequencing revealed 1 silent mutation in the F17 expression construct and one coding mutation in each of the following expression plasmids: pCMV-HA-K2, pCMV-HA-A57 and pCMV-HA B11. A new expression plasmid pCMV-HA-A57 was thus generated, however pCMV-HA-K2 and B11 were excluded from this study due to the lack of expression in HEK 293T cells (see below).

HEK 293T cells were then transfected with 3 µg VV-WR expression plasmid to verify expression (Figure 3-2B). Expression of HA-tagged VV-WR proteins could only be detected for 17 of the 40 tested expression constructs even after repeated attempts with concentration of cellular material for weakly expressed proteins. Failure to detect these proteins may be due to a variety of reasons including very low expression levels, alternative splicing, post-transcriptional modifications that altered the HA-tag, or instability resulting from individual expression or addition of an HA-tag.

During the ORF assembly phase of this study the number of viral candidates to be screened was significantly reduced due to failure to generate the expression plasmids pCMV-HA C7 and VV-WR 208, and due to the lack of detection of expression of pCMV-HA C20, VV-WR 014, VV-WR 015, C8, C5, C1, K5, F3, F5, F6, F7, F10, F11, F14, F15, E2, A51, A55, B4, B11, B17, VV-WR 203 and C14. Only the following 17 VV-WR proteins were screened as potential inhibitors of cGAS-mediated type I IFN induction: VV-WR 003, C19, C9, N2, M1, F16, F17, A47, A57, B1, B2, B3, B6, B10, B12, B13 and VV-WR 204.

3.2.4. Screen of VACV inhibitors of IRF and NFκB pathways

The potential importance of the recently discovered cGAS as a poxviral DNA sensor initially led us to investigate the VACV genome for potential inhibitors of the cGAS signalling pathway, which signals through STING (Ishikawa & Barber 2008; Sun et al. 2013). Additional innate immunity signalling pathways, such as TLRs and RLRs, were included due to their importance in sensing poxviral nucleic acids (Smith et al. 2013; Brady & Bowie 2014). The screening process consisted of a series of reporter gene assays carried out in HEK 293T cells. A firefly luciferase reporter plasmid under the control of either an IFNβ-promoter, an ISRE-element, or NFκB-elements was activated by overexpression of adaptor proteins. The inhibitory potential of the 17 previously selected VV-WR proteins was evaluated by co-transfection of selected ORFs with RGA constructs. To facilitate the selection of the most likely viral inhibitors resulting from the initial screening process, a 50 % inhibition threshold criterion was applied, with viral candidates eliminated if promoter activation was not reduced by at least 50 % in the presence of the VV-WR protein. This threshold value was

based on a power calculation to determine the minimum detectable difference in the present experimental settings ($\alpha=0.05$, $\beta=0.1$, $N=3$). The obtained difference was 52 % for a two-tailed student's t-test.

3.2.4.1. Inhibition of cGAS/STING-driven IFN β and NF κ B

The endogenous expression of cGAS and STING is negligible in HEK 293T cells (Burdette et al. 2011; Sun et al. 2013; Orzalli et al. 2015). Neither STING nor cGAS activated an IFN β -promoter or NF κ B-dependent reporter gene, when individually transfected at suboptimal concentrations into 293T cells (see Appendix 1 for activation levels of all RGAs presented in this study). In contrast, co-transfection of cGAS and STING resulted in activation of both reporters (Appendix 1C,Q). cGAS generates cGAMP, presumably in response to transfected expression plasmid DNA (Orzalli et al. 2015), and thereby activates STING and downstream signalling processes.

Seven of the seventeen screened VV-WR proteins demonstrated significant impairment (>50 % reduction of activation levels) of cGAS and STING-driven IFN β promoter- and/or NF κ B-dependent reporter gene activity: N2, F17, A47, A57, B2, B12, and VV-WR 204 (Figure 3-3A,B). It has to be noted that parts of this screen were initially carried out only in duplicate and replication of the screen at a later time introduced large variation to the inhibition levels exerted by certain VACV proteins. Notably, upon re-screening B13 and M1 no longer passed the threshold value for IFN β and NF κ B, respectively, for which they were initially chosen for more detailed characterisation. Inhibition levels might be dependent on the activation levels of individual reporter plasmids. Therefore it might be possible that varying activation levels caused the variation in inhibition levels, however counterexamples are presented in this study (see 4.2.1.)

N2, (initially) B13, and VV-WR 204 seemed to target only IFN β -promoter activation (Figure 3-3A), whereas (initially) M1, and A47 appeared to selectively impair NF κ B activation (Figure 3-3B). The proteins F17, A57, B2 and B12 inhibited both pathways, which suggests either a strong inhibition of NF κ B activation resulting in reduced IFN β -gene reporter activation, inhibition proximal to cGAS or STING, or impairment of one or more host proteins involved in signal transduction to IRFs and NF κ B. Remarkably, B2 inhibited both pathways by at least 88 %. After B2, the second and third best inhibition of both pathways was detected for B12 and A57, respectively. To ensure that the observed impairment was not due to a general cytotoxic effect, an alternative independent pathway, namely MAPK/ERK activation, was tested as a control (Figure 3-3C). This pathway was analysed using an ELK-Gal4 fusion protein, which was activated by overexpression of the upstream signalling molecule RAS. Active, phosphorylated ELK-Gal4 fusion proteins then transactivated a firefly luciferase reporter under the control of Gal4-binding elements. None of the viral candidates reduced RAS-driven ELK transactivation, and therefore it was determined that the observed inhibition was due to specific inhibition of PRR activation.

3.2.4.2. Inhibition of cGAS/STING, TRIF and MAVS-mediated ISRE and NFκB activation

We next investigated the specificity of inhibition regarding the receptor type and regarding the activated transcription factors. Given the amount of previously discovered VACV inhibitors that inhibit TLR and RLR signalling pathways (Smith et al. 2013; Brady & Bowie 2014), inhibition of these pathways was also assessed. Furthermore, a comparison was drawn between impairment of ISRE and NFκB activation to decipher the aforementioned dual inhibition of IFNβ-promoter- and NFκB-element-dependent reporter activity. The latter is under the control of a series of NFκB response elements whereas the IFNβ-promoter contains an IRF-binding motif in addition to the NFκB response element. IRFs are also able to bind to ISRE elements to induce gene expression. Hence, the effect on ISRE-element activation was evaluated to determine whether ORFs that block both IFNβ-promoter and NFκB did so due to NFκB pathway inhibition alone.

The nine potential IFNβ-promoter and/or NFκB inhibitors found during the cGAS/STING screen were evaluated according to their ability to reduce ISRE- and NFκB-mediated reporter gene activation by overexpression of either cGAS and STING, TRIF (for TLR pathway activation), or MAVS (for RLR pathway activation) (Figure 3-4A,B). None of the studied VACV proteins, with the exception of M1, demonstrated inhibition exclusively restricted to either NFκB or ISRE. Interestingly, B12 was able to efficiently impair cGAS/STING-, TRIF- and MAVS-mediated NFκB activation to nearly identical extents, yet was only able to inhibit MAVS-driven, and not cGAS/STING-, or TRIF-driven ISRE activation. The same “inhibition pattern” as B12 became apparent for F17, A47 and A57. The observed inhibitory effect on ISRE activation may contribute to the inhibition of IFNβ-dependent reporter activation detected for F17, A57 and B12 in the cGAS/STING screen. Hence, these proteins could not be categorised as NFκB specific inhibitors, suggesting the inhibition of a downstream target involved in signal transduction to IRF3 and NFκB, or simultaneous inhibition of different host targets. A47 did not appear to be a specific NFκB inhibitor, as indicated in the cGAS/STING screen and given that MAVS-mediated ISRE activation was reduced by approximately 70 %. Generally, no ISRE specific inhibitors were found as all viral candidates impaired NFκB activation through at least one of the tested pathways (i.e. cGAS, TRIF, MAVS). This result is consistent with the larger number of known NFκB-specific poxviral inhibitors compared to IRF-specific immunomodulators (Smith et al. 2013). However, a technical explanation for the discovery of these NFκB inhibitors might be the greater activation of reporter plasmids dependent on the IFNβ-promoter and ISRE-elements compared to NFκB elements (see Appendix 1C-H, Q-S). A more potent inhibition might be the result of a lower activation of the reporter which might facilitate inhibition.

The protein M1 appeared to target the NFκB pathway specifically impairing activation mediated by MAVS and (prior to this study) cGAS/STING, supporting the observation that it was unable to inhibit IFNβ-promoter activation. Consistent with the previous screen, the VV-WR protein B2 was clearly the most potent inhibitor of ISRE- and NFκB activation. Furthermore, B2 was the only protein able to restrict ISRE activation stimulated by cGAS/STING, TRIF as well as MAVS overexpression. The second best inhibitor was B12, with A47 and A57 displaying similar potency in third position.

3.3. Discussion

To identify and characterise novel VACV encoded cGAS/STING signalling pathway inhibitors, 42 viral ORFs were selected from the VV-WR genome to be subjected to a screen to assess the potency of inhibition. After the assembly phase of VV-WR protein expression plasmids, 17 viral candidates remained to be screened in a series of reporter gene assays. Eight viral proteins were confirmed as strong inhibitors of cGAS/STING-, TRIF- and/or MAVS-driven IFN β -promoter-, ISRE-, and NF κ B-element-dependent reporter activation (Table 3-2). The absence of inhibition specificity regarding the type of PRR signalling pathway indicates these immunomodulators inhibit downstream at activation proximal events or at multiple points on the signalling pathways. The aim of the present study was therefore broadened to the identification of PRR signalling pathway and pro-inflammatory cytokine inhibitors that use overlapping downstream components.

3.3.1. General aspects of the inhibitor candidate ranking

In order to determine the best immunomodulators for further study, the data of each screen were integrated, and ranked according to the following criteria, which are given in order of priority (Table 3-2):

- (1) Inhibitory potential: A threshold of 50 % inhibition was set to identify the most promising viral candidates for characterisation in more detail. Viral proteins failing to impair at least one promoter activation mediated by the different PRR signalling pathways (i.e. cGAS/STING, TRIF or MAVS) were excluded from further screening. We considered the possibility that variation in expression levels could account for differences in inhibition potency. However, B3, B6 and B10 demonstrated good expression in HEK 293T cells (Figure 3-2), but failed to efficiently restrict IFN β - and NF κ B-dependent activation (Figure 3-3A,B). Nonetheless, this explanation cannot be neglected in some cases. Table 3-2 lists the IFN β -specific, ISRE-specific and NF κ B-specific inhibition ranks. The difference in total number of inhibitors for each rank is accounted for by the discovery of M1 as an NF κ B-specific inhibitor and the observation that VV-WR A47 was able to impair MAVS-mediated ISRE activation, yet not cGAS/STING-driven IFN β activation (Figure 3-3A, Figure 3-4A).
- (2) Predicted motifs and functions: Despite being a less important factor than inhibitory potential, this criteria could modify the overall rank of a viral candidate, if the data were compelling. For example, the shared amino acid sequence identity of B12 with the host protein VRK1 appears intriguing (see 3.3.2.2). In contrast, the potential lack of expression of VV-WR 204 gave it a lower ranking (Yang et al. 2015). Another noteworthy case is the protein N2, which was recently demonstrated to be an IFN β inhibitor (Ferguson et al. 2013), confirming our observations, and was therefore excluded from the present screen.
- (3) The presence of the ORF in the VACV Copenhagen strain vv811ΔA49: Previous investigations identified ten non-redundant VV-WR NF κ B inhibitors (Bowie et al. 2000; DiPerna et al. 2004; Chen et al. 2008; Hinthong et al. 2008; Schröder et al. 2008; Myskiw et al. 2009; Willis et al. 2011; Ember et al. 2012; Stack & Bowie 2012; Mansur et al. 2013). Surprisingly, the present study revealed an

additional eight. While it is currently unclear why VACV requires such a large number of intracellular NFκB inhibitors, the presence of at least one additional inhibitor was postulated in a study on an A49 deletion mutant of the VACV Copenhagen strain vv811 (Sumner et al. 2014), which lacks all published NFκB pathway inhibitors. Although this virus was unable to prevent nuclear translocation of p65 in IL-1- and TNFα-stimulated cells, it did significantly inhibit NFκB-dependent promoter activation, suggesting the presence of additional inhibitors acting in the cytoplasm in a translocation-independent manner on the transcription factor, or acting directly in the nucleus. All of the top ranked inhibitors were still present in the vv811ΔA49 genome (Table 3-2).

3.3.2. Detailed description of the three most promising inhibitor candidates

3.3.2.1. B2

VV-WR B2 is overall the most potent inhibitor of the IRF and NFκB pathway activation irrespective of the investigated signalling pathway (Figure 3-3, Figure 3-4). Therefore, a more detailed study of the signalling events following the adaptor proteins STING, TRIF and MAVS would be necessary to decipher the mechanism of inhibition. In support of its high rank is the fact that this protein was reported to be one of the most highly transcribed early genes during viral infection of HeLa cells (third after VV-WR 018 and E3), suggesting an important role in innate immune evasion (Assarsson et al. 2008). However, a recent transcriptome study assigned B2 a lower, yet still noteworthy rank (31st) amongst the most highly translated mRNA transcripts after E3, N2, A46, C6, M2 and N1 (Yang et al. 2015). Unfortunately, the similarity to the *Baculovirus* p26 domain cannot help infer the role of B2, as p26 is only known to form dimers and to localise in the cytosol of infected SF-21 cells and has no known function (Goenka & Weaver 2008).

3.3.2.2. B12

The second best inhibitor was B12, which is also an early gene and which displayed a broad inhibitory potential for the NFκB pathway similar to B2 (Figure 3-4B) (Howard & Smith 1989). However, its potency was inferior to B2 and it was only able to restrict MAVS-driven ISRE activation, whereafter it was placed below B2 in the ranking (Figure 3-4A). This result was unexpected as B12 appeared to be a pseudokinase, the absence of which did not seem to affect viral replication during infection in vitro or in vivo (Banham & Smith 1993; Tschärke et al. 2002). However, the lack of impact on viral titres in vitro has been reported for other well established virulence genes such as C6 and B14 (Chen et al. 2006; Unterholzner et al. 2011). Furthermore, the attenuation observed for intradermally and intranasally infected mice with deletion mutant viruses lacking C6 appears to be virus dose-dependent (Sumner et al. 2013). B12 shares approximately 35 % a.a. sequence identity with B1, and these proteins might have arisen through gene duplication. It may therefore be a possibility that B1 can rescue virus deletion mutants lacking B12. Nonetheless, it is intriguing to investigate B12, as it also shares an approximately 30 % a.a. sequence identity with the nuclear host protein VRK1, which is known to phosphorylate numerous transcription factors (Klerks et al. 2009). An examination of downstream signalling events in presence of B12 could clarify these issues, and address the question of whether B12 does indeed have a role in immunomodulation.

3.3.2.3. F17

Although the rank of F17's inhibitory potential is very similar to that of A47 and A57 (Table 3-2), it has been placed in the third position due to its abundance in viral particles (Zhang & Moss 1991). F17 was able to impair IFN β -dependent reporter activity effectively (Figure 3-3A). It seems that this effect was mainly due to its ability to potently restrict NF κ B-mediated reporter activation, as only MAVS-driven ISRE activity was impaired by F17 (Figure 3-4). F17 also appeared to augment the transactivation of ELK by RAS, however this phenomenon was not further investigated (Figure 3-3C).

Virion morphogenesis, and in particular protein processing prior to crescent formation are dependent on late expressed F17 and this role has been intensively investigated (Zhang & Moss 1991; Pedersen et al. 2000; Wickramasekera & Traktman 2010; Schmidt et al. 2013). It is packaged into nascent virions and constitutes the majority of the lateral body mass (Schmidt et al. 2013), which may allow F17 to have an additional role very early during infection. The dual-specificity phosphatase H1, another late expressed viral protein, is also found in lateral bodies, and after entry is released from lateral bodies in a proteasome-dependent manner (Schmidt et al. 2013). H1 then inhibits interferon signalling by dephosphorylating STAT1 (Najarro et al. 2001). Similarly, F17 dissociates from disassembled lateral bodies after cell entry (Schmidt et al. 2013). Analysis of F17 protein levels at 2 h post fusion of MVs with cells in presence and absence of proteasome inhibitors revealed that 3 times less F17 was present in untreated cells (Schmidt et al. 2013). However, F17 is the third most abundant protein in the virion (Chung et al. 2006; Resch et al. 2007) with an estimated 27.000 copies of F17/MV (Sarov & Joklik 1972), and is one of the 10 most prevalent virion proteins, which constitute together 80 % of the virion mass (Resch et al. 2007). In comparison, only 220 copies of H1 were detected per virion (Liu et al. 1995), and it was ranked on position 15 (Resch et al. 2007), or 28 in term of abundance in the virion (Chung et al. 2006). It may therefore be possible that F17 has a role early in infection, as suggested by the ability of transiently expressed F17 to impair both IFN β -promoter- and NF κ B-element-dependent reporter activity (Figure 3-3A,B). Interestingly H1 is able to dephosphorylate F17 (Liu et al. 1995), whereas cellular kinases, namely ERK, CDK1 and JNK, have been shown to phosphorylate F17 *in vitro* (Wickramasekera & Traktman 2010).

3.3.3. Summary of initial screen

The results of this screen for inhibitors of cGAS-mediated type I IFN responses have revealed eight candidate proteins. These did not only impair the cGAS/STING signalling axis, but also TLR and RLR signalling pathways. As a result of these findings, the aim of this study was changed to focus on the characterisation of VACV proteins able to restrict multiple PRR and pro-inflammatory cytokine signalling.

The VV-WR proteins B2, B12 and F17 were identified as the most promising novel PRR signalling pathway and pro-inflammatory cytokine inhibitors, whilst N2 was excluded from the screen. A total of eight viral candidates remain for further investigations in the screen.

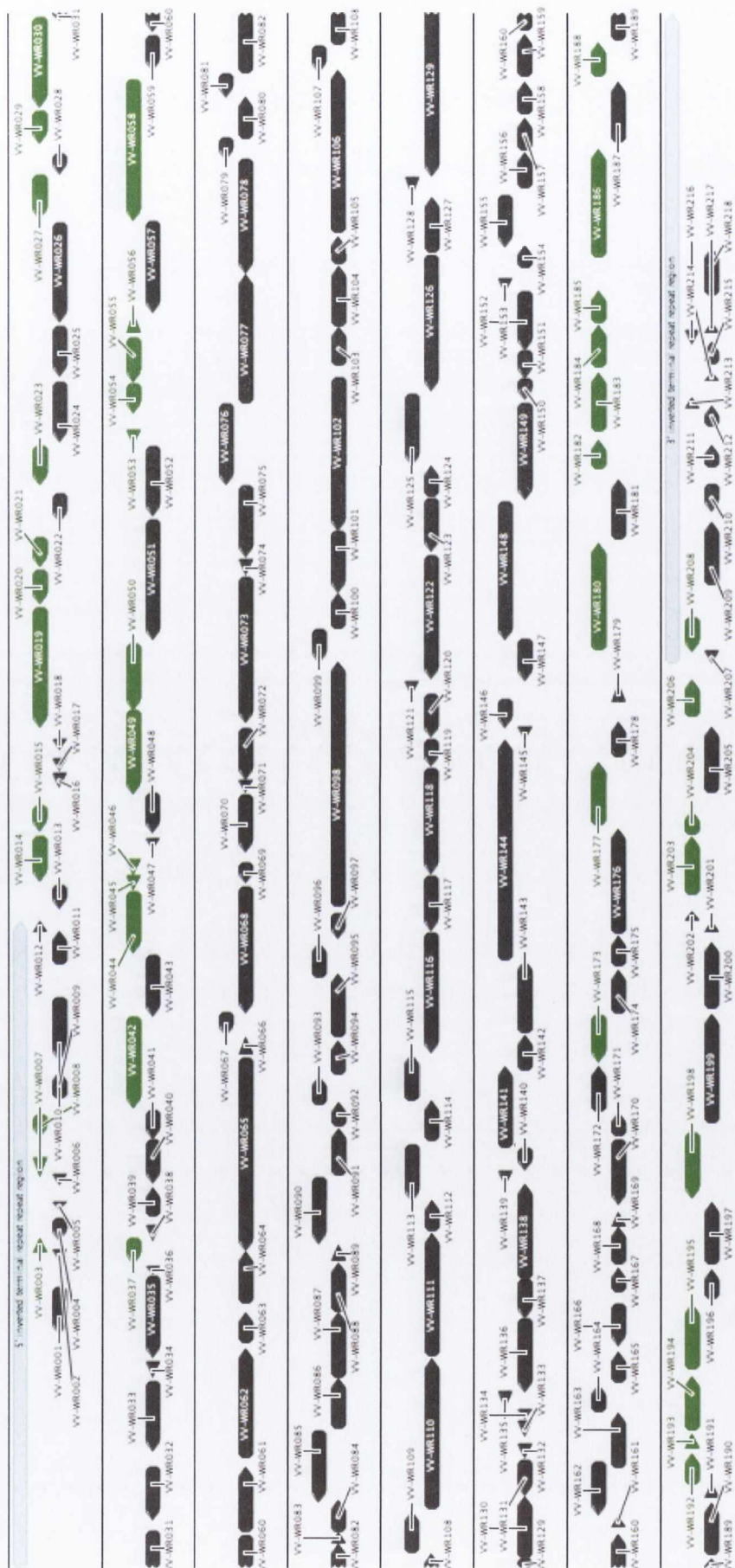


Figure 3-1 Schematic representation of the VV-WR genome (AY2433312)

The genome is composed of linear dsDNA of 195 kb with closed hairpin termini. The DNA strands contain inverted terminal repeats (light blue arrows). Forty-two genes (green arrows) were selected amongst 223 predicted viral genes (grey arrows) to be screened for their ability to impair intracellular innate immune signalling. A truncated arrow indicates an ORF is split over two lines of the schematic.

VV ORF name		Uniprot ID	Predicted motif	Established function	Comments	Reference
WR	COP					
003	-	Q805P9				
007	C20L	Q805M1	Ankyrin-like			
008	C19L	Q805H0	Ankyrin-like			
014	-	P17369	Ankyrin-like			
015	-	P17360	Ankyrin-like			
019	C9L	P17372	Ankyrin-like			
020	C8L	P17364	Ankyrin-like			
021	C7L	P68600		SAMD9 antagonist	Host range determinant, anti-apoptotic effects, reduced PKR and eIF2 α phosphorylation, inconclusive effects on IRF1, N-terminal tag interferes with function	(Oguiura et al. 1993; Oseroff et al. 2005; Nájera et al. 2006; Meng et al. 2008; Backes et al. 2010; Liu et al. 2012; Meng et al. 2012; Terajima et al. 2013; Liu & McFadden 2015; Meng et al. 2015; Sivan et al. 2015)
023	C5L	P17367	Kelch-like (Krp-1, sarcosin)			
027	C1L	P17368	Bcl-2 like			
029	N2L	P14357	Alpha-aminitin, Bcl-2 like	Interferon inhibitor	Nuclear early expressed protein, deletion improves immunogenicity of MVA vaccine vector (HIV)	(Ferguson et al. 2013; García-Arriaza et al. 2014)
030	M1L	P14356	Ankyrin-like			
037	K5L	P18380	Putative monoglyceride lipase			
042	F3L	P24357	BTB/Kelch-like		No effect on viral replication <i>in vitro</i> and plaque morphology, less lesions and increased number of NK and TCDy δ cells observed in mice intradermally inoculated with deletion mutant virus compared to wild-type virus	(Froggatt et al. 2007)

Table 3-1 Overview of candidate VV-WR ORFs for immune modulator and inhibitor screen (see page 75 for legend)

VV ORF name		Uniprot ID	Predicted motif	Established function	Comments	Reference
WR	COP					
044	F5L	P24358	36 kDa major membrane protein		No effect on viral replication <i>in vitro</i> , enhanced plaque size, no effect on disease progression (intradermal/nasal inoculation)	(Dobson et al. 2014)
045	F6L	P68603				
046	F7L	P24359				
049	F10L	Q89121	Ser/Thr kinase	Phosphorylates various viral proteins involved in virion membrane generation	Late expressed and packaged into virion, localised to membranes through association with phosphoinositides	(Lin & Broyles 1994; Traktman et al. 1995; Betakova et al. 1999; Wang & Shuman 1995; Derrien et al. 1999; Doglio et al. 2002; Szajner et al. 2004a; Szajner et al. 2004b; Punjabi & Traktman 2005)
050	F11L	Q80HX7	PBZ-fold, PBZ-binding motif, Rho-associated kinase similarity	RhoA signalling inhibitor by juxtaposing RhoA and Myosin9A	Early expressed, required for actin remodelling at cellular cortex during virus particle release, deletion mutant virus is less virulent in intranasally inoculated mice than wild-type virus	(Valderrama et al. 2006; Arakawa et al. 2007; Morales et al. 2008; Cordeiro et al. 2009; Handa et al. 2013)
053	F14L	P68707				
054	F15L	Q80HX4				
055	F16L	Q80HX3	Prokaryotic serine recombinase		Early expressed, no effect on viral replication <i>in vitro</i> or plaque morphology	(Senkevich et al. 2011)

Table 3-1 Overview of candidate VV-WR ORFs for immune modulator and inhibitor screen (see page 75 for legend)

VV ORF name		Uniprot ID	Predicted motif	Established function	Comments	Reference
056	F17R	P07396	Superhelical DNA-binding phosphoprotein		Encoded in all chordopoxviruses, late expressed and packaged into virions (69 % of lateral body mass), required for protein processing and virion maturation, associated with actin tails during late infection, but not involved in actin tail formation, phosphorylated at Ser53, Ser2 and Ser40 by cellular kinases (potentially ERK, JNK or CDK1), dephosphorylated by VV H1	(Kao et al. 1981; Kao & Bauer 1987; Zhang & Moss 1991; Liu et al. 1995; Reckmann et al. 1997; Pedersen et al. 2000; Gubser et al. 2004; Wickramasekera & Traktman 2010; Schmidt et al. 2013)
058	E2L	P21604		F12 recruitment to microtubules	Intermediate-late expression, not essential for <i>in vitro</i> growth, deletion mutant virus severely impaired in <i>in vitro</i> spread, required for IEV formation, associates and recruits F12 to IEVs and to kinesin-1 motor complex (KLC2 binding) on microtubules	(Domi et al. 2008; Dodding et al. 2009; Carpentier et al. 2015)
173	A47L	P26673			No effect on viral replication, plaque morphology or disease progression in intranasally or intradermally infected mice, immunoprevalent for CD8 ⁺ T cells	(Yuen et al. 2010)

Table 3-1 Overview of candidate VV-WR ORFs for immune modulator and inhibitor screen (see page 75 for legend)

VV ORF name		Uniprot ID	Predicted motif	Established function	Comments	Reference
WR	COP					
177	A51R	Q01219			Virulence gene (deletion virus has reduced titre <i>in vitro</i> , less virulent in intranasally infected mice), forms aggregates and filaments in cytosol, associates and stabilises tubulin, binds to myosin II essential light chain and ubiquitin (mass spectrometry analysis)	(Gammon et al. 2014)
180	A55R	P24768	BTB/Kelch-like		Late expressed, dimerises, no effect on viral replication <i>in vitro</i> , altered plaque morphology and less virus induced cell adhesion observed for deletion mutant virus compared to wild-type virus, larger lesions at site of injection with deletion mutant virus (intradermal inoculation), but no effect in intranasal infections, probably not secreted or packaged into virion	(Beard et al. 2006)
182	A57R	Q01230	Guanylate kinase			
183	B1R	P16913	Ser/Thr kinase	BAF inhibitor (via phosphorylation), clun activator (via phosphorylation), p53 inhibitor (via hyper-phosphorylation)	early cytosolic VV protein, packaged into virions, essential for viral DNA synthesis, intermediate gene transcription and virion maturation, auto-phosphorylation, phosphorylates VV H5, ribosomal S40 subunits Sa and S2 and JNK, binds JIP1 and JNK, synergises with TAK1 and MEKK7	(Howard & Smith 1989; Traktman et al. 1989; Banham & Smith 1992; Rempel & Traktman 1992; Lin et al. 1992; Banham et al. 1993; Beaud et al. 1994; Beaud et al. 1995; Domi & Beaud 2000; Brown et al. 2000; Kovacs et al. 2001; Santos et al. 2004; Boyle & Traktman 2004; Nichols et al. 2006; Santos et al. 2006; Wiebe & Traktman 2007; Ibrahim et al. 2013; Jamin et al. 2015)

Table 3-1 Overview of candidate VV-WR ORFs for immune modulator and inhibitor screen (see page 75 for legend)

VV ORF name		Uniprot ID	Predicted motif	Established function	Comments	Reference
WR	COP					
184	B2R	Q01225	<i>Baculovirus</i> p26 domain		p26 of <i>Autographa californica nucleopolyhedrovirus</i> : cytoplasmic, dimerises	(Goenka & Weaver 2008)
185	B3R	Q01226	Murine Schlafen (Slfn)		Recombinant VV expressing CMLV vSLFN: no effect <i>in vitro</i> or <i>in vivo</i> in intradermal model, but attenuation in intranasal model compared to WT virus	(Gubser et al. 2004)
186	B4R	P24769	Ankyrin-like		Putatively late expressed, virulence gene, deletion mutant virus (VV-COP, w811 or ECTV) forms smaller plaques <i>in vitro</i> , w811 (not VV-COP) deletion virus releases less virus compared to WR, deletion mutant virus is attenuated in intranasally infected mice (ECTV)	(Burles et al. 2014)
188	B6R	P68442	Ankyrin-like			
192	B10R	Q76ZL3	Kelch-like			
193	B11R	Q01229				
194	B12R	P24362	hVRK1, Ser-Thr kinase		Early expressed, not essential, potentially non-functional kinase (lacks obvious ATP-binding site and various key kinase residues, unable to phosphorylate caseins or histones <i>in vitro</i>), no effect on viral replication <i>in vitro</i> or disease progression (intradermal and intranasal infection)	(Howard & Smith 1989; Banham & Smith 1993; Tschärke et al. 2002)

Table 3-1 Overview of candidate VV-WR ORFs for immune modulator and inhibitor screen (see next page for legend)

VV ORF name		Uniprot ID	Predicted motif	Established function	Comments	Reference
WR	COP					
195	B13R	P15059	Serpin, SPI-2, CrmA (92 % a.a. identity)	Caspase-inhibitor	Not essential early gene, prevents PARP cleavage and IL-1 β processing, anti-apoptotic, no effect on viral replication, plaque morphology and disease progression in intra-nasally infected mice, larger lesions, but no effect on humoral and CTL responses and T cell activation observed for deletion mutant in intradermally infected mice	(Smith et al. 1989; Blake et al. 1995; Dobbelsstein & Shenk 1996; Kettle et al. 1997; Li & Beg 2000; Tscharke et al. 2002; Legrand et al. 2004; Veyer et al. 2014)
198	B17L (B16L)	Q01221				
203	-	Q01223	Ankyrin-like			
204	-	P17373	Kelch-like, influenza NS1A-binding protein, EV-M-167			
206	C14L (C13L)	P17365				
208	-	P17366	Zinc-finger-like protein			

Table 3-1 Overview of candidate VV-WR ORFs for immune modulator and inhibitor screen

From the 223 genes listed on NCBI (AY243312), forty-two viral proteins were selected for a innate immune inhibitor screening. The remaining 181 were excluded for fulfilling the following criteria: Their location in the central genomic region; secreted proteins; small gene fragments; well characterised proteins with unrelated functions; duplicates with near 100 % identity. Protein motifs were analysed with NCBI standard protein BLAST. ORF names are given according to the annotation of the VV Copenhagen strain if possible, and ordered in the table according to their order in the genome.

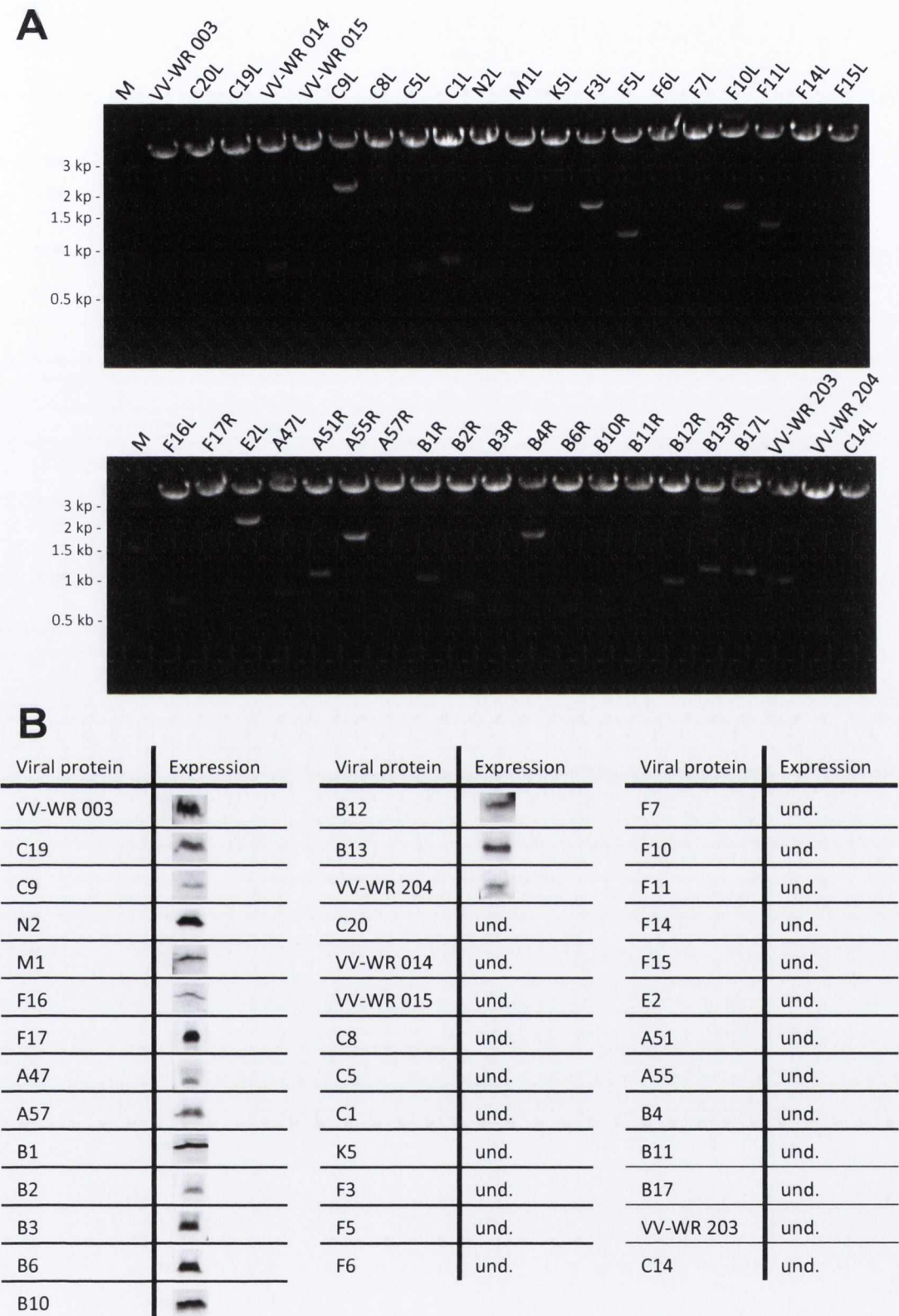


Figure 3-2 Assembly of VV-WR protein expression constructs

(A) VV-WR protein expression plasmids were constructed by inserting VV-WR ORFs into a pCMV-HA expression vector. To verify the successful insertion 2 µg of double digested expression constructs were run on a 1 % agarose gel. Size of the DNA ladder (M) is given to the left of gels. (B) HEK 293T cells were transfected with 3 µg of VV-WR protein expression construct. Proteins from cell extracts were harvested 24 hpt, separated by SDS-PAGE and immunoblotted with an anti-HA antibody. Repeatedly undetected proteins are labelled 'und.' (undetected).

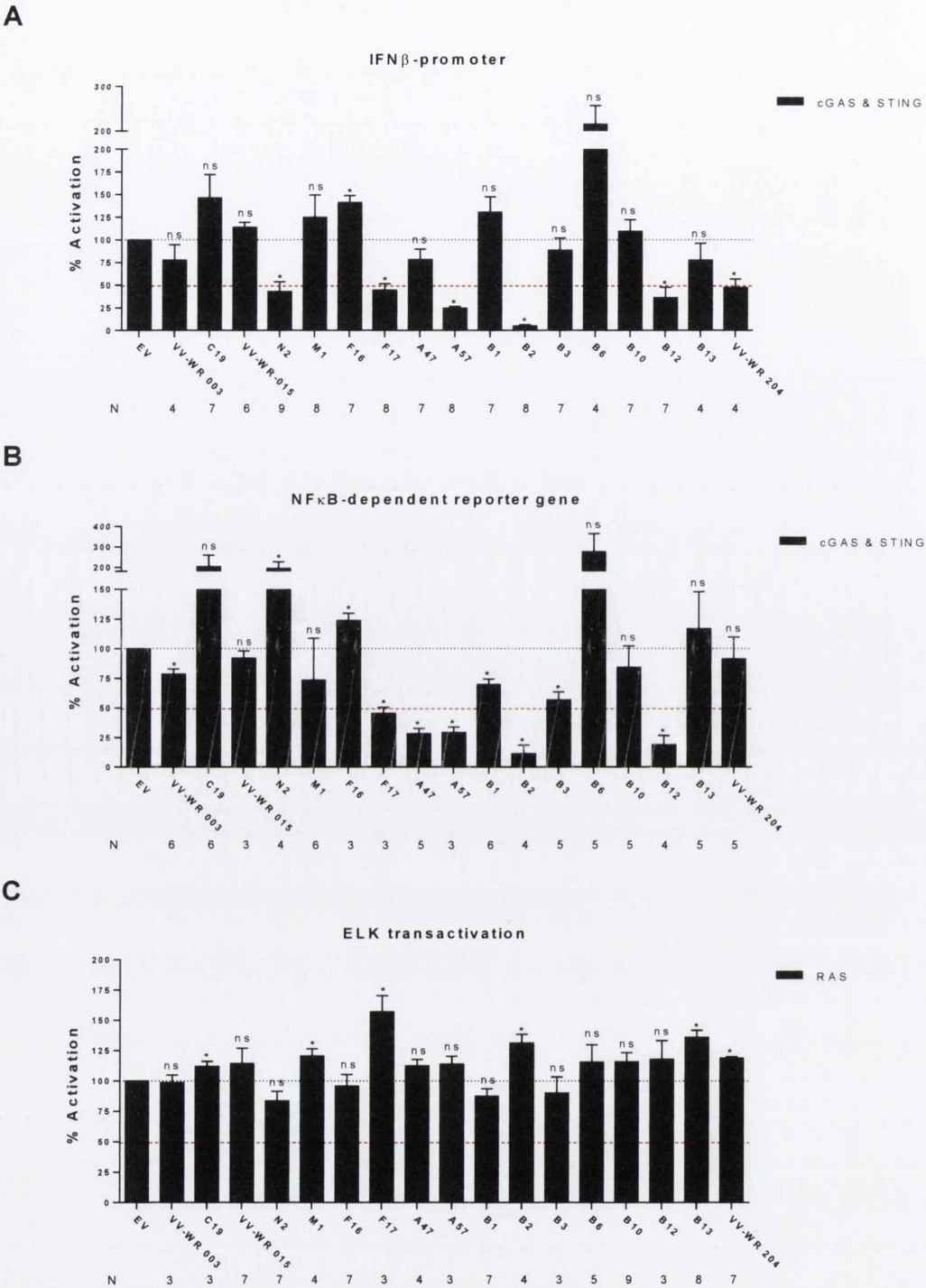
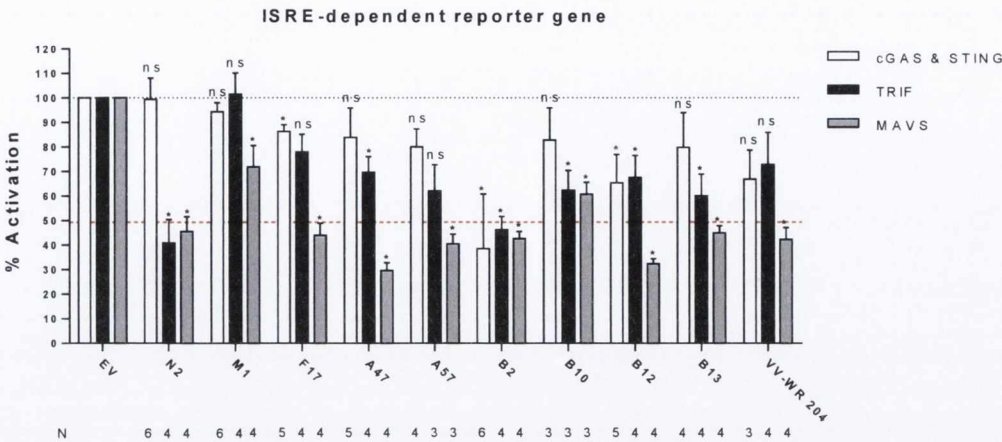


Figure 3-3 Evaluation of inhibitory effects of VV-WR proteins on cGAS/STING-driven IFN β and NF κ B activation

HEK 293T cells were co-transfected with a firefly luciferase reporter plasmid under the control of either (A) an IFN β -promoter, or (B) NF κ B-elements, along with a renilla luciferase control, and to drive promoters: STING and cGAS expression constructs, and indicated VV-WR protein expression plasmids or empty vector control (EV). (C) HEK 293T cells were co-transfected with a pFA-firefly luciferase reporter plasmid under the control of a Gal4-promoter, and an ELK-Gal4 fusion protein, activated by transfection of a RAS expression construct. Cells were harvested 24 hpt, firefly luciferase activity was measured and normalised to renilla luciferase activity. VV-WR protein assay values were scaled relative to EV, which was set to 100 % activation (black dotted line). The red dotted line represents 50 % inhibition. Means of at least three independent experiments with triplicate determination are shown with their SEMs. Exact number of independent experiments (N) is given below each graph. * $p < 0.05$, ns (not significant).

A



B

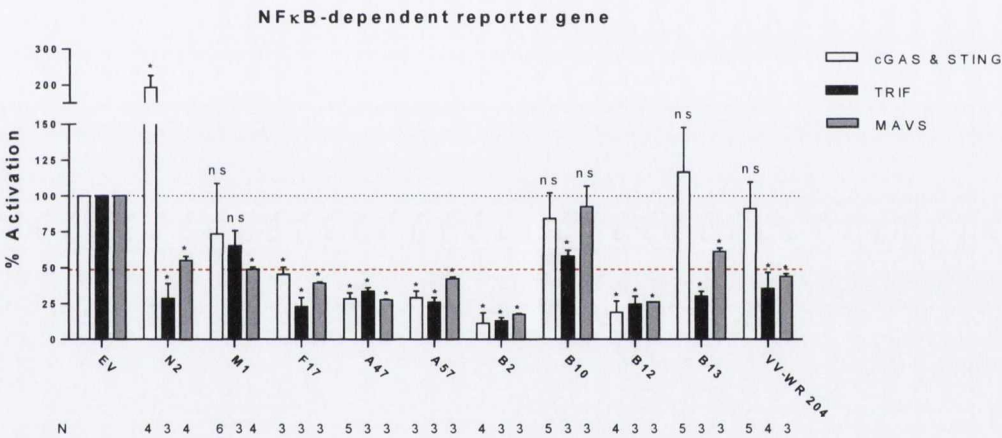


Figure 3-4 Dissection of inhibitory effects on ISRE and NFκB activation of previously identified VV-WR proteins

HEK 293T cells were co-transfected with a firefly luciferase reporter plasmid under the control of either (A) an ISRE element or (B) NFκB-elements, and a renilla luciferase control. Promoters were activated by transfection of either of cGAS and STING expression constructs, TRIF expression plasmid, or MAVS expression construct. These cells were also transfected with the indicated VV-WR protein expression constructs or empty vector control (EV). VV-WR B10 was included to serve as a negative control for inhibition of NFκB activation. Cells were harvested 24 hpt, firefly luciferase activity was measured and normalised to renilla luciferase activity. VV-WR protein assay values were scaled relative to EV, which was set to 100 % activation (black dotted line). The red dotted line represents 50 % inhibition. Means of at least three independent experiments with triplicate determination are shown with their SEMs. Exact number of independent experiments (N) is given below each graph. *p<0.05, ns (not significant).

VV-WR protein	Size	Motifs & functions	Rank	IFN β inhibition rank	ISRE inhibition rank	NF κ B inhibition rank	Present in vv811 Δ A49
B2	24.6 kDa	<i>Baculovirus</i> p26 domain	1	1/6 (95 %)	1/8 (58 %)	1/9 (86.3 %)	Yes
B12	33.3 kDa	hVRK1, Ser/Thr kinase	2	3/6 (64 %)	3/8 (45 %)	2/9 (77.3 %)	Yes
F17	11.4 kDa	Major virion component	3	4/6 (56 %)	7/8 (31 %)	5/9 (61.3 %)	Yes
A57	17.4 kDa	Guanylate kinase	4	2/6 (76 %)	5/8 (39 %)	3/9 (68 %)	Yes
A47	29.2 kDa		5	> 6/6 (22 %)	4/8 (39 %)	3/9 (70.6 %)	Yes
B13	38.5 kDa	Serpin, CrmA, Apoptosis inhibitor	6	> 6/6 (23 %)	6/8 (39 %)	8/9 (36.6 %)	No
M1	54.3 kDa	Ankyrin-like	7	> 6/6 (0 %)	> 8/8 (11 %)	9/9 (37.6 %)	No
VV-WR 204	16 kDa	Kelch-like	8	6/6 (53 %)	8/8 (40 %)	6/9 (43.6 %)	No
N2	20.9 kDa	Interferon inhibitor	10	5/6 (58 %)	2/8 (38.6 %)	7/9 (39 %)	No

Table 3-2 Ranking of identified immune inhibitor candidates

The two initial screens identified 10 potential ISRE/IFN β and/or NF κ B inhibitors, which are ranked according to overall, IFN β -specific, ISRE-specific, and NF κ B-specific inhibition. Inhibition occurred if a given viral protein antagonised at least one activator (i.e. cGAS/STING, TRIF, MAVS) in its ability to drive a promoter by more than 50 % activation in presence of the viral inhibitor. A rank of > N/N signifies that no inhibition >50 % occurred. Overall reduction of reporter activity is given in percent for each rank in brackets. VV-WR protein size, predicted motifs and established functions are listed as well as their presence in the VV-Cop mutant virus strain vv811

Chapter 4 : Results II – Characterisation of identified viral inhibitors of innate immune signalling

4.1. Introduction

4.1.1. Relevance of VACV research to virology and immunology

The study of VACV virulence genes has led to numerous discoveries in the past advancing the fields of basic and applied virology and immunology, which are illustrated through examples in the following section.

The VACV protein A52 is able to prevent IRAK2 from transducing signals received from TLRs and IL-1R to downstream kinases, which would otherwise lead to activation of NF κ B (Harte et al. 2003). These results demonstrated the importance of IRAK2 in host antiviral defences in human cells in particular, a role which was, prior to these studies, solely attributed to IRAK1 (Keating et al. 2007). In addition, K7 impairs DDX3 function, which led to the discovery of DDX3 and its involvement in IKK ϵ (and TBK1)-dependent activation of the transcription factors IRF3 and IRF7 (Schröder et al. 2008). Hence, characterisation of A52 and K7 not only revealed new aspects of VACV immune evasion strategies, but also improved our understanding of innate immune signalling pathways.

Recruitment of the sorting adaptors TRAM and MAL to TLR4 is directly blocked by A46 (Stack & Bowie 2012). Another study subsequently reported the generation of an 11 a.a. peptide called viral inhibitory peptide of TLR4 (VIPER), which was derived from A46 (Lysakova-Devine et al. 2010). VIPER was shown to efficiently reduce TLR4 activation in response to LPS *in vitro* and *in vivo* (Lysakova-Devine et al. 2010). It has recently been shown that VIPER conferred protection of mice infected with lethal doses of a mouse-adapted influenza strain, when administered in therapeutic doses 2 days after virus challenge (Shirey et al. 2016), providing a proof of principle that virus-derived peptides have the potential to be developed into drugs.

The VACV protein C6 was shown to bind the TBK1 complex scaffold proteins TANK, SINTBAD, and NAP1, thereby reducing TBK1- and IKK ϵ -mediated activation of IRF3 and IRF7 (Unterholzner et al. 2011). Deletion of C6 from an MVA based vaccine vector encoding HIV-1 proteins resulted in an ameliorated T-cell response, in particular CD8⁺T-cells, as well as increased antibody production against the HIV-1 envelope protein env (García-Arriaza et al. 2011). Similarly, VACV deletion mutant viruses lacking C6 elicited higher cytotoxic T cell responses compared to WT virus *in vivo*, although no difference in humoral responses was observed (Sumner et al. 2013). Study of VACV proteins is of continuous interest as VACV and its derived virus strains are used as potential vaccine vectors. Identification of virulence genes can help improve the safety and immunogenicity of these vectors.

4.1.2. Experimental strategy for characterisation of VACV candidates

Eight viral proteins emerged from a screen for inhibitors of PRR signalling pathways (see 3.3). In the past, various techniques have been employed to strategically elucidate the mechanism of inhibition of poxviral virulence genes (Schröder et al. 2008; Unterholzner et al. 2011; Mansur et al. 2013; Brady et al. 2015), and a similar approach will be taken in this study to characterise the selected candidates.

Reporter gene assays are informative and rapid tools to map the effect exerted on different signalling pathways (Schröder et al. 2008; Unterholzner et al. 2011; Mansur et al. 2013; Brady et al. 2015). They have already been proven useful for the identification M1, F17, A47, A57, B2, B12, B13 and VV-WR 204 as potential immunomodulators (see 3.3). The advantage of RGAs is that activation of the reporter can be elicited at

virtually any step in the signalling pathway, for example, by use of ligands at the receptor level (e.g. TNF α , 3'3'-cGAMP), overexpression of pathway components (e.g. MAVS, TRIF, IKK α , TBK1), or of transcription factors (e.g. IRF7, p65), and hereby the potential function of the viral proteins can be narrowed down.

Impairment of the NF κ B signalling pathway appeared to be the common target of the eight potential immunomodulators, and therefore a time-course analysis of the events prior to NF κ B nuclear translocation (e.g. I κ B α degradation, p65 phosphorylation) may map the point of inhibition (Mansur et al. 2013; Brady et al. 2015).

Analysis of cellular localisation of viral proteins further inform on the mechanism of inhibition. Study of nuclear translocation of transcription factors (e.g. IRF3, p65) which are activated by PRR and pro-inflammatory signalling can link the cellular localisation of the potential inhibitors and their mechanism of action (Unterholzner et al. 2011; Mansur et al. 2013; Brady et al. 2015).

In addition to understanding how VACV proteins are able to prevent induction of type I IFN and activation of NF κ B signalling, it is important to investigate their physiological consequences. The result of IRF and NF κ B signalling is the production of type I IFNs and pro-inflammatory cytokines (see 1.2.1), which can be measured by means of bioassays and ELISAs (Stack et al. 2005; Schröder et al. 2008; Unterholzner et al. 2011; Mansur et al. 2013; Brady et al. 2015).

The sum of the experiments described above should guide the identification of the most promising viral candidate(s) and its/their host target(s). The latter can, then, be identified through co-immunoprecipitations and mass spectrometry (see Chapter 5).

4.2. Results

4.2.1. Mapping inhibition of NFκB by VACV ORFs

The transcription factor NFκB is activated by stimulation of PRR signalling pathways and leads to expression of pro-inflammatory cytokines such as TNFα and IL-1, which in turn can result in further NFκB activation. IL-1R and TLRs signal through TRAF6, whilst TNFR uses TRAF2 (see 1.2.1.2.2 and 1.2.1.3.2). In order to determine inhibition of PRR-independent NFκB-dependent promoter activation, reporter activity was driven by stimulation with TNFα or IL-1. Although cells were stimulated with less TNFα than IL-1, reporter activation was induced three times more potently by TNFα, than by IL-1 (Appendix 1Y,Z). In contrast, the impairment of NFκB-dependent reporter activation exerted by all of the candidates was greater in TNFα-stimulated cells (Figure 4-1).

The reporter activation in response to either cytokine was reduced by M1, A47, B2 and B12, whilst F17 and to a lesser extent B13 were only able to impair TNFα-mediated NFκB activation. These results suggest a role downstream of pro-inflammatory cytokine receptors for these candidates (M1, F17, A47, B2, B12, B13). A57 and VV-WR 204 were not able to suppress NFκB activation by either TNFα or IL-1.

To support the impairment of proinflammatory cytokine signalling observed for the three most promising candidates (F17, B2 and B12) additional RGAs were performed. TRAF2 and TRAF6 are components of the TNFR and IL-1R signalling cascades, and overexpression of these host proteins activates NFκB. B2 and B12 greatly reduced NFκB activation, as anticipated from the ligand stimulation assays (Figure 4-2). However, F17 was able to impair both TRAF2- and TRAF6-driven reporter activation, which was unexpected given its inability to restrict IL-1-driven NFκB activation.

Upon receptor stimulations a signalling cascade is initiated which leads to the activation of the IKK complex (see 1.2.1.2.2). Given the potent inhibition of PRR and pro-inflammatory cytokine receptor signalling, the effect on IKK complex activation was determined by overexpression of the different IKK family members (the kinases that directly regulate NFκB activation). All of the studied viral proteins were able to reduce NFκB-dependent reporter activation driven by at least one kinase, suggesting a role downstream of PRRs and pro-inflammatory cytokine receptors for each candidate. B12 and F17 potently impaired NFκB activation by IKKα, IKKβ and IKKε, whereas A47, B2 and A57 were no longer able to prevent reporter activation mediated by IKKβ after repetition of the assay at a later time. VV-WR 204, B13 and M1 were only able to restrict IKKε-driven NFκB activation. It is worth noting, that experiments evaluating the effects on IKKβ-driven NFκB activation were repeated at a later time, which introduced large variations for the following candidates F17, A47, A57, B2 and B12. These initially had demonstrated very potent reduction of reporter activity (Figure 4-3).

4.2.1.1. NFκB activation by the IKK complex is inhibited by VACV proteins

The activated IKK complex phosphorylates the NFκB repressor IκBα (Traenckner et al. 1995), as well as NFκB (Buss et al. 2004). Following phosphorylation IκBα is degraded in a proteasome-dependent manner (see 1.2.1.2.2, (Henkel et al. 1993; Spencer et al. 1999)). To analyse the effect of the viral candidates on these events, cells stably transfected with CdCl₂-inducible VV-WR expression constructs were stimulated with TNFα,

and protein levels of I κ B α and p65, and phosphorylation of p65 at Ser536 (pp65) were monitored over the course of 2 h (Figure 4-4). An inducible system was chosen, because of the potential growth-inhibitory effect of stable expression of the selected viral proteins. Inducible VV-WR expression constructs were generated by amplifying HA-tagged ORFs from their respective pCMV-HA expression plasmid, and were inserted into a pMEP4-vector. HEK 293T cells were transformed with expression plasmids, and selected using Hygromycin. The number of viral candidates was then reduced to 7, as the cloning of pMEP4-HA M1 was abandoned after numerous unsuccessful attempts. Transformation with empty pMEP vector served as a negative control for potential CdCl₂-induced effects. It has to be noted that expression levels varied significantly from one construct to another, and certain constructs “lost” expression over the course of a few passages, despite maintenance at high hygromycin levels. The difficulty of culturing stably transformed HEK 293Ts could be explained by 1) ‘leaked’ expression in the absence of CdCl₂ and 2) a harmful effect of the viral protein on the cell given its prolonged expression. Due to difficulties in viral protein expression and protein detection (e.g. viral proteins, pp65), further experiments will be necessary to elucidate the mechanism of inhibition for the following candidates: A47, A57, B13 and VV-WR 204 (marked by ‘†’).

The CdCl₂ concentration used in these assays did not have an impact on the studied events, as no effects were observed in induced cells transfected with empty pMEP-vector compared to non-induced cells (Figure 4-4A). Interestingly, F17 appeared to delay the initial phosphorylation of p65 by 10 min (Figure 4-4B). In non-induced cells, p65 phosphorylation was readily detected 5 min post-stimulation and started to lessen by 15 min post-stimulation, whereas in CdCl₂-induced cell, stably transfected with an F17 expression vector pp65 levels were notably lower at 5 min post-stimulation, peaked at 15 min post-stimulation, and lessened by 30 min post-stimulation. No effect was observed on total p65 levels, or I κ B α degradation. This transitory effect may represent a very early effect of F17, once it is released from lateral bodies and prior to its degradation and the transcription of early genes. Despite good and stable expression of B2 and B12 (Figure 4-4E,F), no effects on either of the studied host proteins were observed, suggesting a role following the release of p65 from its repressor I κ B α . Unfortunately, phosphorylation of p65 was only detected in one experiment for the following proteins, and no conclusions could be drawn: A47, A57, B13 and VV-WR 204 (Figure 4-4C,D,G,H, marked by ‘†’). In each case no difference was observed between CdCl₂-induced and non-induced cells. Although total p65 and I κ B α levels were detected in at least 3 experiments for B13 and VV-WR 204, the presence of viral proteins had no impact on either of these host proteins (Figure 4-4G,H).

4.2.1.2. NF κ B inhibition exerted by VACV proteins does not impair p65 translocation

Once NF κ B is activated and released from its repressor, it translocates to the nucleus to bind to the promoter of NF κ B-dependent genes such as type I IFN and pro-inflammatory cytokines (see 1.2.1.2.2). After establishing that most of the viral candidates had no apparent effect on I κ B α degradation or p65 phosphorylation at Ser536, nuclear translocation of p65 in response to TNF α was analysed in the presence or absence of transiently transfected viral candidates. Preliminary results are shown in Figure 4-5. M1, B13 and VV-WR204 were not included in this experiments as they were unable to impair IKK α - and IKK β -mediated NF κ B activation (Figure 4-3). The lack of effect of the potential viral immunomodulators, with exception of F17, on I κ B degradation, phosphorylation and nuclear translocation of p65 might be due to the nature of the inhibitors,

which might act following the release of p65 phosphorylated at Ser536 or may impair p65 activation independent of these events. The amount of TNF α (50 ng/ml) chosen to activate the NF κ B signalling cascade in these experiments was greater than the quantity used for RGAs (12.5 ng/ml), therefore the absence of an effect might be due to this technical difference.

Transfection of cells with empty vector control did not impair the nuclear translocation of the NF κ B subunit p65 in response to TNF α (Figure 4-5). In unstimulated cells, p65 was predominantly localised in the cytoplasm and only a few cells were detected with nuclear stain for p65. In contrast, cells stimulated with TNF α showed a clear nuclear stain for p65. Presence of viral inhibitors did not appear to affect nuclear translocation of p65 in response to TNF α . This result may indicate that the candidates target NF κ B activity after its phosphorylation at Ser563 and its nuclear translocation. In support of this hypothesis, these experiments also showed that A47, A57, B2 and particularly B12 are present in the nucleus. However, the lack of effect may also result from the low transfection efficacy (Figure 4-5A), or an S563 phosphorylation- and I κ B α -independent impairment of NF κ B activity.

To distinguish between an effect prior to or following nuclear translocation of p65, NF κ B-dependent reporter activation was driven by transfection of a p65 expression construct (Figure 4-6). Increasing amounts of p65 were transfected to determine if impairment exerted by viral proteins could be overcome by saturating the activation of the reporter. Indeed, when p65 was transfected in a dose-dependent manner reduction of reporter activity mediated by F17 was reverted. A similar, yet not as dramatic result was obtained for A47. In contrast, A57, B2, and B12 potentially limited reporter activation, suggesting a role in the nucleus for these candidates after translocation of p65. The presence of M1, B13, and VV-WR 204 did not affect reporter activation as anticipated, due to their inability to impair IKK α - and IKK β -mediated NF κ B activation.

Following nuclear translocation of NF κ B, this transcription factor and others induce the expression of pro-inflammatory cytokines such as IL-6 and IL-8 (see 1.2.1). In attempt to correlate the impairment detected in RGAs with a physiological response, CdCl₂-inducible stably transfected HEK 293T cells were assayed for IL-8 production in response to IL-1 (Figure 4-7). Although no differences in growth were apparent in previous experiments using these cell lines (see above), differences in the acidity of the supernatants were observed in response to CdCl₂ (data not shown). The reduced cell growth and less acidic supernatants may be more readily detected in wells of 96-well plates, which were used to perform ELISAs, whereas previous experiments were carried out in 6-well plates. However no effect on actin protein levels was observed in earlier experiments (Figure 4-4). The effect of CdCl₂ was not consistently present, however it did impede the feasibility of these experiments, as they were only carried out if the acidity of the supernatants in absence and presence of CdCl₂ was equal, which was assessed by colour change of the growth medium (single experiments and experiments which were only repeated once are marked by '†').

Empty vector control cells treated with CdCl₂ appeared to produce more IL-8 in response to IL-1 compared to untreated cells, however this difference was not significant (Figure 4-7A). CdCl₂-stimulated expression of F17 and B12 had no statistically significant effect on cytokine production (Figure 4-7B,E), whilst B12 may increase the production of IL-8 following IL-1 stimulation (Figure 4-7D). This result does not correlate with its ability to impair NF κ B activation and is more likely attributable to the presence of CdCl₂. It appears therefore that these

cells are not suitable for determining the effect of viral proteins on chemokine production. Transient transfection of HEK293T cells with candidates did not demonstrate any effects of viral proteins on IL-8 production, however this may be due to low transfection efficiency (data not shown).

4.2.2. Characterisation of inhibition of type I interferon induction by VACV ORFs

Stimulation of PRRs leads to the activation of a signalling network, resulting in the activation of transcription factors such as IRF3, IRF7, and NFκB. The effects of the previously discovered viral candidates showed that all were potent at impairing NFκB activation (see 3.3). In addition, B2 demonstrated the ability for effective reduction of ISRE activation mediated by transfection of cGAS/STING, TRIF and MAVS, whilst this effect was less evident for other candidates. Following PRR activation and mobilisation of adaptor proteins (i.e. STING, TRIF, MAVS), TBK1 is recruited to the latter. TBK1 was shown to phosphorylate adaptor proteins, which in turn facilitates recruitment of IRF3 to the adaptor protein, thereby juxtaposing TBK1 with its primary target IRF3 (Liu, Cai, et al. 2015).

To evaluate the effect of viral candidates on IRF signalling pathways in particular, TBK1 was transfected into cells to drive ISRE-element dependent reporter activation (Figure 4-8A). The role of M1 as an NFκB-specific inhibitor was supported as this candidate was not seen to prevent reporter activity. Additionally, B13 and VV-WR 204 were not able to reduce reporter activation. In contrast, B12, B2 and A47, which became already apparent as potential IFNβ-promoter inhibitors in previous RGAs, reduced TBK1-mediated reporter activation by at least 70 %. F17 and A57 also appeared to restrict TBK1-mediated ISRE activation, however to a lesser extent than the aforementioned viral candidates. The target of these proteins may therefore lie between the adaptor and the downstream signalling proteins of the IRF pathway.

The transcription factors IRF3 and IRF7 can be phosphorylated by TBK1 and IKKε leading to transactivation ability. A transactivation reporter gene assay similar to the ELK transactivation assay was used to determine an effect of VV-WR proteins on TBK1- and IKKε-mediated IRF transactivation. This system uses an IRF3- or IRF7-Gal4 fusion protein activated through phosphorylation by TBK1 or IKKε, which once phosphorylated transactivates a firefly luciferase reporter under the control of Gal4-binding elements (Figure 4-8B,C).

Almost all of the studied viral proteins appeared to target either kinases that activated IRFs, or the transcription factors themselves, with the notable exception of M1, which recurrently presented with NFκB-specific inhibition. Interestingly, two different mechanisms of inhibition manifested themselves in this study: The above described inhibitors of TBK1-mediated ISRE activation (i.e. B12, B2, A47, F17) primarily or exclusively impaired IRF3 and IRF7 activation induced by TBK1, whereas B13 and VV-WR 204 preferentially suppressed IKKε-stimulated IRF3 and IRF7 transactivation ability. The viral candidate A57 inhibited both IRF3 and IRF7 transactivation by TBK1 or IKKε equally.

Once endogenous IRF3 and IRF7 are phosphorylated, these transcription factors homodimerise, translocate to the nucleus and induce the production of type I IFNs. Given the potent impairment of IRF3- and IRF7-transactivation, direct activation of an ISRE-element dependent reporter by transfection of IRF7, and constitutively active IRF3 and IRF7 was analysed in the presence of viral candidates (Figure 4-8D).

Viral proteins were only partially able to reduce reporter activation, compared to a similar experiment assaying NFκB activation by p65 (see 4.2.1). Notably, A47 appeared most effective of all candidates, able to

reduce reporter activation induced by both IRF3 and IRF7, whereas B2, F17 and VV-WR 204 only impaired IRF7 activity. Unexpectedly, M1 presented with some ability to limit IRF7 activity, which did not correlate with previous results. Interpretation of these results is more difficult as the overall inhibition levels were much lower than the ones observed in previous experiments. Either these viral proteins did indeed target host proteins upstream of IRF activation or the use of constitutively active transcription factors may have saturated reporter activity before sufficient amounts of viral proteins were present.

Despite the lack of strong impairment of ISRE-element dependent reporter activation, the effect of viral proteins on production of type I IFNs was evaluated in CdCl₂-inducible stably transfected HEK 293T (Figure 4-9A-E). In addition, the most promising inhibitors identified in previous RGAs were evaluated for their ability to transiently suppress SeV-mediated IFN β -promoter activation (Figure 4-9F). These experiments aimed to correlate a physiological role with the results obtained in previous RGAs. To evaluate type I IFN production, cells were transfected with TRIF and MAVS, and supernatants of transfected cells were analysed by a type I IFN bioassay (Figure 4-9A,E). It is worth noting that in spite of numerous attempts, activation of type I IFN production by transfection of cGAS and STING did not yield any detectable amounts of type I IFN (data not shown). Furthermore, the difficulties encountered previously with distinct supernatant acidification and cell growth in response to CdCl₂ treatment also interfered with these experiments (single experiments and experiments which were only repeated once are marked by '†').

Empty vector control cells produced reduced levels of type I IFNs when grown in presence of CdCl₂ (Figure 4-9A), and the usefulness of these cells for this assay is therefore questionable. B12 greatly suppressed TRIF and MAVS-driven type I IFN production (Figure 4-9E). F17 was also able to limit type I IFN secretion in response to MAVS overexpression (Figure 4-9B). It may be argued that the difference observed for B12 and to a lesser extent F17 is greater than the difference observed for EV stably transfected cells. Transient expression of candidates did not demonstrate any reduction of type I IFN production, however this may be due to low transfection levels.

Stimulation of cells with SeV (Figure 4-9F), which were transiently transfected with F17, showed reduced IFN β -promoter activity compared to cells transfected with empty vector control. Unfortunately, the large variation observed in cells transfected with B2 and B12 did not give a significant result.

4.2.3. Examining inhibition of cGAS-STING signalling

Despite the previous results indicating that viral ORFs inhibit downstream of receptor activation, inhibition of cGAS activity was evaluated in attempt to elucidate whether any of the candidates also directly target STING. To this end, HEK 293T cells stably expressing STING were used to investigate the cGAS/STING pathway through direct activation of STING with 3'3'-cGAMP. We did not attempt to drive NF κ B-reporter gene activity using 3'3'-cGAMP in STING-293Ts, because the ligand only poorly activated the IFN β -promoter (Appendix 1B). As a general finding, the IFN β reporter construct was activated to higher levels than the NF κ B reporter gene. Pathway activation through overexpression of cGAS was preferred, because cGAS synthesises the natural STING ligand 2'3'-cGAMP and activation levels were more pronounced. However, stimulation of STING-293Ts with 3'3'-cGAMP could indicate whether inhibitors act at the level of STING downstream of cGAS, and it was

therefore included in the present study (Figure 4-10B - Figure 4-17B). The results of the 3'3'-cGAMP study were not comparable with previous results of the different inhibitors. Except in the case of M1, none of the viral proteins were able to impair 3'3'-cGAMP-mediated IFN β -promoter activation, despite their inhibition of cGAS/STING-mediated pathway activation. M1 was the only candidate able to moderately reduce IFN β reporter gene activity induced by 3'3'-cGAMP. However, M1 was unable to impair cGAS/STING-mediated IFN β -promoter activation (Figure 4-10B). A possible technical explanation for this observation is that not all cells were stimulated with 3'3'-cGAMP, resulting in a lower activation level. The general assumption for reporter gene assays is that reporter, expression and control plasmids are transfected with equal transfection efficiency, and that therefore the control construct will transfect with a lower efficiency. This could explain the differences observed in cells activated by overexpression with cGAS and STING, and cells stimulated with 3'3'-cGAMP.

4.2.4. Summary of VACV protein characterisations

Eight putative VACV immunomodulators were chosen to be characterised in detail (Figure 4-10 - Figure 4-17). Inhibition for each protein was mapped in the respective networks through pathway activation by ligand stimulation (i.e. TNF α , IL-1, cGAMP), overexpression of receptor/adaptor proteins (i.e. cGAS/STING, TRIF, MAVS), signalling proteins (i.e. TBK1, IKKs) and transcription factors (i.e. IRF3, IRF7 and p65). Cellular localisation of overexpressed viral proteins was then evaluated. Mapping of the inhibition profiles of the individual viral candidates not only narrowed down the potential targets of these viral proteins, but also supported their ranking in terms of inhibition potency. The VV-WR proteins B2 and B12 were repeatedly confirmed as the most potent inhibitors of both the IRF and the NF κ B pathway, followed by F17, A47 and A57. In addition, specific inhibitory mechanisms were elucidated, e.g. the selective inhibition of IKK ϵ -mediated IRF3, IRF7 and NF κ B activation by B13 and VV-WR 204.

4.3. Discussion

In this study, 42 VV-WR proteins were selected to be screened for potential as PRR signalling pathway and pro-inflammatory cytokine inhibitors. After a rigorous screening process, which included DNA sensing, TLR, RLR, as well as TNFR, and IL-1R signalling pathways, eight viral candidates were identified as good inhibitors of pathways to NF κ B or IRF activation. These results demonstrate that the study of VACV proteins continues to lead to the discovery of novel potential immunomodulators, knowledge of which may improve VACV based vaccine vectors and the understanding of signalling pathways. These results also confirmed that the approach taken (i.e. rapid screen of viral proteins using RGAs, followed by more functional studies) was useful in rapidly identifying and characterising potential viral inhibitors of PRR and pro-inflammatory cytokine signalling pathways. However, the choice of CdCl₂-inducible cell lines does not appear suitable for analysis of cytokine production as CdCl₂ appears to effect normal cell growth. A summary of the results obtained for each protein are shown in Figure 4-10 - Figure 4-17 and will be discussed in the remainder of this chapter.

4.3.1. Detailed characterisation of novel VACV immunomodulators

Identification of potential immunomodulators can lead to a better understanding of innate immune signalling pathways, as previous studies of several VACV proteins have demonstrated (Bowie et al. 2000; Keating et al. 2007; Schröder et al. 2008). Such studies may also contribute to research into the use of VACV as a vaccine vector. The presence or absence of the viral candidates in the VACV Copenhagen derived strain vv811ΔA49 was already discussed in the previous chapter. shows a representation of the vv811ΔA49 genome, with white arrows marking ORFs removed from the parental genome, and red arrows indicating the position of the studied candidates. As previously mentioned, M1 and B13 are located within the deleted regions. The VACV Cop genome does not encode a VV-WR 204 homologue. F17, A47, A57, B2 and B12 are encoded by the vv811ΔA49 genome. These proteins may account for the impairment of NF κ B pathway signalling reported for this virus (Sumner et al. 2014). The eradication of smallpox was brought about by the utilisation of so-called 'first generation' VACV vaccines. The occurrence of adverse events ranging from sores at the vaccine site and fever to harmful, but rare reactions such as eczema vaccinatum, progressive vaccinia and postvaccinal encephalitis spurred the development of a safer vaccine strain (Jacobs et al. 2009). Third generation vaccine strains include LC16m8 and MVA, which were generated by serial passage in cell culture of parental virus strain VACV Lister and VACV Copenhagen, respectively (Antoine et al. 1998; Morikawa et al. 2005). This process attenuated the virus strains through fragmentation and deletion of viral ORFs, which, in the case of MVA, resulted in an approximately 15% loss of genomic information (Antoine et al. 1998). In contrast, the attenuated VACV strain NYVAC was obtained by targeted deletion of 18 viral genes associated with virulence from the parental VACV-Cop strain (Tartaglia et al. 1992). These attenuated VACV strains were and are still investigated for their capacity to function as poxvirus vaccines, vaccine vectors for other infectious diseases, and also in oncolytic virotherapy (Verardi et al. 2012). B13 and VV-WR 204 are not present in either strain (Tartaglia et al. 1992; Antoine et al. 1998; Morikawa et al. 2005). In contrast, both NYVAC and LC16m8 still encode F17, A47, A57, B2 and B12. The latter also encodes M1. The MVA genome only harbours F17, A47 and B12, whilst A57 is truncated, and B2 is fragmented (Antoine et al. 1998). Deletion of these viral candidates

may improve the safety and immunogenicity of these VACV strains, as was reported for removal N2, and C6 from an MVA-based HIV vaccine vector (García-Arriaza et al. 2011; García-Arriaza et al. 2014).

4.3.1.1. M1

The approximately 55 kDa protein encoded by the VV-WR ORF M1L is a putative NF κ B pathway inhibitor, which probably exerts its action downstream of the receptor adaptor proteins and upstream of the IKK proteins, as depicted in Figure 4-10A. Belonging to the group of early expressed VACV genes, this protein is likely to be involved in immune evasion (Assarsson et al. 2008; Yang et al. 2015). An ankyrin repeat domain was predicted for this protein, which is known to mediate protein-protein interactions, suggesting that M1 has the potential to interact directly with its target. A series of RGAs demonstrated M1's selectivity for NF κ B inhibition, as it was unable to impair IFN β -promoter dependent and ISRE activation driven by overexpression of the receptor and adaptor proteins cGAS/STING and TRIF, as well as by overexpression of the signalling protein TBK1 (Figure 4-10B,C). Additionally, IRF3 and IRF7 transactivation assays revealed M1's inability to restrict TBK1- and IKK ϵ -mediated activation of these transcription factors (Figure 4-10D). As noted before (see 4.2), IFN β -promoter activation stimulated by 3'3-cGAMP in STING-293Ts was inhibited, yet cGAS/STING mediated promoter activation remained unaltered (Figure 4-10B). ISRE-element dependent reporter activation mediated by IRF3 and IRF7 was reduced by M1, however these results do not correlate with its lack of inhibition on other IRF signalling pathway events (Figure 4-10C).

The potential NF κ B inhibition was mapped downstream of the adaptor protein MAVS, and upstream of the IKK proteins, as M1 was able to impair MAVS-, IL-1- and TNF α -driven NF κ B, yet failed to inhibit IKK complex-mediated activation (Figure 4-10E). This mechanism of inhibition is further supported by its cytosolic localisation, as the above mentioned signalling events take place in the cytoplasm (Figure 4-10F). Despite numerous attempts, a CdCl₂-inducible expression construct could not be generated, which impeded the characterisation of its effects on I κ B α degradation and p65 phosphorylation.

The absence of M1 in the genome of the VACV Copenhagen strain vv811 Δ A49 does not render the discovery of its inhibitory effects less important, as the candidate may still contribute to NF κ B inhibition (Sumner et al. 2014). No studies on its role as a potential virulence factor (i.e. disease progression in an appropriate animal model) have been carried out to date. Such studies may clarify this issue. M1 was not further characterised due to the lack of an inducible stable cell line and as other candidates appeared more promising.

4.3.1.2. F17

The VV-WR ORF F17R encodes an approximately 11.5 kDa cytoplasmic protein, which was shown to efficiently restrict IRF and NF κ B pathway activation at the level of TBK1 and the IKKs (Figure 4-11A,F). Interestingly, this protein may be one of the few immunomodulators encoded by all chordopoxviruses besides D9, D10 and H1 (Gubser et al. 2004). F17 is a late gene, and was shown to rank amongst the ten most highly expressed intermediate and late genes during viral infections (Yang et al. 2015). Here, we propose a role for F17 early during infection in addition to its established essential role in virion morphogenesis (Zhang & Moss 1991;

Pedersen et al. 2000; Wickramasekera & Traktman 2010; Schmidt et al. 2013). Although the majority of immunomodulators are expressed early during infection (Assarsson et al. 2008), there are exceptions to this general assumption, such as the secreted semaphorin-like protein A39 (Gardner et al. 2001), the soluble IL-1 β receptor B15 (Alcami & Smith 1992), the intracellular protein B7 (Price et al. 2000), and H1 (Liu et al. 1995), whose expression is controlled by late promoters. The latter, like F17, is associated with virion assembly, however the impact of H1 is more subtle. H1 appears to be involved in virion core stability (Condit et al. 2006). Absence of H1 leads to hyperphosphorylation of F17, A14, and A17; proteins which are required for virion assembly (Liu et al. 1995; Derrien et al. 1999). H1 is packaged into virions, with a density of approximately 200 copies/particle, where it is localised in lateral bodies (Liu et al. 1995; Schmidt et al. 2013). Upon entry, lateral bodies disassemble, H1 is released and can impair STAT1 signalling (Schmidt et al. 2013). It is estimated that the number of F17 protein vastly outnumber that of H1, at around 27,000 per virion (Sarov & Joklik 1972). Indeed F17 has been shown to be the most (Resch et al. 2007), or the third most abundant protein (Chung et al. 2006) in the virion. Although a recent report has suggested that F17 is degraded upon cell entry (Schmidt et al. 2013), the large number of F17 proteins may account for a very early role in infection targeting NF κ B activation, as hinted by the transient effect observed on p65 by ectopically expressed F17 (Figure 4-4B). A delay of p65 phosphorylation may interfere with subsequent post-translational modifications, which are required for full activation of NF κ B (Huang et al. 2010). F17 may also be able to intervene in IRF signalling as indicated by the reduced production of type I IFNs in cells stably expressing F17 (Figure 4-9B). However, these results are difficult to interpret due to the possible effect of CdCl₂ on cell growth. Transient expression of F17 did impair SeV-driven IFN β -promoter activation (Figure 4-9F). Despite its ability to impede the IRF signalling pathway as evidenced by reduction of ISRE-element dependent reporter activity driven by MAVS, TBK1 and IRF7 (Figure 4-11C), F17 appears to more potently interfere with NF κ B activation (Figure 4-11E). This viral protein impairs NF κ B-dependent reporter activation in response to stimulation with ligands (i.e. TNF α (Figure 4-11E)), overexpression of receptor and adaptor proteins (i.e. cGAS/STING, TRIF, MAVS (Figure 4-11E)), and of downstream signalling pathway components (i.e. TRAF2, TRAF6 (Figure 4-2), IKKs (Figure 4-11E)). In contrast, F17's ability to reduce p65-driven NF κ B activation is overcome by increasing the amount of transfected p65 (Figure 4-6). In accordance with the transient effect observed for F17 on p65 phosphorylation, and the lack of impact on I κ B α degradation (Figure 4-4B), preliminary data (N=1) have shown that transient transfection of F17 had no influence on nuclear translocation of p65 in response to TNF α (Figure 4-5). The VV-WR ORF F17L is still encoded in the genome of VACV Copenhagen strain vv811 Δ A49, indicating a putative contribution to the NF κ B inhibition reported for the mutant virus occurring in the cytoplasm, which may be independent of NF κ B nuclear translocation.

Early studies of F17 showed that this viral protein was preferentially binding to superhelical DNA. This trait was attributed to its basic character, as no apparent function in genome packaging during virion assembly was identified (Kao et al. 1981). Whilst several studies have demonstrated the binding of IFI16 to naked linear dsDNA (Jin et al. 2012; Morrone et al. 2014), one report showed that IFI16 could also bind to superhelical DNA (Brázda et al. 2012), which may be prevented by F17. No studies of cGAS associating with superhelical DNA have been reported to date. The above suggested early role in NF κ B-proximal activation poses difficulties to addressing the possibility of F17 intervening in viral DNA detection by cGAS and IFI16. Activation

of both IFN β -promoter- and NF κ B-dependent reporter genes through transfection of cGAS and STING was impaired by transient expression of F17 (Figure 4-11B,E). In contrast, F17 did not reduce 3'3'-cGAMP-mediated activation of IFN β -promoter in STING-293T cells (Figure 4-11B). The latter would favour a role of F17 targeting DNA sensing. RGAs used as a method to study viral proteins cannot be used to identify upstream roles of F17, as F17 appears to impair signalling events proximal to NF κ B.

4.3.1.3. A47

The VV-WR ORF A47L is highly conserved amongst orthopoxviruses, and encodes a 29 kDa protein that does not possess any known motifs. Yuen and co-authors revealed that this early expressed gene was not a virulence gene (Yuen et al. 2010; Yang et al. 2015). In this study, the authors demonstrated that deletion of A47 from the viral genome had no effect on viral replication *in vitro* and *in vivo*, on plaque morphology, or disease progression in intradermally and intranasally infected mice (Yuen et al. 2010). However, the potency of inhibition of the investigated PRR and pro-inflammatory cytokine signalling pathways and its partial nuclear localisation led us to further characterise this protein as detailed in the following section.

Cellular localisation studies revealed the presence of A47 in the cytoplasm and in the nucleus (Figure 4-12F). A47 was observed to form distinct foci in each compartment, and was also observed to be expressed without any particular pattern (Figure 4-12F, data not shown). Amongst the candidates, A47 was one of the strongest inhibitors of TNF α - and IL-1-mediated NF κ B-dependent reporter activation (Figure 4-1). For this reason, and for its partial nuclear localisation and its presence in the vv811 Δ A49 genome, we postulate that A47 may play an important role in NF κ B pathway signalling inhibition. Although IFN β inhibition appeared to be largely mediated through NF κ B inhibition, as A47 was unable to interfere with cGAS/STING- and TRIF-mediated ISRE-element activation (Figure 4-12C), the protein demonstrated great potential in restricting TBK1- and IKK ϵ -driven IRF3- and IRF7-transactivation (Figure 4-12D). The observed NF κ B inhibition was mapped to the level of either the IKK complex (Figure 4-12E), or possibly NF κ B itself, as inhibition of NF κ B-dependent promoter activation was only surpassed by saturating the reporter activation (Figure 4-6). Preliminary data (N=1) for events following IKK complex activation, i.e. degradation of I κ B α , p65 phosphorylation and nuclear translocation, and following NF κ B activation, i.e. IL-8 and type I IFN production did not suggest that A47 affected these processes (Figure 4-4, Figure 4-5, Figure 4-7C, Figure 4-9C).

4.3.1.4. A57

The protein blast search for the approximately 17.5 kDa protein encoded by the early-late VV-WR gene A57R (Assarsson et al. 2008), revealed a predicted guanylate kinase region. In context with the recent discovery of cGAS as an important sensor of poxvirus-derived, cytosolic DNA (Schoggins et al. 2014), we postulated a role for this protein in regulating this signalling cascade. As described in 1.2.2.3.3, cGAS synthesises cGAMP from ATP and GTP in response to DNA binding. Guanylate kinases catalyse ATP-dependent phosphorylation of GMP, and therefore have a direct influence on nucleotide levels (Fitzgibbon et al. 1996; Zhang et al. 2015). Protein sequence alignment of human GUK1 and A57, however, suggested that A57 lacks key residues to function as a kinase (see 3.2.2.1). A57 strongly impaired the cGAS/STING signalling cascade, when IFN β -

reporter activity was driven by overexpression of STING and cGAS (Figure 4-13B). This inhibition was abolished when 3'3'-cGAMP was used for pathway activation (Figure 4-13B), indicating that cGAS activity may be the target of A57. Many VACV proteins have multiple roles in immune evasion, and the effect on nucleotide levels or cGAS might be masked by the targeting of downstream signalling proteins. Unfortunately, the experimental setup used to screen the viral candidates did not allow us to distinguish between these two possibilities. In order to assess an effect on nucleotide levels, these would have to be examined early during infection in the presence and absence of A57 by a cGAMP activity co-culture assay (Ablasser et al. 2014).

The aforementioned effect on IFN β -promoter activation was mapped to the level of IRF3 and IRF7 activation by TBK1 and IKK ϵ (Figure 4-13A), as ISRE activation by overexpression of these transcription factors was not impaired (Figure 4-13C,D). Inhibition of NF κ B activation appeared to be restricted to PRR signalling pathway activation as neither activation by TNF α nor IL-1 was reduced (Figure 4-13E). However, A57 potentially inhibited IKK α -, IKK β - and p65-driven NF κ B-dependent promoter activation, which are all part of the TNFR and IL-1R signalling cascades (Figure 4-13E). Preliminary data (N=1) suggested that this viral protein had no effect on TNF α -mediated I κ B α degradation, p65 phosphorylation and nuclear translocation (Figure 4-4D, Figure 4-5), which correlates with its inability to reduce reporter activation in response to TNF α . A57 was found to localise in both the cytoplasm and the nucleus in distinct foci (Figure 4-13F), and it was able to impair reporter activation despite transfection of increasing amounts of p65 (Figure 4-6), which may indicate that A57 targets NF κ B activity in the nucleus. Its presence in the VACV Copenhagen strain vv811 Δ A49 further supports a putative role in NF κ B inhibition. This candidate demonstrated its potential to be an IRF and NF κ B inhibitor, yet no studies have been undertaken to this date to investigate its potential as a virulence factor.

4.3.1.5. B2

The VV-WR ORF B2R is an early gene, suggesting an important role in immune evasion, as it has been shown for many genes belonging to this group (Assarsson et al. 2008; Yang et al. 2015). Interestingly, quantification of B2R RNA levels during viral infection of HeLa cells ranked it at third position after VV-WR 018 and E3 at early (0.5 hours post-infection, 1 hpi and 2 hpi), and intermediate (4 hpi) and late (8 hpi) timepoints (Assarsson et al. 2008). However, a recent transcriptome study assigned a much lower, yet nonetheless important rank to B2 (26st) after E3, C6, A46, N2, M2, and N1 (Yang et al. 2015). Both studies showed that B2 transcription levels are highest immediately after infection and decline over time. It is possible that the experimental protocol used by Yang and co-authors did not allow the detection of high levels of translated B2 RNA due to the choice of the first timepoint (2 hpi), whereas Assarsson *et al.* included earlier timepoints (0.5 hpi and 1 hpi).

The results of the present study showed that B2 was the most potent novel inhibitor of IRF and NF κ B pathway activation overall (Figure 4-14). This protein was the only candidate able to efficiently impair NF κ B, as well as ISRE-element activation irrespective of the activating pathway (Figure 4-14C,E). B2 was also one of the few studied VACV proteins able to impair TNF α - and IL-1-driven NF κ B-element dependent reporter activation (Figure 4-14E). This observation was further supported by reduction of TRAF2- and TRAF6-mediated NF κ B activation (Figure 4-2). NF κ B activity is likely impaired at the level of the IKKs or NF κ B itself, as increasing amount of p65 did not surpass the B2-mediated restriction of reporter activation (Figure 4-6). In addition,

TNF α -stimulated degradation of I κ B α , p65 phosphorylation and possibly nuclear translocation (N=1) were not affected by B2 (Figure 4-4E, Figure 4-5). These results, its nuclear as well as cytoplasmic localisation (Figure 4-14F), and its presence in the mutant virus vv811 Δ A49 genome suggest a role targeting the activity of activated NF κ B in the nucleus. Inhibition of the IRF pathway was mapped to the level of transcription factor activation (Figure 4-14A), as targeting of TBK1 and IKK ϵ resulted in decreased transactivation of IRF3 and IRF7 (Figure 4-14D). Furthermore, ISRE activation by overexpression of IRF7 was reduced by B2 (Figure 4-14C). Despite its overall ability to restrict both IFN β -promoter and NF κ B activation, B2 was not found to interfere with type I IFN production following transfection of a MAVS expression construct (Figure 4-9D), or IFN β -promoter activation in response to SeV (Figure 4-9F). The lack of effect may be due to technical difficulties such as effect of CdCl₂ on cell growth and low transfection efficiency. Unfortunately, its similarity to the *Baculovirus* p26 domain did not provide any indications regarding its function. Although confirming the cytosolic localisation, nothing other than the potential to form dimers is known about p26 (Goenka & Weaver 2008). Given its great inhibitory potential, an assessment of its effect on disease progression in an appropriate animal model may determine its role as a virulence factor.

4.3.1.6. B12

The VV-WR CRF B12R belongs to the early genes, suggesting an involvement in immune evasion, as demonstrated for many other proteins belonging to this group (Assarsson et al. 2008; Yang et al. 2015). It encodes an approximately 33.5 kDa nuclear protein, which could account for the residual NF κ B inhibition observed in the vv811 Δ A49 study (Figure 4-15) (Sumner et al. 2014). In the present screen, it manifested as the second best PRR and pro-inflammatory cytokine signalling inhibitor, probably targeting the activation or action of transcription factors in the nucleus (Figure 4-15A). NF κ B-dependent reporter activation was reduced by this viral candidate in response to TNF α and IL-1 stimulation, overexpression of cGAS/STING, TRIF and MAVS, of TRAF2 and TRAF6 (Figure 4-2), of IKK α , IKK β , and IKK ϵ , and of p65 (Figure 4-15E). Consistent with the hypothesis that this viral candidate affects NF κ B activity in the nucleus, NF κ B-dependent promoter activation was impaired, despite use of increasing amounts of p65 expression construct (Figure 4-6). Furthermore, B12 expression had no effect of TNF α -induced degradation of I κ B α degradation, p65 phosphorylation and possibly on p65 nuclear translocation (N=1) (Figure 4-4F, Figure 4-5). IRF pathway restriction was observed at the level of TBK1-mediated transactivation of IRF3 and IRF7 (Figure 4-15D). Although B12 was unable to prevent ISRE activation through overexpression of these transcription factors (Figure 4-15C), it was able to impair type I IFN production in cell lines stably transfected with B12 (Figure 4-9E). The reduction observed in B12 expressing cells may be due to the effect of CdCl₂, but it was greater than the reduction seen in empty vector control cells (Figure 4-9A). Taken together, this data indicates that B12 may be a very potent nuclear inhibitor of PRR and pro-inflammatory cytokines signalling pathways. However, it was shown to have no impact on viral replication *in vitro* and on disease progression *in vivo* (Banham & Smith 1993; Tschärke et al. 2002).

B12 is one of three Ser/Thr kinases encoded in the VV-WR genome. However, an early study demonstrated its inability to act as a kinase, because it lacked key residues in conserved regions of the active site, an ATP-binding site could not be identified, and B12 was unable to phosphorylate α - and β -casein, as well as calf

thymus histones (Howard & Smith 1989; Banham & Smith 1993). Although these proteins are not the natural substrates of B12, which could account for the lack of kinase activity, they were phosphorylated by the other VV-WR kinases F10 and B1 (Banham & Smith 1992; Lin & Broyles 1994). Interestingly, a 30 % a.a. sequence identity with the host protein VRK1 was discovered as part of the protein BLAST search (Table 3-1). VRK1 is a nuclear kinase, and it is involved in cell cycle progression. It is known to phosphorylate a series of transcription factors, yet no role in innate immunity has been suggested to this date (Klerkx et al. 2009). Consistent with the hypothesis that VRK1 is a target of B12, B12 was found almost exclusively expressed in the nucleus (Figure 4-15F). Co-immunoprecipitation of B12 with VRK1 could clarify whether VRK1 is indeed a target of B12.

4.3.1.7. B13

The VV-WR ORF B13R is an early gene and encodes an approximately 38.5 kDa protein (Assarsson et al. 2008; Yang et al. 2015). B13 shares nearly 90 % a.a. sequence identity with the CPVX protein CrmA, and was reported as an efficient apoptosis inhibitor (Table 3-1). Studies demonstrated B13-mediated inhibition of pro-IL-1 β processing by caspase-1 and inhibition of caspase-3 and -9 activity (Kettle et al. 1997; Veyer et al. 2014). Its potential role as a virulence factor has been investigated in two reports, revealing 1) its expendability for viral replication *in vitro*, and for disease progression in intranasally infected mice; and yet, 2) its negative effect on lesion size in intradermally infected mice (Kettle et al. 1997; Tschärke et al. 2002). In this study, an involvement of B13 in PRR signalling pathway and pro-inflammatory cytokine inhibition was investigated and although it did not appear to be the most promising candidate, it revealed a possible specific inhibition for IKK ϵ -mediated signalling (Figure 4-16A).

B13 was unable to restrict TBK1-mediated activation of IFN β -promoter, and ISRE-element dependent reporter genes (Figure 4-16B,C). Intriguingly, it was not only able to impair IKK ϵ -induced IRF3 and IRF7 transactivation, but also IKK ϵ -mediated NF κ B-dependent promoter activation (Figure 4-16D,E). However, it appeared unable to inhibit any of the other investigated kinases (Figure 4-16E), confirming its specific inhibition of IKK ϵ . Inhibition was not observed downstream of IKK ϵ , as overexpression of IRF3, IRF7 or p65 lead to unrestricted promoter activation (Figure 4-16C,E). Similarly, events leading to NF κ B activation, i.e. I κ B α degradation and possibly p65 phosphorylation were not affected by the candidate (Figure 4-4G). B13 was found to localise in the nucleus and the cytosol, which was consistent with the localisation of its potential targets (Figure 4-16A,F). The absence of B13R in the genome of the VACV Copenhagen strain vv811 Δ A49 does not exclude a role for B13 in NF κ B inhibition, it implies that it cannot account for restriction of nuclear translocation of NF κ B (Sumner et al. 2014).

4.3.1.8. VV-WR 204

VV-WR 204 is present in the genome of HSPV, CPXV, TATV, ECTV, CMLV and *Yoka poxvirus*, yet absent in the VACV Copenhagen strain. It was reported to be an early gene, which encodes a 16 kDa protein (Assarsson et al. 2008). However, a recent study revealed that VV-WR 204 may not be expressed during viral infection (Yang et al. 2015). A kelch motif was predicted for VV-WR 204, which are known to mediate direct protein-protein

interaction, and therefore VV-WR 204 could interact directly with its target (Table 3-1). Intriguingly, the impairment of activated IRF and NF κ B pathways seemed mainly restricted to IKK ϵ (Figure 4-17A).

VV-WR 204 inhibited both IRF and NF κ B activation in the context of PRR pathway signalling, but not pro-inflammatory cytokine receptor signalling, as neither TNF α - nor IL-1-mediated NF κ B activation was impaired by VV-WR 204 (Figure 4-17E). This protein reduced IRF3 and IRF7 transactivation driven by IKK ϵ , as well as NF κ B activation by IKK ϵ (Figure 4-17D,E). Accordingly, VV-WR 204 was not able to affect IKK complex activation of NF κ B, or TNF α stimulated degradation of I κ B α , and possibly phosphorylation of p65 (Figure 4-4H). It also impaired TBK1-mediated IRF3 transactivation (Figure 4-17D). Activation of promoters through overexpression of transcription factors (i.e. IRF3, IRF7, p65) was not prevented (Figure 4-17C,E). Taken together, these findings suggest that VV-WR 204 may restrict IRF and NF κ B signalling in the cytosol at the level of TBK1 and the IKK complex when expressed transiently (Figure 4-17A,F).

Overall, the results in this chapter provide initial mechanistic insights into how the selected VV-WR ORFs impair PRR and pro-inflammatory signalling pathways. The following chapter expands on these results by attempting to identify potential host proteins interacting with the viral proteins.

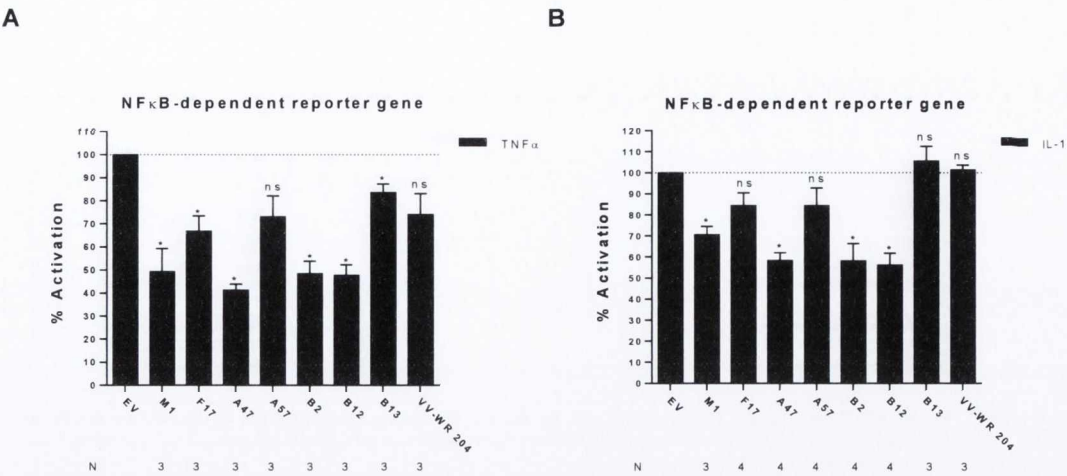


Figure 4-1 Inhibition of TNFα and IL-1 induced NFκB-dependent reporter gene activation

HEK 293T cells were co-transfected with a renilla luciferase control, a firefly luciferase reporter plasmid under the control of NFκB-elements, and indicated VV-WR protein expression plasmids or empty vector control (EV). Cells were stimulated with (A) 12.5 ng/ml TNFα or (B) 20 ng/ml IL-1 at 24 hpt. Cells were harvested 24 hps, firefly luciferase activity was measured and normalised to renilla luciferase activity. VV-WR protein assay values were scaled relative to EV, which was set to 100 % activation (black dotted line). Means of at least three independent experiments with triplicate determination are shown with their SEMs. Exact number of independent experiments (N) is given below each graph. *p<0.05, ns (not significant).

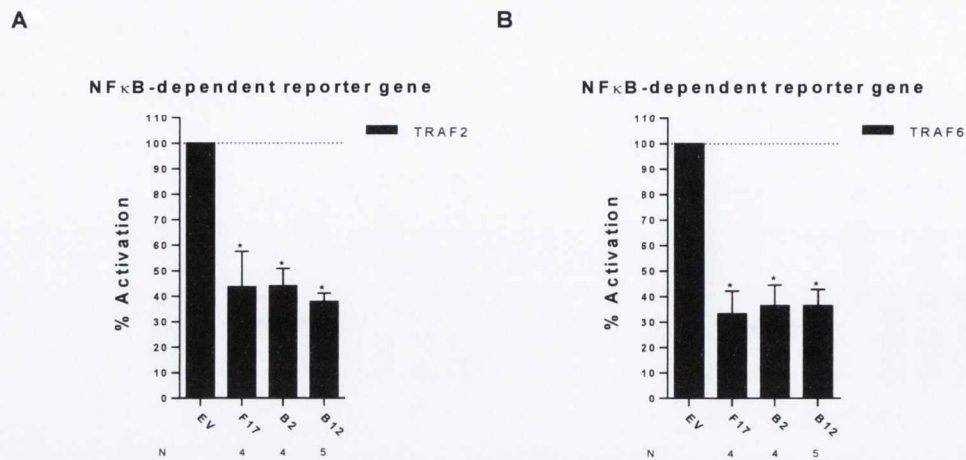


Figure 4-2 Inhibitory effect on TRAF2- and TRAF6-mediated NFκB activation by selected viral proteins

HEK 293T cells were co-transfected with a firefly luciferase reporter plasmid under the control of NFκB-elements, a renilla luciferase control and (A) TRAF2 or (B) TRAF6 expression constructs. These cells were also transfected with the indicated VV-WR protein expression plasmids or empty vector control (EV). Cells were harvested 24 hpt, firefly luciferase activity was measured and normalised to renilla luciferase activity. VV-WR protein assay values were scaled relative to EV, which was set to 100 % activation (black dotted line). Mean of at least three independent experiments with triplicate determination are shown with their SEMs. Exact number of independent experiments (N) is given below each graph.*p<0.05, ns (not significant).

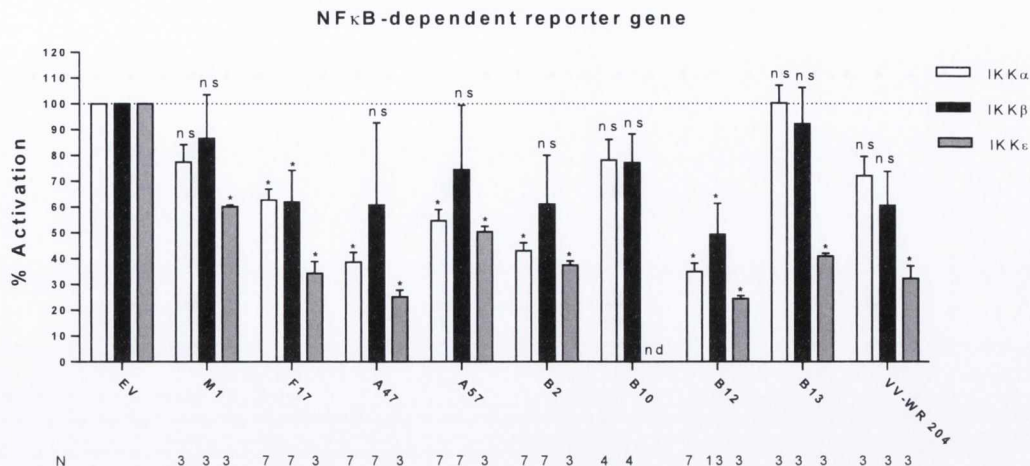


Figure 4-3 Detailed mapping of NFκB inhibition downstream of PRRs

HEK 293T cells were co-transfected with a firefly luciferase reporter plasmid under the control of NFκB-elements, a renilla luciferase control along with either IKKα, IKKβ, or IKKε. In addition, cells were transfected with the indicated VV-WR ORF expression constructs or empty vector control (EV). Cells were harvested 24 hpt, firefly luciferase activity was measured and normalised to renilla luciferase activity. VV-WR protein assay values were scaled relative to EV, which was set to 100 % activation (black dotted line). Means of at least three independent experiments with triplicate determination are shown with their SEMs. Exact number of independent experiments (N) is given below each graph. *p<0.05, ns (not significant). nd (not determined).

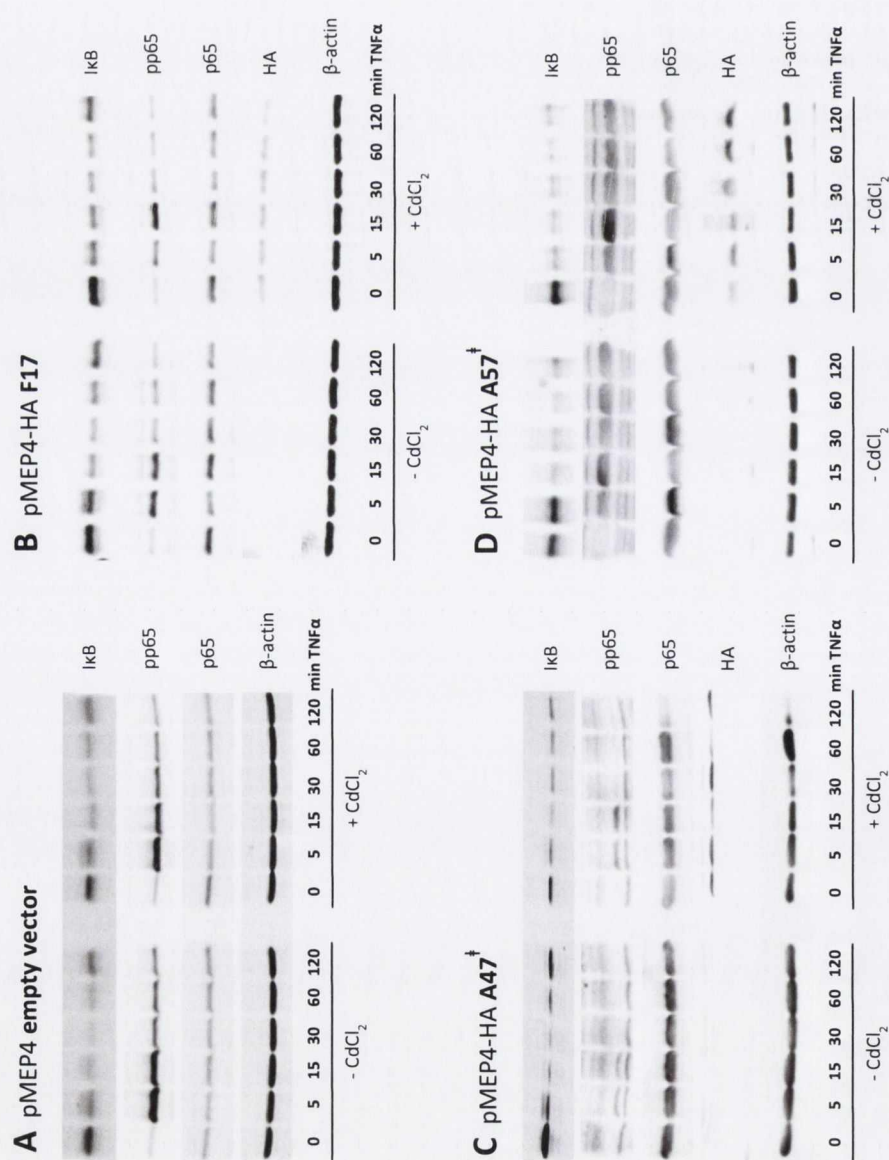


Figure 4-4 Inhibition of TNFα induced NFκB signalling cascade (see next page for legend)

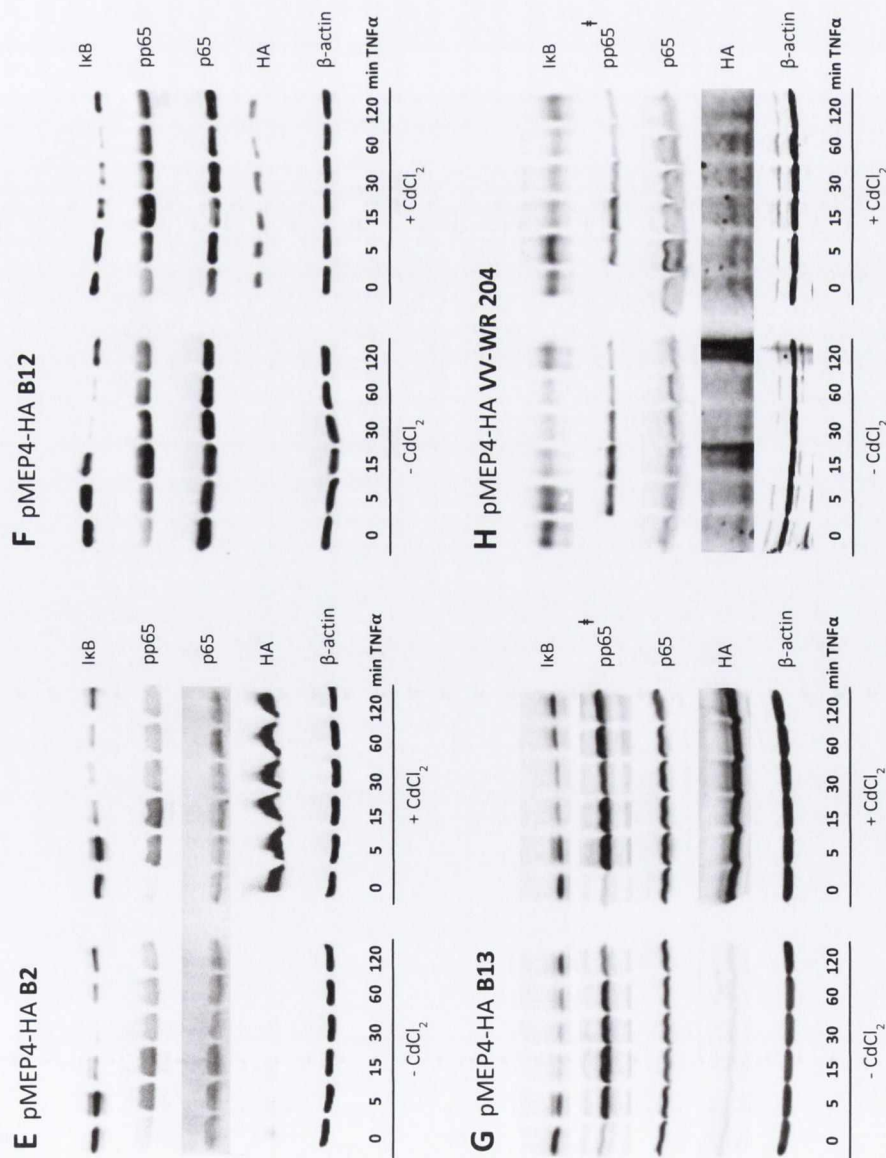


Figure 4-4 Inhibition of TNFα induced NFκB signalling cascade

(A-H) HEK 293T cells were transfected with 8 μg of a CdCl₂ inducible VV-WR protein expression construct (pMEP4HA-VV-WR ORF), and selected and maintained in 300 μg/ml Hygromycin. Forty-eight hours after induction with 1 μM CdCl₂, stably transformed cells were stimulated with 50 ng/ml TNFα at 0, 5, 15, 30, 60 and 120 min. Proteins from cell extracts were separated by SDS-PAGE and analysed by immunoblotting with anti-IκB, anti-pp65, anti-p65, anti-HA, and anti-β-actin antibodies. Cells that were not induced with CdCl₂ served as internal negative controls, whilst cells containing empty pMEP4 vector control (EV) served as CdCl₂ negative controls. Unless indicated by †, a representative of at least 3 independent experiments is shown.

A

	Total cell number	Nuclear p65	VV-WR ORF positive cells	VV-WR ORF positive cells: nuclear p65
EV: NS	577	194	-	-
EV: TNFα	137	136	-	-
F17: TNFα	582	578	47	47
A47: TNFα	1003	997	46	44
A57: TNFα	626	612	24	24
B2: TNFα	587	580	41	41
B12:TNFα	915	907	53	53

B

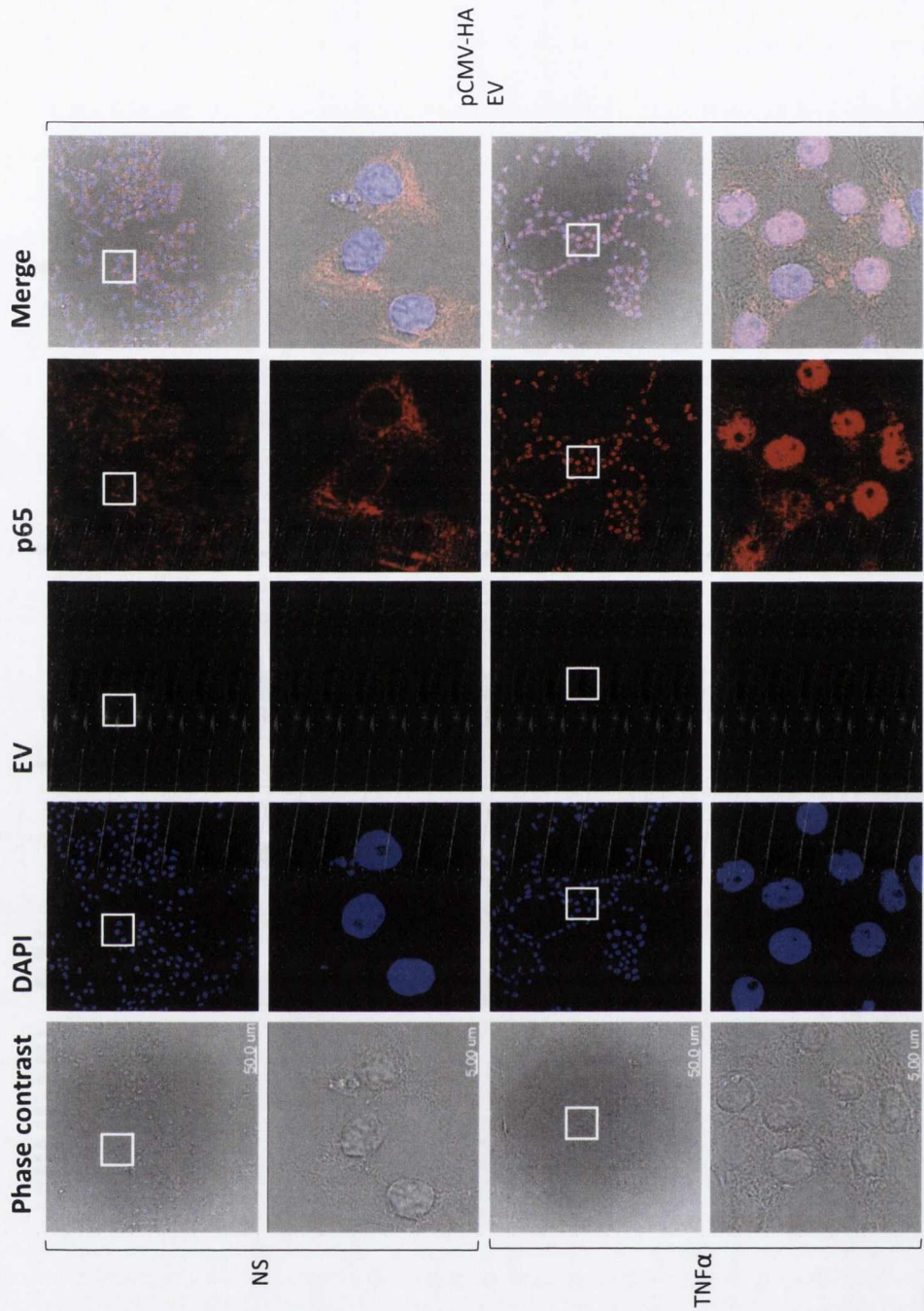


Figure 4-5 NFκB inhibitory VV-WR proteins do not prevent TNFα-stimulated nuclear translocation of p65 (see page 107 for legend)

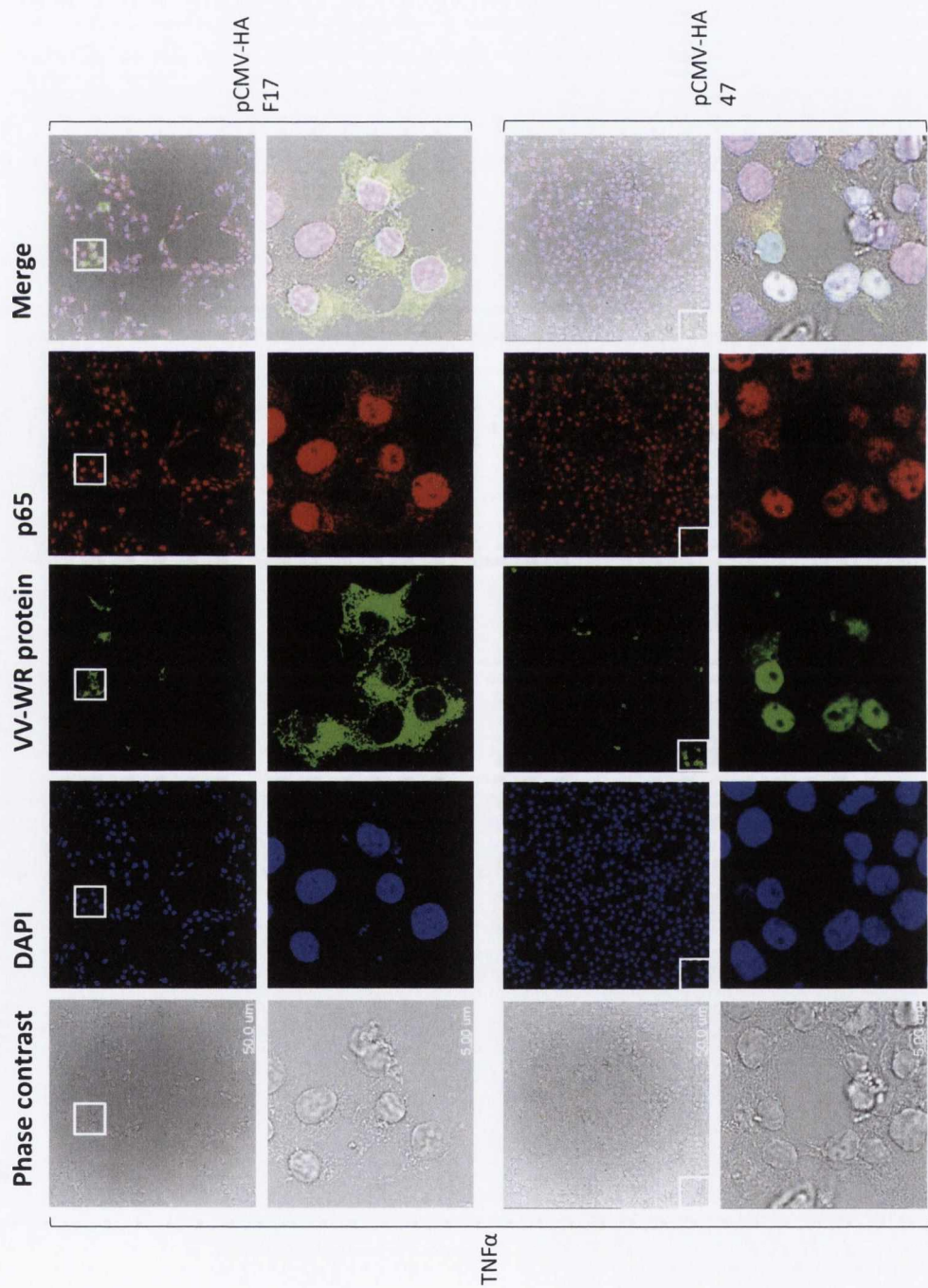


Figure 4-5 NFκB inhibitory VV-WR proteins do not prevent TNFα-stimulated nuclear translocation of p65 (see page 107 for legend)

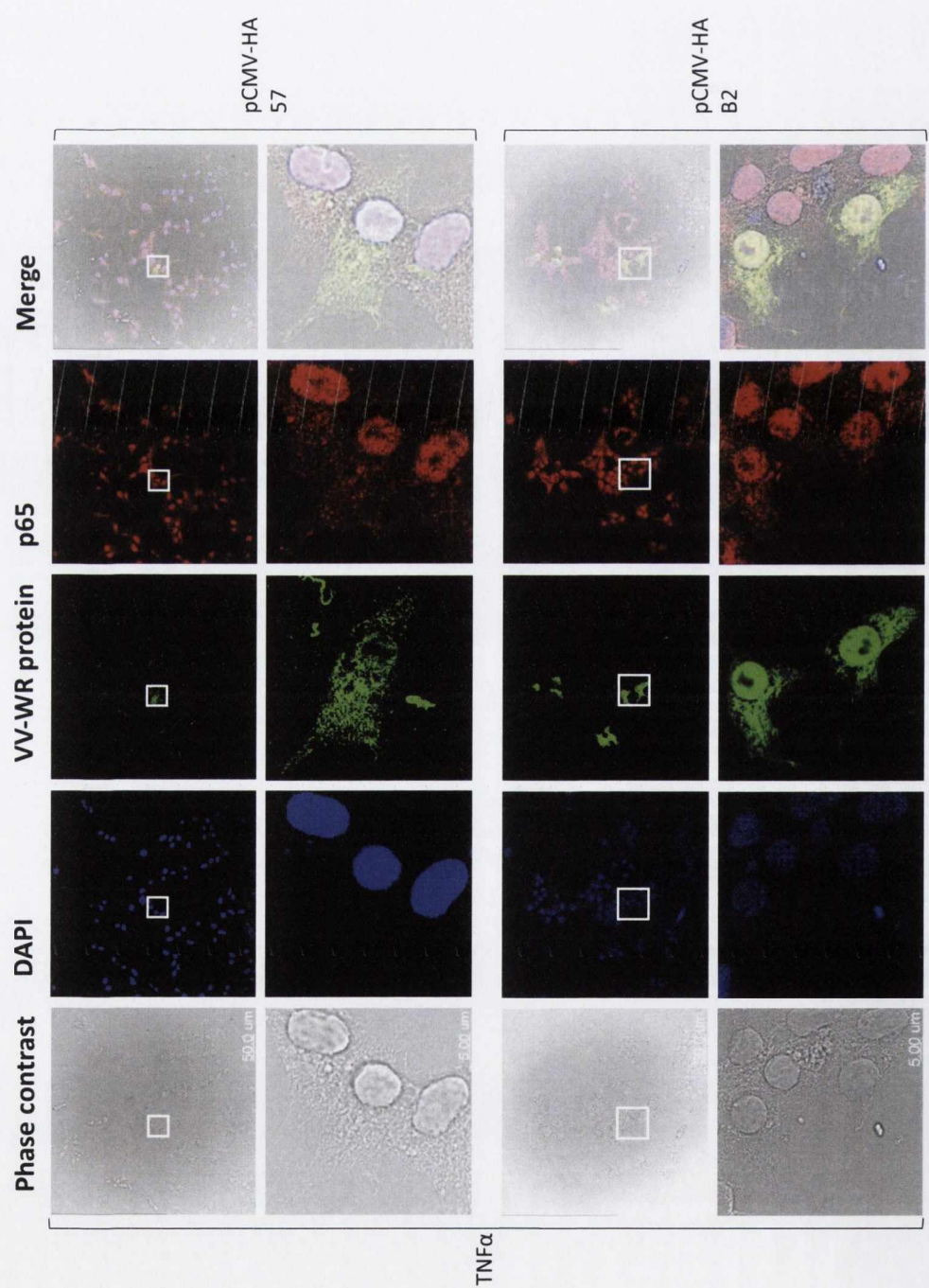


Figure 4-5 NFκB inhibitory VV-WR proteins do not prevent TNFα-stimulated nuclear translocation of p65 (see next page for legend)

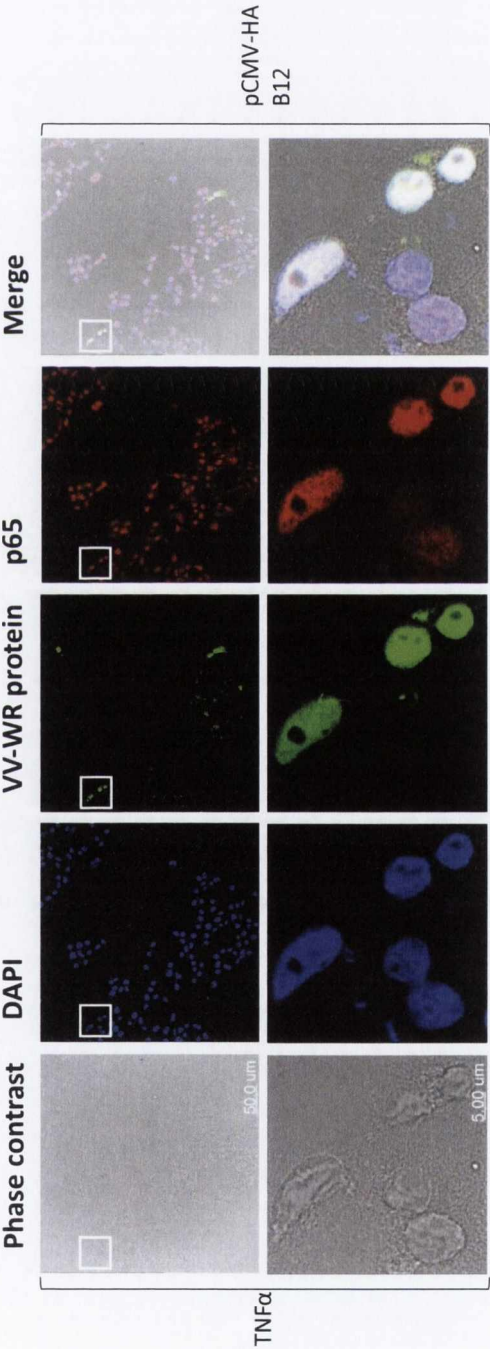


Figure 4-5 NFκB inhibitory VV-WR proteins do not prevent TNFα-stimulated nuclear translocation of p65

HEK 293T cells grown on coverslips were transfected with the indicated VV-WR expression plasmids or empty vector control (EV) and stimulated 24 hpt with 50 ng/ml TNFα. Fixed cells were stained with anti-HA (green) and anti-p65 (red) antibodies. Nuclear DNA was stained with DAPI (blue). (A) Table listing total number of cells, total number of cells with nuclear p65, total number of VV-WR-positive cells and total number of VV-WR positive cells with nuclear p65 was based on a single experiment. (B) White rectangle in upper panel indicates positions of magnified section shown in lower panel. NS (not stimulated).

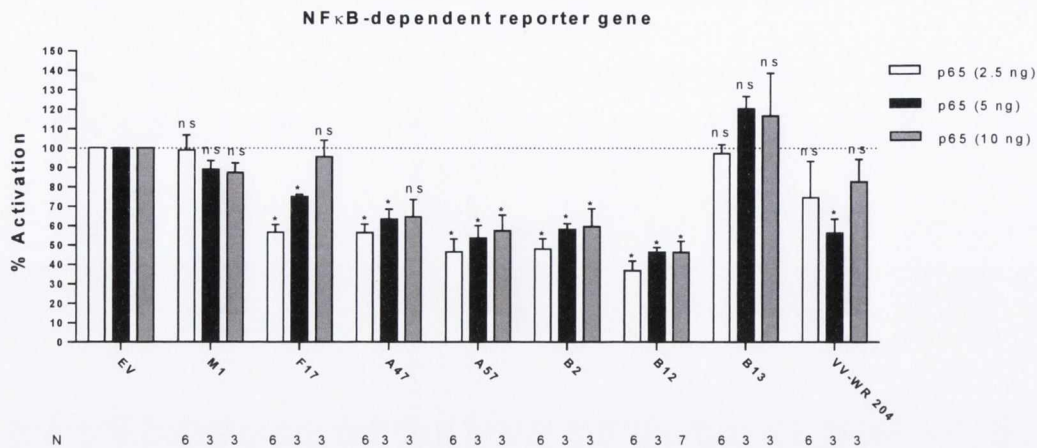


Figure 4-6 Direct effect of NFκB inhibiting VV-WR proteins on p65-depdenent NFκB activation

HEK 293T cells were co-transfected with a firefly luciferase reporter plasmid under the control of NFκB-elements, a renilla luciferase control, and increasing amounts of p65 expression plasmid. In addition, cells were transfected with the indicated VV-WR ORF expression constructs or empty vector control (EV). Cells were harvested 24 hpt, firefly luciferase activity was measured and normalised to renilla luciferase activity. VV-WR protein assay values were scaled relative to EV, which was set to 100 % activation (black dotted line). Mean of at least three independent experiments with triplicate determination are shown with their SEMs. Exact number of independent experiments (N) is given below each graph.*p<0.05, ns (not significant).

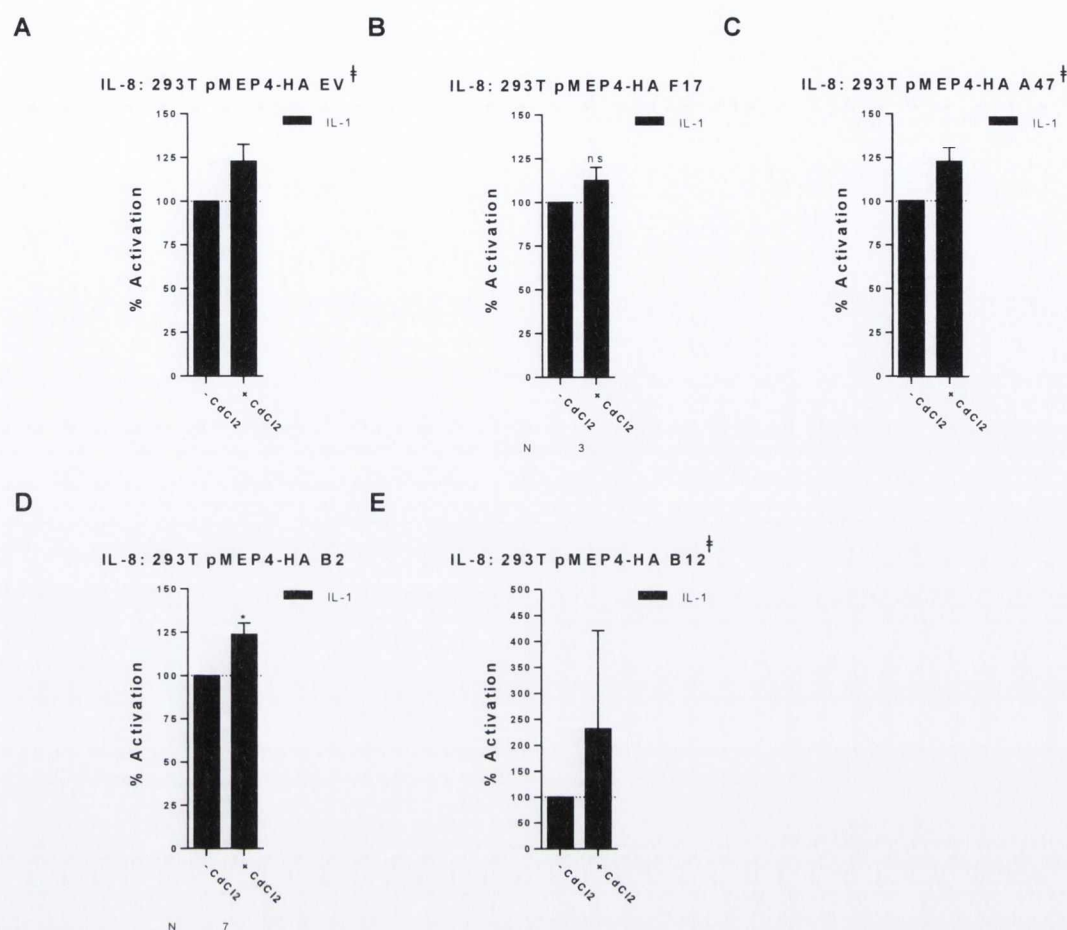
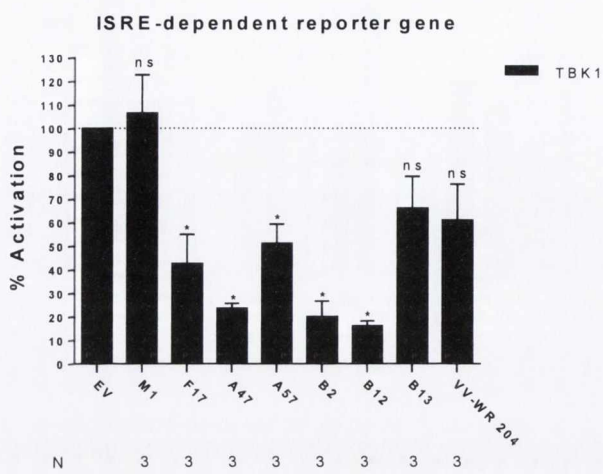


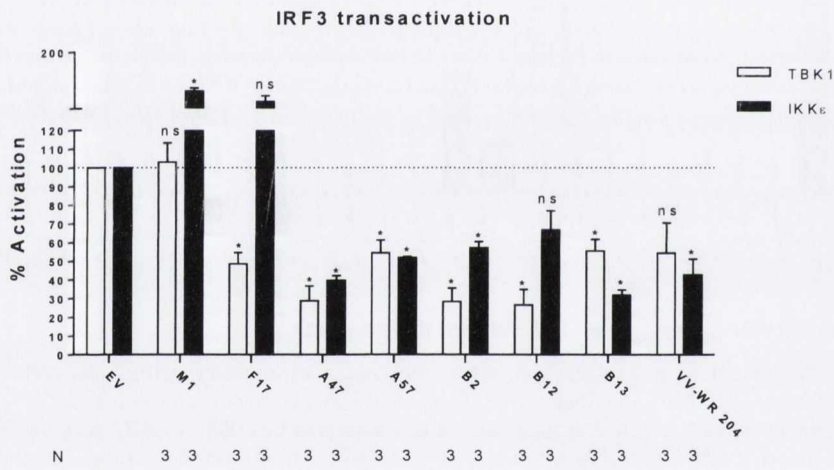
Figure 4-7 Effect of VV-WR ORF expression on IL-1 induced IL-8 production

HEK 293T cells were transfected with 8 µg of a CdCl₂ inducible VV-WR protein expression construct (pMEP4HA-VV-WR ORF), and selected and maintained in 300 µg/ml Hygromycin. Forty-eight hours after induction with 1 µM CdCl₂, stably transformed cells were stimulated with IL-1. IL-8 in supernatants was measured by ELISA. '+ CdCl₂' assay values were scaled relative to corresponding '-CdCl₂' assay value, which was set to 100 % activation (black dotted line). Cells that were not induced with CdCl₂ served as internal negative controls, whilst cells containing empty pMEP4 vector control (EV) served as CdCl₂ negative controls. Unless marked by '†', means of at least three independent experiments with triplicate determination are shown with their SEMs. Exact number of independent experiments (N) is given below each graph. *p<0.05, ns (not significant). If marked with '†', mean of a single or two independent experiments with triplicate determination is shown with SD.

A



B



C

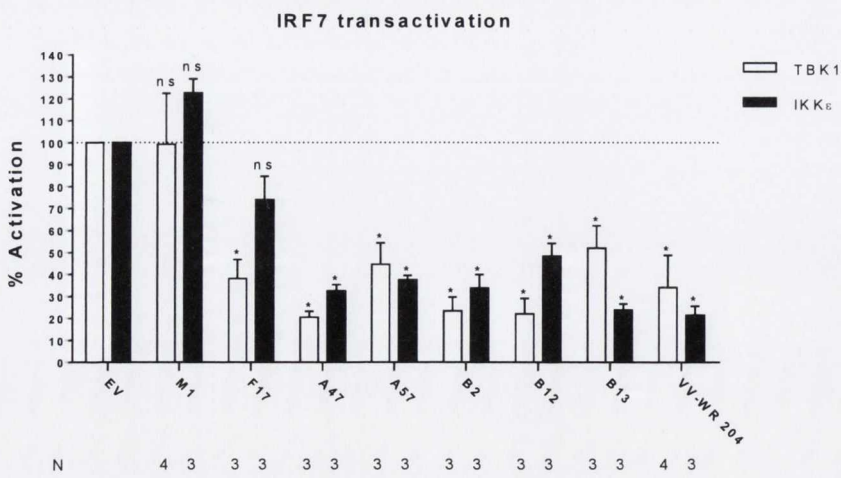


Figure 4-8 Inhibition of IRF activity by VV-WR proteins (see next page for legend)

D

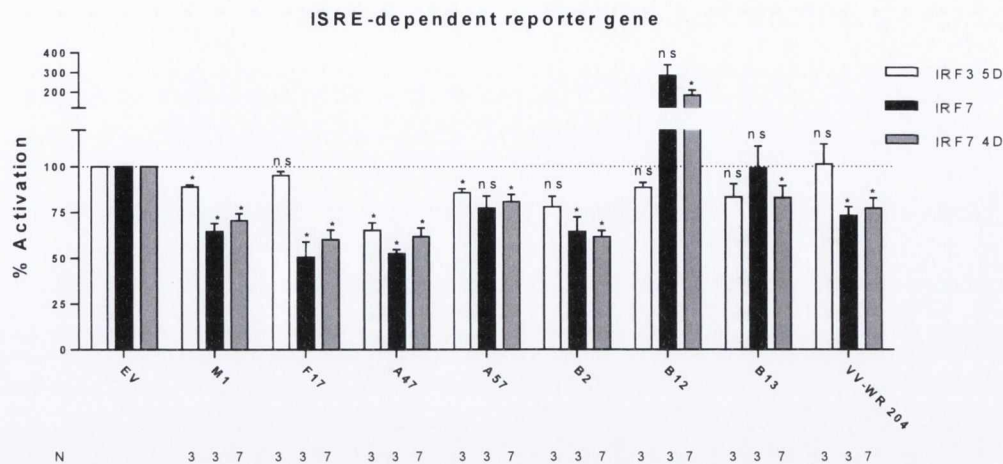


Figure 4-8 Inhibition of IRF activity by VV-WR proteins

(A,D) HEK 293T cells were co-transfected with a renilla luciferase control, a firefly luciferase reporter plasmid under the control of an ISRE element, and TBK1, IRF3 5D, IRF7 or IRF7 4D expression plasmids to activate the promoter. (B,C) Cells were transfected with a renilla luciferase control, a pFA-firefly luciferase reporter plasmid under the control of a Gal4-promoter; (B) an IRF3-Gal4 or, (C) an IRF7-Gal4 fusion protein, which was driven by transfection of either TBK1 or IKKe expression constructs. In addition, cells were transfected with the indicated VV-WR ORF expression constructs or empty vector control (EV). Cells were harvested 24 hpt, firefly luciferase activity was measured and normalised to renilla luciferase activity. VV-WR protein assay values were scaled relative to EV, which was set to 100 % activation (black dotted line). Means of at least three independent experiments with triplicate determination are shown with their SEMs. Exact number of independent experiments (N) is given below each graph. * $p < 0.05$, ns (not significant).

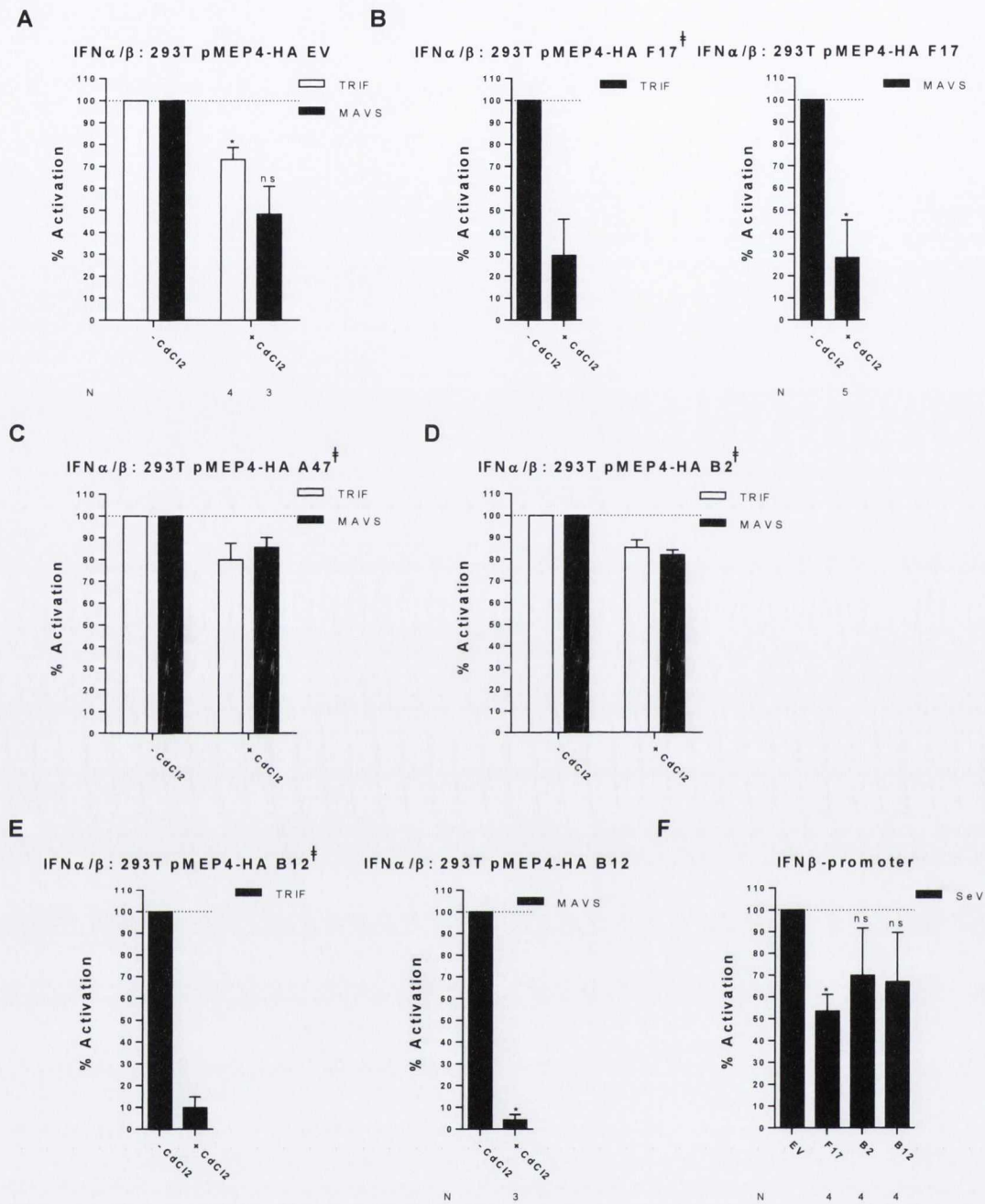


Figure 4-9 Evaluation of inhibitory effect of VV-WR proteins on type I interferon production

(A-E) HEK 293T cells were transfected with 8 μ g of a CdCl₂ inducible VV-WR protein expression construct (pMEP4HA-VV-WR ORF), and selected and maintained in 300 μ g/ml Hygromycin. Forty-eight hours after induction with 1 μ M CdCl₂, stably transformed cells were transfected with TRIF or MAVS expression constructs. Type I IFN bioactivity in supernatants was measured using HEK-Blue™ IFN α/β cells 24 hpt. '+CdCl₂' assay values were scaled relative to '-CdCl₂', which was set to 100 % activation (black dotted line). Cells that were not induced with CdCl₂ served as internal negative controls, whilst cells containing empty pMEP4 vector control (EV) served as CdCl₂ negative controls. Unless marked by '†', means of at least three independent experiments with triplicate determination are shown with their SEMs. *p<0.05, ns (not significant). If marked with '†', mean of a single or two independent experiments with triplicate determination is shown with SD. (F) HEK 293T cells were co-transfected with a firefly luciferase reporter plasmid under the control of an IFN β promoter, a renilla luciferase control and indicated VV-WR ORF expression constructs or empty vector control (EV). Cells were stimulated with SeV 24 hpt. Cells were harvested 24 h post-stimulation, firefly luciferase activity was measured and normalised to renilla luciferase activity. VV-WR protein assay values were scaled relative to EV, which was set to 100 % activation (black dotted line). Means of at least three independent experiments with triplicate determination are shown with their SEMs. Exact number of independent experiments (N) is given below each graph. *p<0.05, ns (not significant).

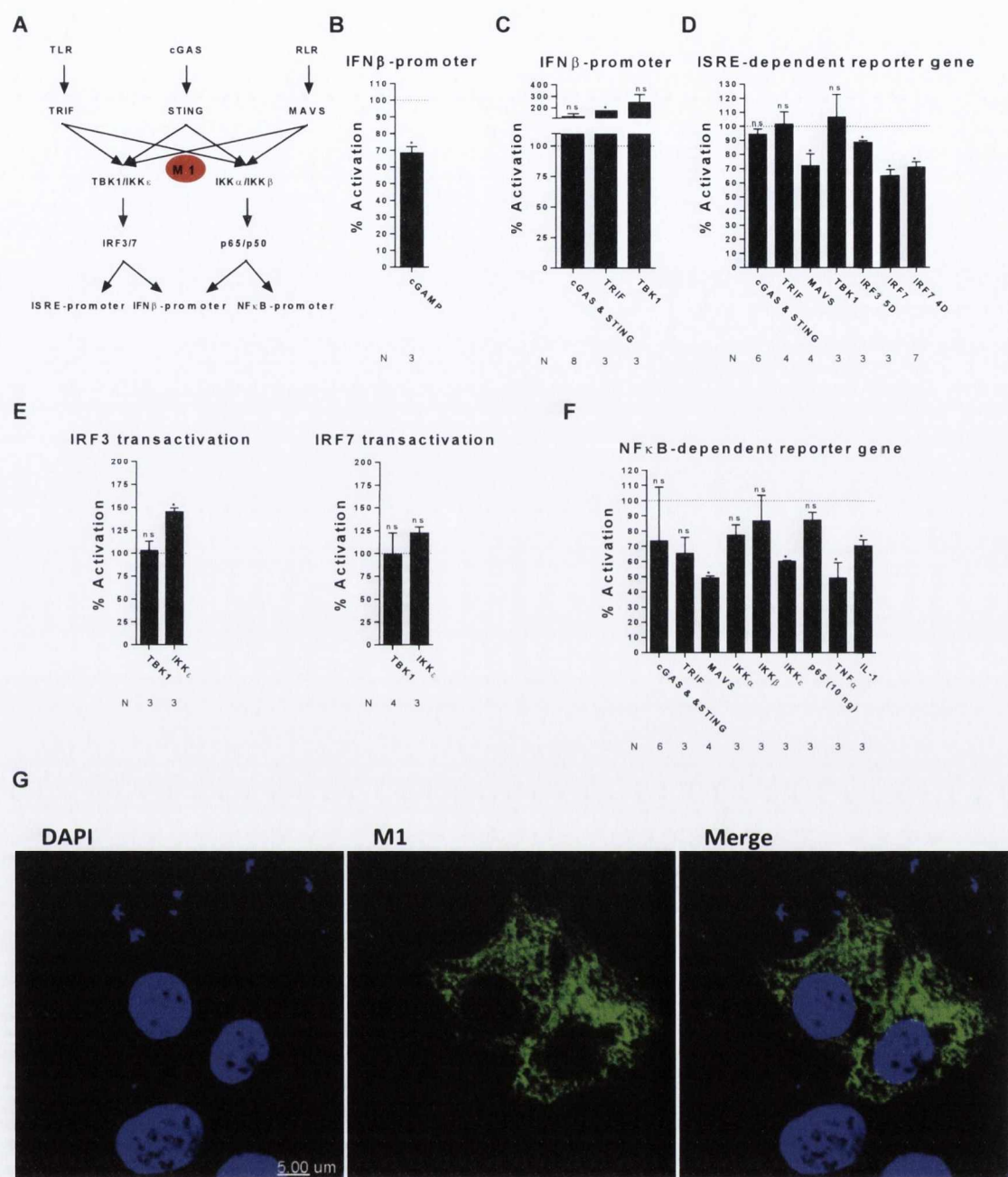


Figure 4-10 M1 - a cytosolic NFκB inhibitor

(A) Schematic outline of screened innate immune signalling pathways. The deduced point of inhibition by M1 is marked with 'M1'. (B) HEK 293T cells stably expressing STING were co-transfected with a firefly luciferase reporter plasmid under the control of an IFN β -promoter, a renilla luciferase control, and M1 expression construct or EV. Cells were stimulated with 3'3'-cGAMP 24 hpt. (C-E) Summary of previously reported data for M1. HEK 293T cells were co-transfected with a renilla luciferase control, a firefly luciferase reporter plasmid under the control of (C) an IFN β -promoter, (D) an ISRE element, or (F) NF κ B-elements; or (E) co-transfected with a pFA-firefly luciferase reporter plasmid under the control of a Gal4-promoter and an IRF3-Gal4 or IRF7-Gal4 fusion protein. Promoters were activated by overexpression of indicated components of the signalling pathways or (F) by stimulation with TNF α or IL-1 24 hpt. In addition, cells were transfected with M1 expression construct or empty vector control (EV). Cells were harvested 24 hpt or 24 h post-stimulation, firefly luciferase activity was measured and normalised to renilla luciferase activity. M1 assay values were scaled relative to EV, which was set to 100 % activation (black dotted line). Means of at least three independent experiments with triplicate determination are shown with their SEMs. Exact number of independent experiments (N) is given below each graph. *p<0.05, ns (not significant). (G) HEK 293T cells grown on coverslips were transfected with M1 expression plasmid. Fixed cells were stained with anti-HA (green) and DAPI (blue).

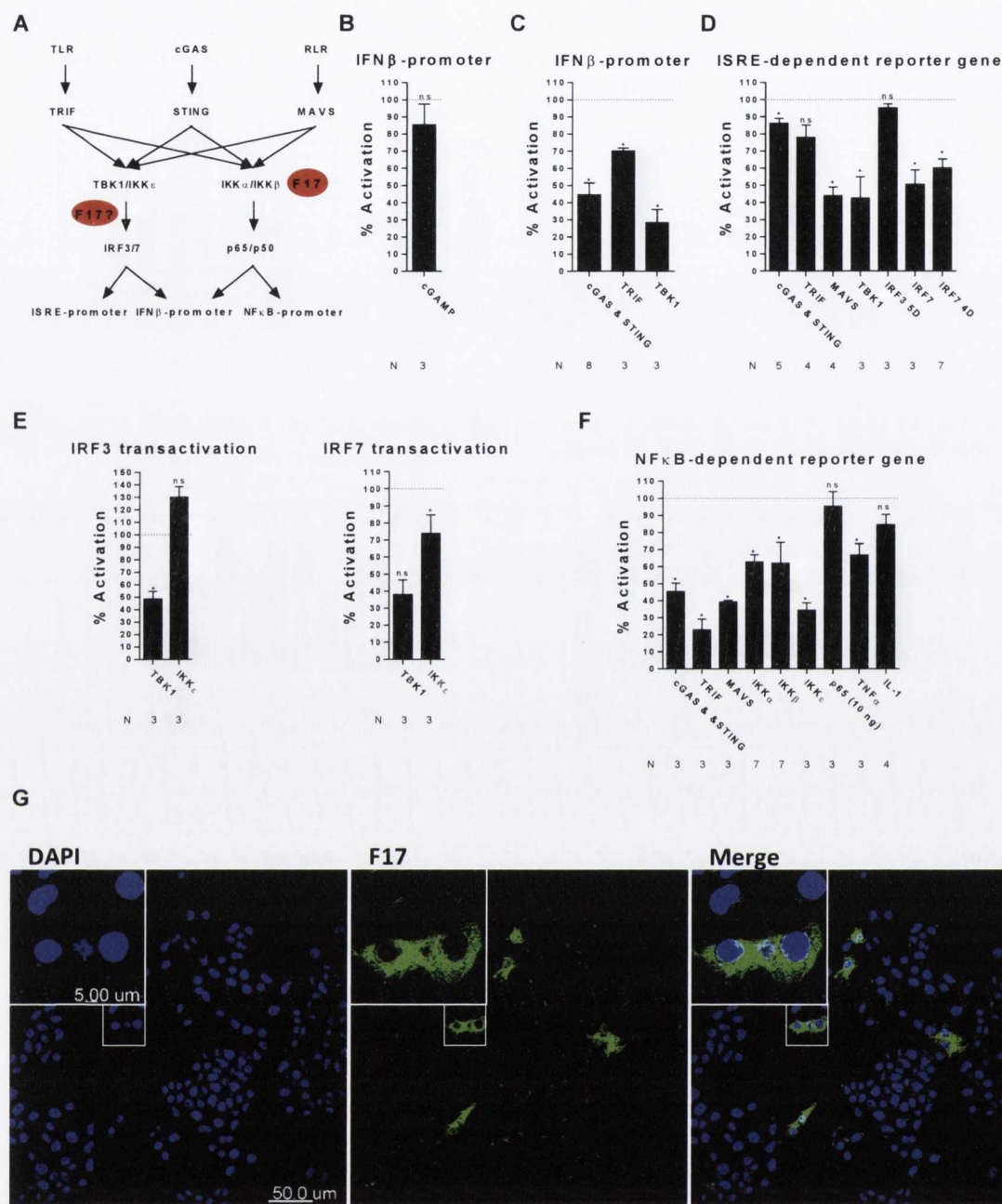


Figure 4-11 F17 - A cytosolic IRF and NF κ B inhibitor packaged into newly generated virions

(A) Schematic outline of screened innate immune signalling pathways. The deduced point of inhibition by F17 is marked with 'F17'. (B) HEK 293T cells stably expressing STING were co-transfected with a firefly luciferase reporter plasmid under the control of an IFN β -promoter, a renilla luciferase control and F17 expression construct or EV. Cells were stimulated with 3'3'-cGAMP 24 hpt. Summary of previously reported data for F17. (C-E) HEK 293T cells were co-transfected with a renilla luciferase control, a firefly luciferase reporter plasmid under the control of (C) an IFN β -promoter, (D) an ISRE element, or (E) NF κ B-elements; or (F) co-transfected with a pFA-firefly luciferase reporter plasmid under the control of a Gal4-promoter, and an IRF3-Gal4 or IRF7-Gal4 fusion protein. Promoters were activated by overexpression of indicated components of the signalling pathways or (F) by stimulation with TNF α or IL-1 24 hpt. In addition, cells were transfected with F17 expression construct or empty vector control (EV). Cells were harvested 24 hpt or 24 h post-stimulation, firefly luciferase activity was measured and normalised to renilla luciferase activity. F17 assay values were scaled relative to EV, which was set to 100 % activation (black dotted line). Mean of at least three independent experiments with triplicate determination are shown with their SEMs. Exact number of independent experiments (N) is given below each graph. * $p < 0.05$, ns (not significant). (G) HEK 293T cells grown on coverslips were transfected with F17 expression plasmid. Fixed cells were stained with anti-HA (green) and DAPI (blue).

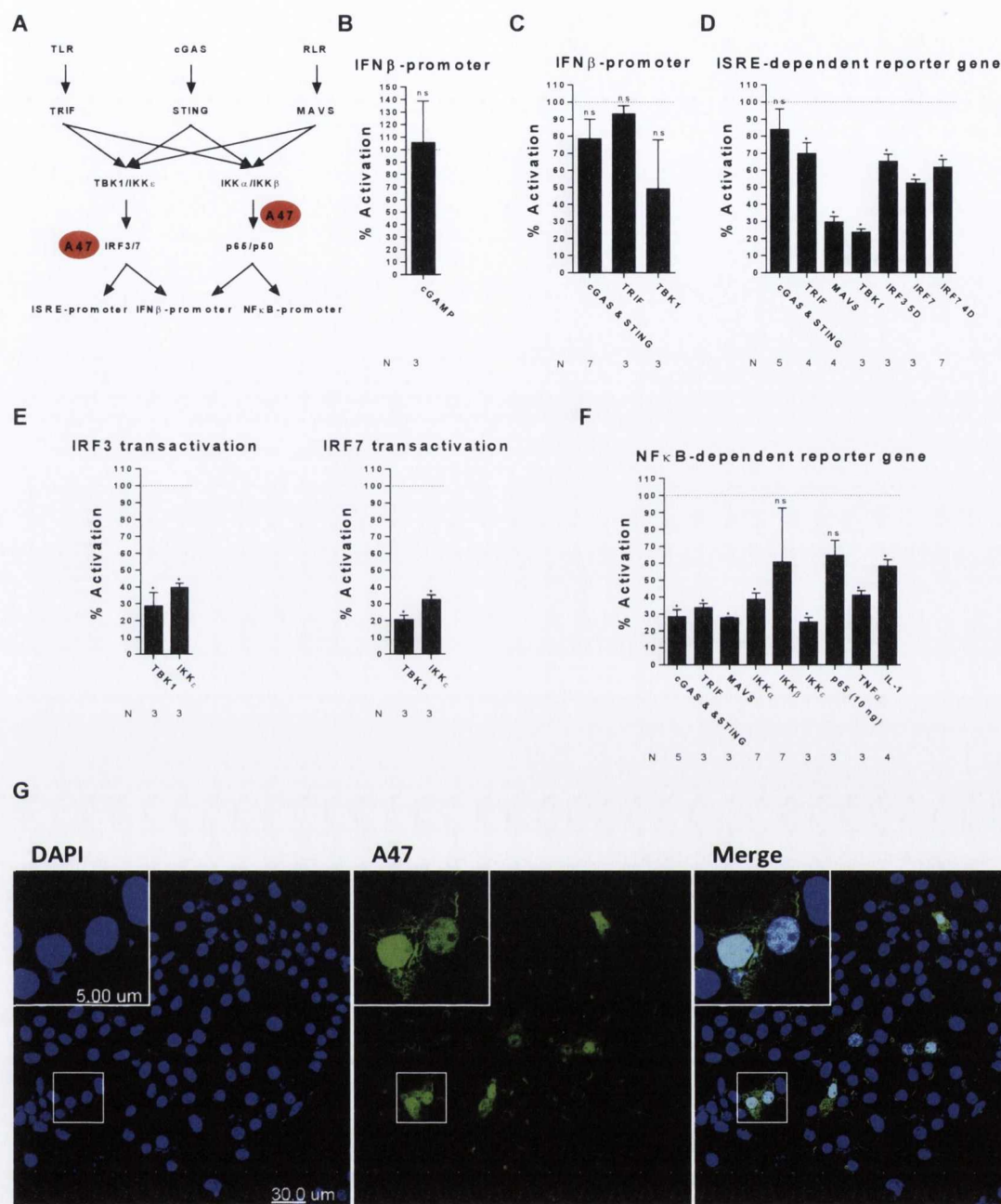


Figure 4-12 A47 - An inhibitor of IRFs and NF κ B localised in the nucleus and the cytoplasm

(A) Schematic outline of screened innate immune signalling pathways. The deduced point of inhibition by A47 is marked with 'A47'. (B) HEK 293T cells stably expressing STING were co-transfected with a firefly luciferase reporter plasmid under the control of an IFN β -promoter, a renilla luciferase control and A47 expression construct or EV. Cells were stimulated with 3'3'-cGAMP 24 hpt. Summary of previously reported data for A47. (C-E) HEK 293T cells were co-transfected with a renilla luciferase control, a firefly luciferase reporter plasmid under the control of (C) an IFN β -promoter, (D) an ISRE element, or (F) NF κ B-elements; or (E) co-transfected with a pFA-firefly luciferase reporter plasmid under the control of a Gal4-promoter, and an IRF3-Gal4 or IRF7-Gal4 fusion protein. Promoters were activated by overexpression of indicated components of the signalling pathways or (F) by stimulation with TNF α or IL-1 24 hpt. In addition, cells were transfected with A47 expression construct or empty vector control (EV). Cells were harvested 24 hpt or 24 h post-stimulation, firefly luciferase activity was measured and normalised to renilla luciferase activity. A47 assay values were scaled relative to EV, which was set to 100 % activation (black dotted line). Means of at least three independent experiments with triplicate determination are shown with their SEMs. Exact number of independent experiments (N) is given below each graph. * $p < 0.05$, ns (not significant). (G) HEK 293T cells grown on coverslips were transfected with A47 expression plasmid. Fixed cells were stained with anti-HA (green) and DAPI (blue).

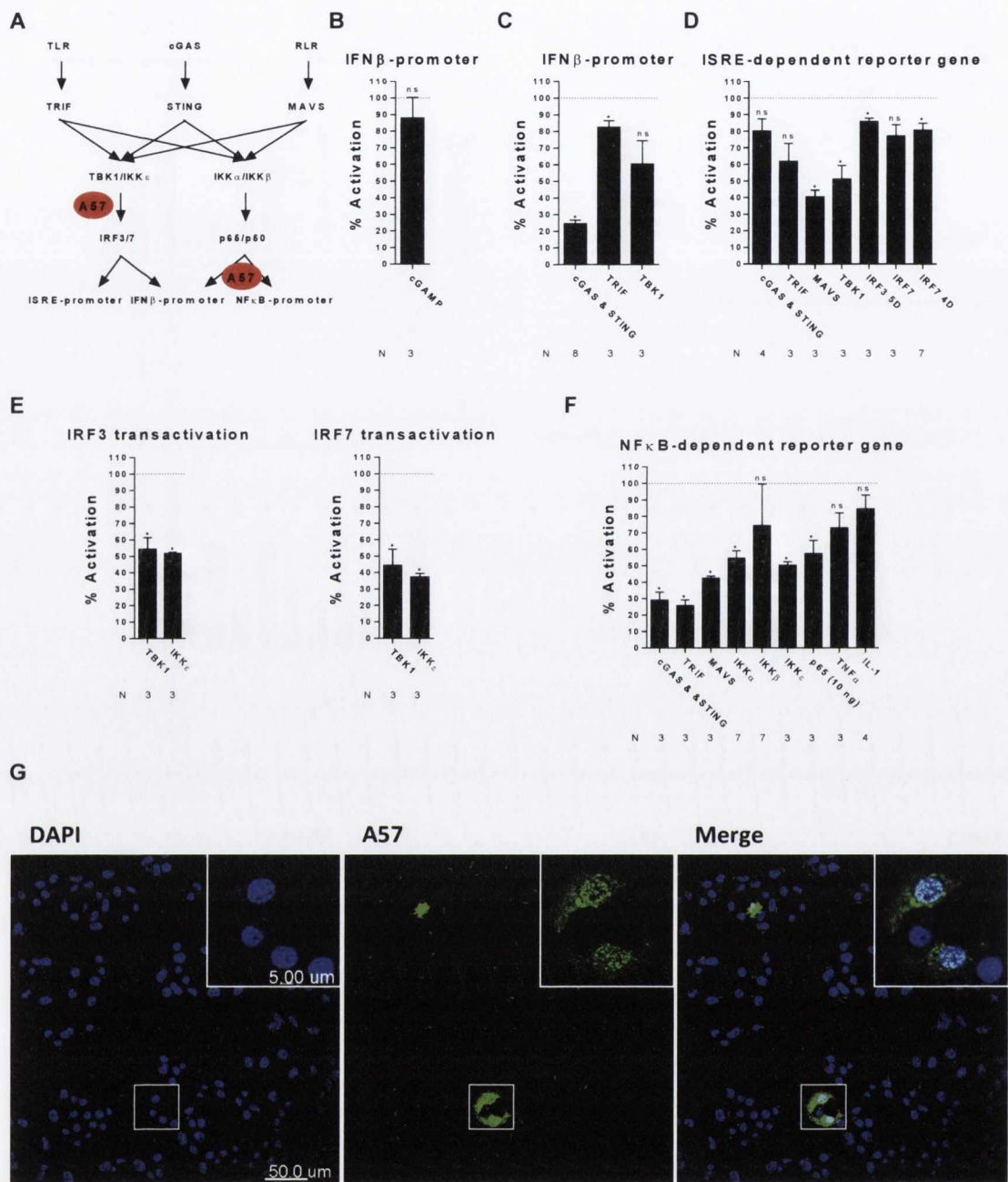


Figure 4-13 A57 – An IRF and NF κ B inhibitor forming distinct foci in the cytoplasm and nucleus

(A) Schematic outline of screened innate immune signalling pathways. The deduced point of inhibition by A57 is marked with 'A57'. (B) HEK 293T cells stably expressing STING were co-transfected with a firefly luciferase reporter plasmid under the control of an IFN β -promoter, a renilla luciferase control and A57 expression construct or EV. Cells were stimulated with 3'3'-cGAMP 24 hpt. Summary of previously reported data for A57. (C-E) HEK 293T cells were co-transfected with a renilla luciferase control, a firefly luciferase reporter plasmid under the control of (C) an IFN β -promoter, (D) an ISRE element, or (F) NF κ B-elements; or (E) co-transfected with a pFA-firefly luciferase reporter plasmid under the control of a Gal4-promoter, and an IRF3-Gal4 or IRF7-Gal4 fusion protein. Promoters were activated by overexpression of indicated components of the signalling pathways or (F) by stimulation with TNF α or IL-1 24 hpt. In addition, cells were transfected with A57 expression construct or empty vector control (EV). Cells were harvested 24 hpt or 24 h post-stimulation, firefly luciferase activity was measured and normalised to renilla luciferase activity. A57 assay values were scaled relative to EV, which was set to 100 % activation (black dotted line). Means of at least three independent experiments with triplicate determination are shown with their SEMs. Exact number of independent experiments (N) is given below each graph.* $p < 0.05$, ns (not significant). (G) HEK 293T cells grown on coverslips were transfected with A57 expression plasmid. Fixed cells were stained with anti-HA (green) and DAPI (blue).

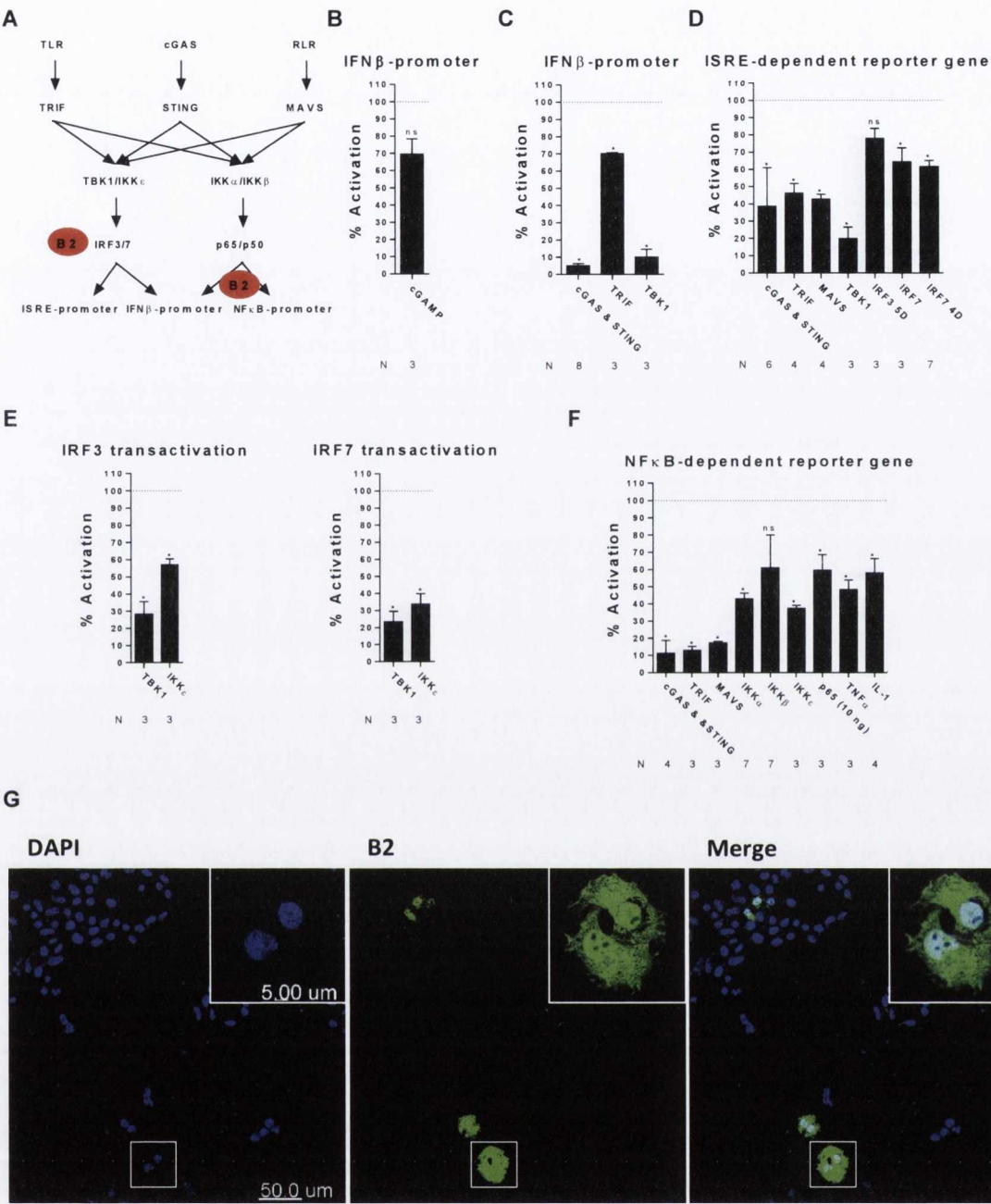


Figure 4-14 B2 - A cytosolic and nuclear IRF and NFκB inhibitor

(A) Schematic outline of screened innate immune signalling pathways. The deduced point of inhibition by B2 is marked with 'B2'. (B) HEK 293T cells stably expressing STING were co-transfected with a firefly luciferase reporter plasmid under the control of an IFNβ-promoter, a renilla luciferase control and B2 expression construct or EV. Cells were stimulated with 3'3'-cGAMP 24 hpt. Summary of previously reported data for B2. (C-E) HEK 293T cells were co-transfected with a renilla luciferase control, a firefly luciferase reporter plasmid under the control of (C) an IFNβ-promoter, (D) an ISRE element, or (F) NFκB-elements; or (E) co-transfected with a pFA-firefly luciferase reporter plasmid under the control of a Gal4-promoter, and an IRF3-Gal4 or IRF7-Gal4 fusion protein. Promoters were activated by overexpression of indicated components of the signalling pathways or (F) by stimulation with TNFα or IL-1 24 hpt. In addition, cells were transfected with B2 expression construct or empty vector control (EV). Cells were harvested 24 hpt or 24 h post-stimulation, firefly luciferase activity was measured and normalised to renilla luciferase activity. B2 assay values were scaled relative to EV, which was set to 100 % activation (black dotted line). Means of at least three independent experiments with triplicate determination are shown with their SEMs. Exact number of independent experiments (N) is given below each graph. *p<0.05, ns (not significant). (F) HEK 293T cells grown on coverslips were transfected with B2 expression plasmid. Fixed cells were stained with anti-HA (green) and DAPI (blue).

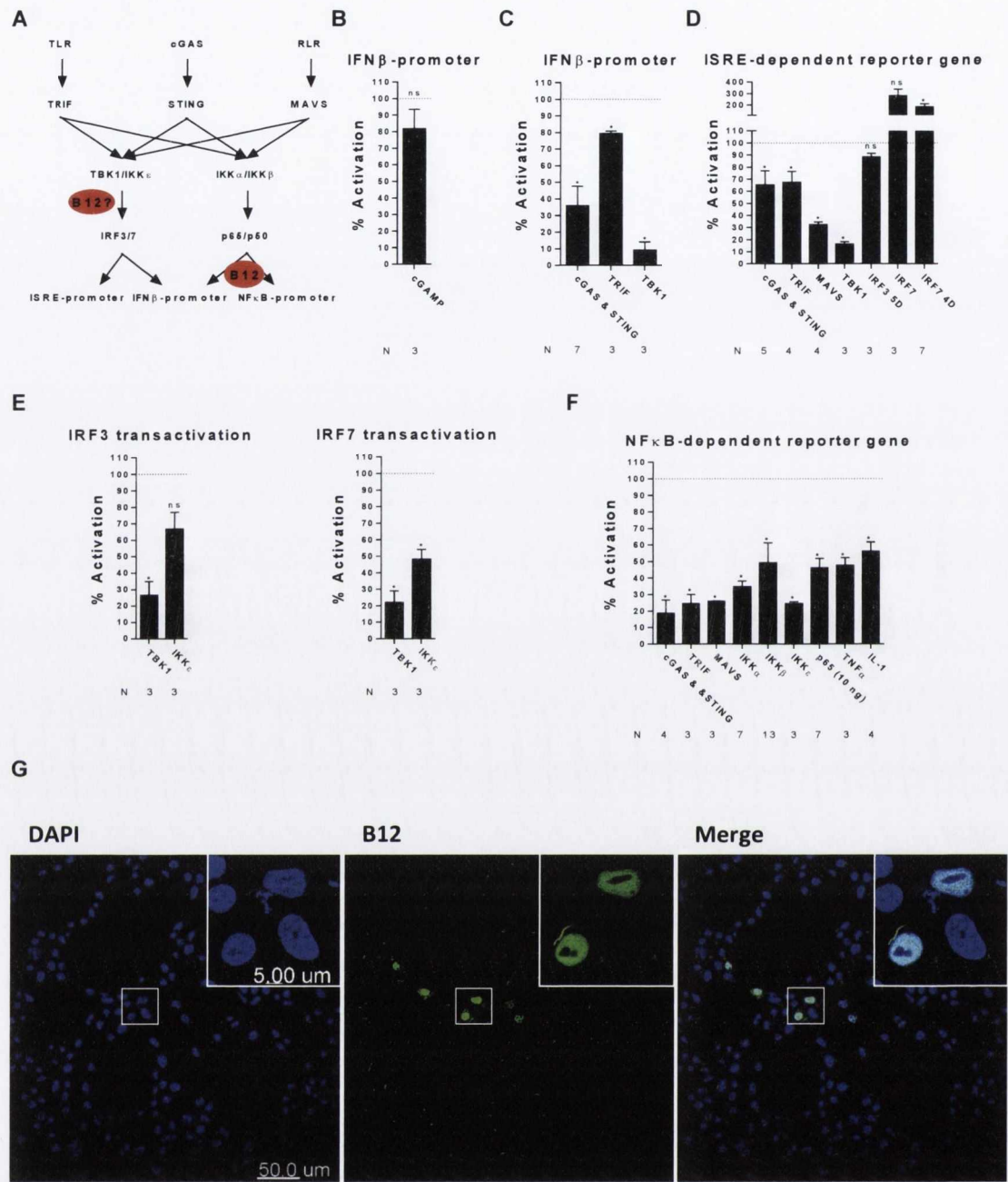


Figure 4-15 B12 - A nuclear inhibitor of IFNβ- and NFκB-promoter activation

(A) Schematic outline of screened innate immune signalling pathways. The deduced point of inhibition by B12 is marked with 'B12'. (B) HEK 293T cells stably expressing STING were co-transfected with a firefly luciferase reporter plasmid under the control of an IFNβ-promoter, a renilla luciferase control and B12 expression construct or EV. Cells were stimulated with 3'3'-cGAMP 24 hpt. Summary of previously reported data for B12. (C-E) HEK 293T cells were co-transfected with a renilla luciferase control, a firefly luciferase reporter plasmid under the control of (C) an IFNβ-promoter, (D) an ISRE element, or (F) NFκB-elements; or (E) co-transfected with a pFA-firefly luciferase reporter plasmid under the control of a Gal4-promoter, and an IRF3-Gal4 or IRF7-Gal4 fusion protein. Promoters were activated by overexpression of indicated components of the signalling pathways or (F) by stimulation with TNFα or IL-1 24 hpt. In addition, cells were transfected with B12 expression construct or empty vector control (EV). Cells were harvested 24 hpt or 24 h post-stimulation, firefly luciferase activity was measured and normalised to renilla luciferase activity. B12 assay values were scaled relative to EV, which was set to 100 % activation (black dotted line). Means of at least three independent experiments with triplicate determination are shown with their SEMs. Exact number of independent experiments (N) is given below each graph. *p<0.05, ns (not significant). (G) HEK 293T cells grown on coverslips were transfected with B12 expression plasmid. Fixed cells were stained with anti-HA (green) and DAPI (blue).

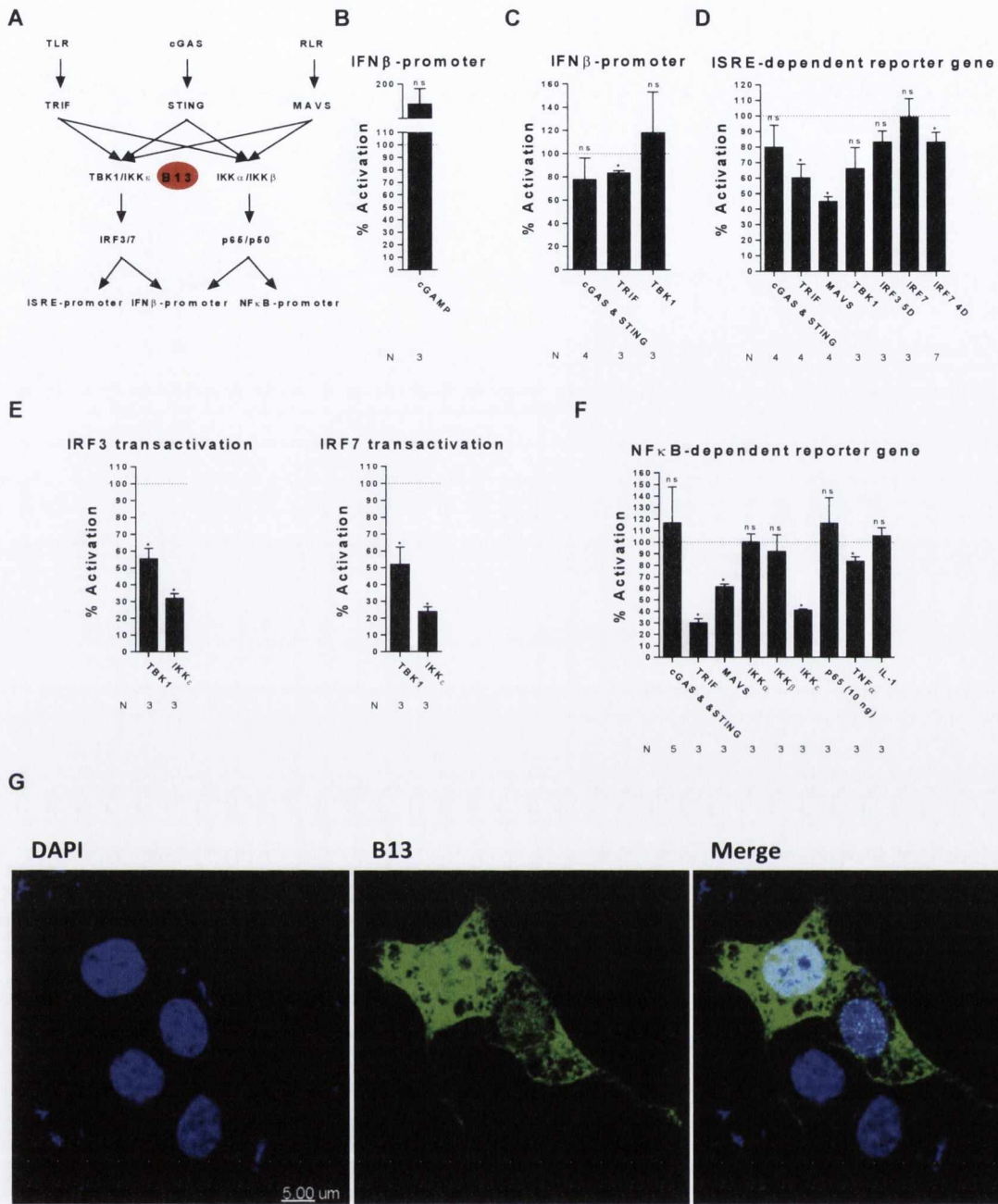


Figure 4-16 B13 - An inhibitor of IRF and NFκB pathways

(A) Schematic outline of screened innate immune signalling pathways. The deduced point of inhibition by B13 is marked with 'B13'. (B) HEK 293T cells stably expressing STING were co-transfected with a firefly luciferase reporter plasmid under the control of an IFN β -promoter, a renilla luciferase control and B13 expression construct or EV. Cells were stimulated with 3'3'-cGAMP 24 hpt. Summary of previously reported data for B13. (C-E) HEK 293T cells were co-transfected with a renilla luciferase control, a firefly luciferase reporter plasmid under the control of (C) an IFN β -promoter, (D) an ISRE element, or (F) NF κ B-elements; or (E) co-transfected with a pFA-firefly luciferase reporter plasmid under the control of a Gal4-promoter, and an IRF3-Gal4 or IRF7-Gal4 fusion protein. Promoters were activated by overexpression of indicated components of the signalling pathways or (F) by stimulation with TNF α or IL-1 24 hpt. In addition, cells were transfected with B13 expression construct or empty vector control (EV). Cells were harvested 24 hpt or 24 h post-stimulation, firefly luciferase activity was measured and normalised to renilla luciferase activity. B13 assay values were scaled relative to EV, which was set to 100 % activation (black dotted line). Means of at least three independent experiments with triplicate determination are shown with their SEMs. Exact number of independent experiments (N) is given below each graph. * $p < 0.05$, ns (not significant). (G) HEK 293T cells grown on coverslips were transfected with B13 expression plasmid. Fixed cells were stained with anti-HA (green) and DAPI (blue).

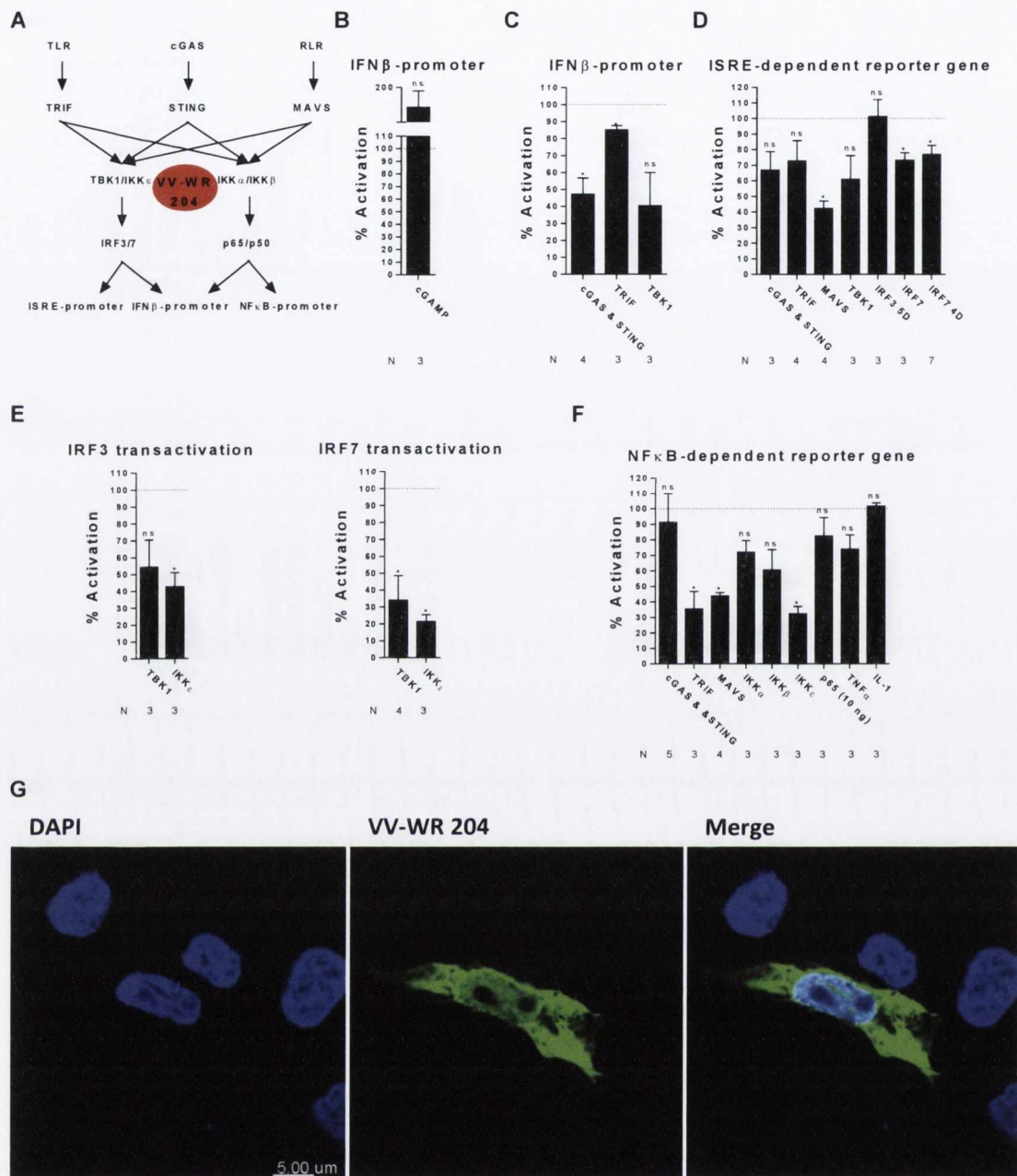


Figure 4-17 VV-WR 204 - An IRF and NFκB inhibitor

(A) Schematic outline of screened innate immune signalling pathways. The deduced point of inhibition by VV-WR 204 is marked with 'VV-WR 204'. (B) HEK 293T cells stably expressing STING were co-transfected with a firefly luciferase reporter plasmid under the control of an IFNβ-promoter, a renilla luciferase control and VV-WR 204 expression construct or EV. Cells were stimulated with 3'3'-cGAMP 24 hpt. Summary of previously reported data for VV-WR 204. (C-E) HEK 293T cells were co-transfected with a renilla luciferase control, a firefly luciferase reporter plasmid under the control of (C) an IFNβ-promoter, (D) an ISRE element, or (F) NFκB-elements; or (E) co-transfected with a pFA-firefly luciferase reporter plasmid under the control of a Gal4-promoter, and an IRF3-Gal4 or IRF7-Gal4 fusion protein. Promoters were activated by overexpression of indicated components of the signalling pathways or (F) by stimulation with TNFα or IL-1 24 hpt. In addition, cells were transfected with VV-WR 204 expression construct or empty vector control (EV). Cells were harvested 24 hpt or 24 h post-stimulation, firefly luciferase activity was measured and normalised to renilla luciferase activity. VV-WR 204 assay values were scaled relative to EV, which was set to 100 % activation (black dotted line). Means of at least three independent experiments with triplicate determination are shown with their SEMs. Exact number of independent experiments (N) is given below each graph. *p<0.05, ns (not significant). (G) HEK 293T cells grown on coverslips were transfected with VV-WR 204 expression plasmid. Fixed cells were stained with anti-HA (green) and DAPI (blue).

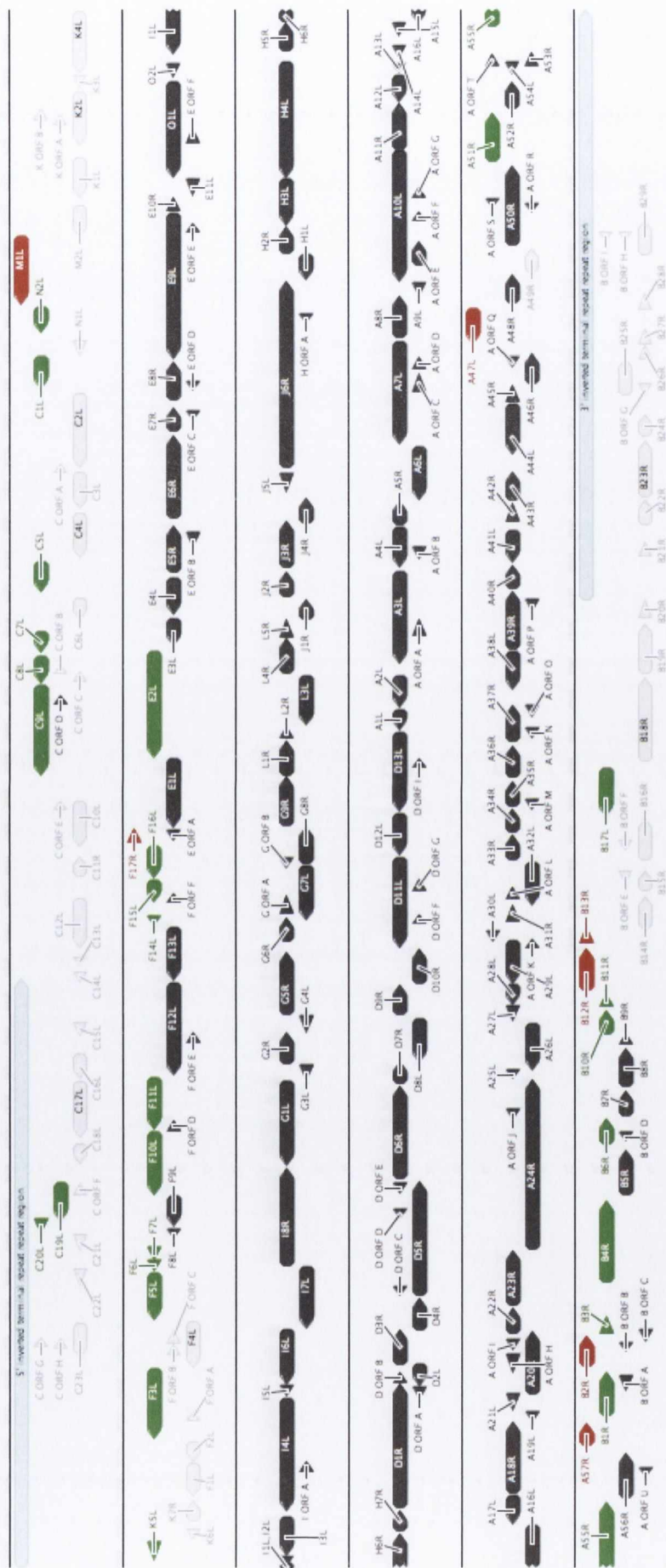


Figure 4-18 Summary of identified viral inhibitors found in w811ΔA49R

The VV strain w811ΔA49R is based on VV strain Copenhagen (VV-Cop, M35027), but 56 ORFs (light grey arrows, light grey dashed line) have been deleted from its genome. A screen for innate immune modulators and inhibitors was performed on forty-two VV-WR ORFs (green arrows), of which eight (red arrows) were identified as potent inhibitors of innate immune signalling. A truncated arrow indicates an ORF is split over two lines of the schematic. VV-WR 003, 014, 015, 203, 204 and 208 are not present in VV-Cop and are therefore not represented in this schematic.

Chapter 5 : Results III – human VRK1 is a target of VACV B12 protein

5.1. Introduction

Eight viral proteins were chosen from a library of 42 VV-WR encoded proteins, compiled for a screen for potential PRR and pro-inflammatory cytokine signalling pathway inhibitors, which was detailed in the previous chapters. Characterisation of these putative viral inhibitors indicated that impairment of the signalling pathways occurred proximal to the transcription factors NF κ B, and IRF3 and IRF7. Lack of effect on nuclear translocation of p65 and a nuclear localisation of all studied viral proteins, with the exception of F17, suggests that these potential inhibitors may have the ability to impair the activity of transcription factors in the nucleus preventing activation of IFN β -promoter- and of NF κ B-dependent genes. Alternatively, viral proteins may affect activation of transcription factors in the cytoplasm in a way that diminishes their activity once they translocated to the nucleus.

The prototype NF κ B dimer is composed of p65 (also called RelA) and p50 and they are characterised by an N-terminal Rel homology domain (RHD), which mediates DNA binding, and homo- and heterodimerisation of the five NF κ B family members (Hayden & Ghosh 2008). In addition, p65 has a transcription activation domain (TAD) at its C-terminus. The NF κ B dimer, (both p65 and p50 harbour a NLS), is sequestered by its repressor I κ B α in the cytoplasm. I κ B α masks the NLS of p65, and itself has a nuclear export signal. Once I κ B α is degraded, NF κ B can translocate to the nucleus, and bind to promoters containing the κ B-consensus site 5'-GGGRNWYYCC-3' (R – purine, N – any nucleotide, W – A/T, Y – pyrimidine) (Kunsch et al. 1992). This sequence is highly degenerate and more than 400 genes involved in innate and adaptive immune responses as well as in cell cycle progression have been reported to be regulated by NF κ B to date (Gilmore 2016). Posttranslational modifications of NF κ B, such as phosphorylations, have been shown to determine selectivity of NF κ B-dependent gene expression (Hochrainer et al. 2013). In addition, stimuli-induced chromatin remodelling by complexes such as the SWI/SNF complex may be necessary to expose promoter regions to binding of transcription factors and RNA polymerase II (Lomvardas & Thanos 2001; Natoli et al. 2005). Saccani and co-authors identified two groups of NF κ B-dependent genes based on the accessibility of the promoter to the transcription factor (Saccani et al. 2001). Genes encoding I κ B α and the chemokine macrophage inflammatory protein 2 (MIP2, also called CXCL2) were bound by NF κ B immediately after nuclear translocation. The rapid association of NF κ B with the MIP2 promoter correlated with high basal levels of histone H4 acetylation for MIP2 indicative of a less densely packed DNA structure readily accessible upon NF κ B activation. In contrast, the promoter region of the genes encoding RANTES and IL-6 were bound solely at 90 to 150 min after LPS stimulation, respectively (Saccani et al. 2001). No basal levels of histone H4 acetylation were detected, however 30 to 90 min post-stimulation acetylation became apparent and increased over the course of 3 h. These findings suggest that in addition to pathway activation and posttranslational modifications (see below), chromatin remodelling plays an important role in regulation of gene expression. This is further supported by the discovery of viral genes which modulate chromatin structures, such as: the IVA protein NS, which harbours a histone H3-like domain and is able to regulate expression of antiviral genes (Marazzi et al. 2012); the *Human adenovirus* protein E1A, which impairs monoubiquitination of histone H2B and expression of ISGs (Fonseca et al. 2012); and the KSHV protein LANA, which is able to bind to the promoter of the TGF- β receptor II (TGF β RII) gene, inducing methylation of the promoter and deacetylation of histone H4, resulting in a reduced expression of TGF- β RII (Di Bartolo et al. 2008).

The NFκB subunit p65 contains twelve phosphorylation sites, five at the RHD (Ser205, Thr254, Ser276, Ser281 and Ser311) and seven at the TAD (Thr435, Ser468, Thr505, Ser529, Ser535, Ser536 and Ser547) (Huang et al. 2010; Hochrainer et al. 2013). Notably, p65 can be phosphorylated by IKKα, IKKβ and IKKε, as well as by TBK1 at Ser365 in the cytoplasm (Buss et al. 2004), but also in a p53-dependent manner by ribosomal subunit kinase 1 in the nucleus (Bohuslav et al. 2004). This modification induces a conformational change which allows the binding of co-activator CREB-binding protein (CBP)/p300 and decreases its affinity for IκBα. Ser276 has been shown to be phosphorylated in the cytoplasm by protein kinase A following IκBα degradation (Zhong et al. 1998), and in the nucleus by mitogen- and stress-activated protein kinase-1 (Vermeulen et al. 2003). Similarly to Ser365, phosphorylation at Ser276 reduces the N- and C-terminal interactions of p65 to favour association with CBP/p300. Phosphorylation of these residues recruits CBP/p300 to p65, and in turn CBP/p300 acetylates p65 at Lys310 (Chen et al. 2005), which is required for full transcriptional activity. Furthermore, acetylation of Lys310 prevents methylation of Lys314 and Lys315 by Set9 (Yang et al. 2010), which otherwise results in ubiquitin-mediated degradation of p65. However, methylation can also promote NFκB activity as reported for methylation of p65 at Lys218 and Lys221 (Zhang et al. 2013). The protein phosphatase 2A (PP2A) inhibitor homeodomain finger protein 20 (PHF20) is recruited to p65 methylated at these two lysine residues, and in turn prevents dephosphorylation of Ser536. Posttranslational modifications are transient and reversible, for example PP2A can dephosphorylate p65 at Ser365 (Yang et al. 2001; Sakurai et al. 2003), NAD-dependent protein deacetylase sirtuin-1 (SIRT1) can deacetylate Lys310 (Yeung et al. 2004), and SIRT6 has been reported to remove acetylation of histone H3, thereby reducing the affinity of p65 to κB sites (Kawahara et al. 2009).

In attempt to determine the host targets of candidate inhibitors, which may explain the previous findings, stably transfected cells expressing VV-WR ORFs were used to identify interactions partners *in vitro* using a previously described strategy that was successful in identifying host target of MCV proteins (Brady et al. 2015).

5.2. Results

5.2.1. Interactome analysis of potential VACV inhibitors

The previous screen mapped the likely inhibition of the different viral candidates, yet no direct interaction partners could be identified using this method. Therefore, stably transfected cells were induced with CdCl₂, and an analysis of binding partners by mass spectrometry was performed by our collaborators Dr. Andreas Pichlmair and Dr. Darya Haas. As noted before, expression levels varied between constructs (Figure 5-1A), yet did not necessarily impact on the results (Figure 5-1B-H). Induction of cells transfected with empty vector served as negative controls for unspecific binding to HA-beads used to isolate immunoprecipitates. The induced viral proteins were only detected in four of the seven cell lines (i.e. F17, B2, B12 and B13), which could be attributed to low expression levels. The results of the remaining proteins (i.e. A47, A57 and VV-WR 204) will also be presented in the following section. The obtained data was initially filtered against contaminant lists provided in the literature to identify and discard potential unspecific interactions (Trinkle-Mulcahy et al. 2008; Mellacheruvu et al. 2013).

Ten potential interaction partners were identified for F17, of which one was involved in cell cycle progression (CDK1), two in metabolism (asparagine synthetase, spermidine synthase), one in cellular traffic (sorting nexin-9), one associated with the proteasome (proteasome subunit beta type-1), and five were found in contaminant lists (Acidic leucine-rich nuclear phosphoprotein 32 family member B (ANP32B), Fructose-bisphosphate aldolase A, RNA-binding protein FUS, D-3-phosphoglycerate dehydrogenase, BAG family molecular chaperone regulator 2) (Figure 5-1B).

The interactome experiments for A47 identified seven potential targets, including: DNA-directed RNA polymerase II subunit RPB11-a/b1/b2, HIST2H3PS2 and cleavage and polyadenylation specificity factor 3 (CPSF3) (transcription); Trifunctional enzyme subunit beta (metabolism); Putative RNA-binding protein 3 and Lysine-rich nucleolar protein 1 (heat/cold shock protein); and Luc7-like protein 3 (contaminant list) (Figure 5-1C). The nuclear protein CPSF3 was identified as a significant result, despite its appearance in a contaminant list. Two studies revealed a link between CPSF3 and apoptosis. One report showed that upregulation of CPSF3 induced pro-apoptotic responses (Zhu et al. 2009), whereas the other study demonstrated that a reduction in CPSF3 levels caused cell death (Calzado et al. 2004). No role in NFκB activation has been reported to this date which could explain the observed inhibition of NFκB activity.

Numerous proteins associated with transcription were found as potential interaction partners of A57 (Figure 5-1D). It has to be noted, that many, i.e. ANP32B (also identified for F17), metastasis-associated protein MTA1 (MTA1), HIV Tat-specific factor 1 (HTATSF1), HNRNPC, MTA2, DNA topoisomerase 2-alpha (TOP2A), protein SET (SET), TOP2B, heterogeneous nuclear ribonucleoprotein U-like protein 2 (HNRNPUL2), transcriptional repressor p66-beta (GATAD2B), Chromodomain-helicase-DNA-binding protein 4 (CHD4) and RNA-binding protein Raly (RALY), were also present in contaminant lists. However, the five proteins Histone-binding protein RBBP7 (also RbAp46), MTA1, MTA2, CHD4 and GATAD2B belong to or are associated with the nucleosome remodeling and deacetylase (NuRD) complex, which is involved in gene regulation (Torchy et al.

2015). Other proteins implicated in transcription include ANP32E, the PP2A inhibitor SET, Nuclear ubiquitous casein and cyclin-dependent kinase substrate 1 (NUCKS1), Beta-catenin-like protein 1, HTATSF1, TOP2A, TOP2B and HNRNPUL2. The remaining proteins, i.e. Eukaryotic translation initiation factor 5B, Single-stranded DNA-binding protein, ANP32B, Centromere protein V, HNRNPC and RALY, are associated with translation or structural functions.

Only two proteins were detected in the interaction partner analysis for B2 (Figure 5-1E). Allograft inflammatory factor 1-like is a structural protein, which can bind actin; and LIM domain only protein 7, which is most likely a contaminant as it was detected in control cells and it was identified as an interaction partner of VV-WR204. Neither of these proteins would explain the observed reduction of PRR and pro-inflammatory cytokine signalling activation.

Interestingly, the only interaction partner that was found for B12 was VRK1, a cellular homologue of VACV B1 and B12, which is a nuclear kinase involved in cell cycle progression, as described in the preceding chapter (see 3.2.2.2, Figure 5-1F).

Similarly, only one interaction partner was identified for B13, i.e. 28S ribosomal protein S18c, which is a ribosomal protein and hence unrelated to regulation of transcription factor activity (Figure 5-1G).

Three DDX helicases (i.e. DDX24, DDX21 and DDX18), four proteins involved in translation (i.e. Signal recognition particle 54 kDa protein, Neuroguidin, Ribosome biogenesis protein BRX1 homolog and RNA methyltransferase-like protein 1), one ribonucleotide associated protein (i.e. H/ACA ribonucleoprotein complex subunit 4), and five proteins present in contaminant lists (i.e. Ribosomal L1 domain-containing protein 1, 40S ribosomal protein S26, Pumilio domain-containing protein KIAA0020, Nuclear fragile X mental retardation-interacting protein 2 and Myb-binding protein 1A) were discovered as potential interaction partners for VV-WR 204 (Figure 5-1H). The relevance of these proteins is questionable as significance thresholds were reduced in attempt to detect VV-WR 204.

5.2.2. B12 targets VRK1 and potentially antagonises its role in AP1 activation

5.2.2.1. B12 interacts with VRK1

The nuclear VACV protein B12 was chosen for further characterisation due to its sequence similarity with the mammalian kinase VRK1 (~30 % a.a. sequence identity, Figure 5-2A), its highly ranked inhibitory potential and the identification of VRK1 as its only interaction partner in HEK 293T cells (Figure 5-1F). In this assay, endogenous VRK1 was co-immunoprecipitated using an HA-tagged B12 expression construct. Here, a further confirmation of this interaction was demonstrated.

The VRK1 expression construct was generated, by amplifying VRK1 sequence from HEK 293T cDNA using primers containing a HA-tag sequence, and inserting the amplicon into a pCEP4-vector. Expression of the

pCMV-HA-B12 and pCEP4-FLAG-VRK1 expression constructs were confirmed in HEK 293T cells (Figure 5-2B). A protein of approximately 50 kDa was observed for the VRK1 expression plasmid.

To assess interaction, HEK 293T cells were transfected with FLAG-tagged VRK1, and HA-tagged B12 expression constructs. Cell lysates were immunoprecipitated using FLAG-beads only, because the heavy chain of anti-HA antibodies ran to the same size as VRK1 on an SDS PAGE gel. HA-tagged B12 and FLAG-tagged VRK1 were detected in lysates, as well as co-immunoprecipitations, confirming the interaction between the two proteins (Figure 5-2C).

Next, co-localisation of B12 and VRK1 was evaluated by confocal microscopy. HEK 293T cells were transfected with HA-tagged B12 and FLAG-tagged VRK1. As previously shown, B12 (green) localised to the nucleus, as did VRK1 (red) (Figure 5-2D upper and middle panels). In addition, cells transfected with both plasmids did not demonstrate expression in different localisations within the nucleus, which confirmed their co-localisation (Figure 5-2D lower panels).

5.2.2.2. Exploring VRK1's possible role in NF κ B signalling

VRK1 phosphorylates various transcription factors and other factors involved in gene expression (Klerkx et al. 2009), however its involvement in NF κ B activation has not yet been studied. An NF κ B-element dependent reporter gene was only marginally activated in response to transfection of a VRK1 expression construct (Figure 5-3A). Repetition of this experiment with greater amounts of VRK1 may show an effect. B12 is able to potentially prevent IKK β - and p65-mediated NF κ B activation, however VRK1 did not appear to alter reporter activation driven by IKK β (Figure 5-3B). There seemed to be an additive trend when VRK1 was co-expressed with p65 (Figure 5-3C), but the difference was not significant compared to NF κ B activation by p65 alone, because of large variations between experiments.

The previous experiments indicated either no impact of VRK1 on NF κ B-dependent reporter activation or a subtle activation at best. If VRK1 was having a positive effect on NF κ B activation, a rescue of the reduction mediated by B12 might be expected. As anticipated, B12 reduced IKK β - and p65-driven reporter activation. However, co-transfection of VRK1 did not reverse the observed impairment of NF κ B activation (Figure 5-3D, E). VRK1 appeared even to further reduce reporter activation in combination with B12, at higher concentrations (Figure 5-3D).

5.2.2.3. The role of VRK1 in MAPK signalling and potential antagonism by B12

VRK1 was identified as a potential target of B12, however this interaction may have no physiological consequence, or reveal another role of B12 distinct from its ability to modulate NF κ B. VRK1 is involved in cell cycle regulation and was shown to phosphorylate p53 and c-Jun (Lopez-Borges & Lazo 2000; Sevilla, Santos, Barcia, et al. 2004). To address the possibility of B12 impairing not only NF κ B signalling but other pathways, where VRK1 is implicated, its effect on p53 and AP1 activation was examined.

B12 expression reduced the basal levels of an unstimulated p53-element dependent report gene significantly (Figure 5-4A). It also impaired p53-mediated activation of this reporter gene (Figure 5-4B), indicating that B12

may affect this function of VRK1. As anticipated VRK1 activated an AP1-dependent reporter, and to similar levels as cells treated with PMA, which served as positive control. Co-transfection of B12 with VRK1 reduced the activation of the AP1-dependent reporter to levels seen in cells transfected with empty vector control (Figure 5-5).

Cell cycle regulation is one of the functions of VRK1, wherefore it cannot be deleted from the genome without deleterious consequences (Valbuena et al. 2011). Transient knockdown of VRK1 by siRNA, however, allows an examination of the role of VRK1 in innate signalling pathways without interfering with cell growth as indicated by equal actin levels (Figure 5-6A). Given the effect of B12 on AP1-dependent reporter activation, phosphorylation of the transcription factor c-Jun (pc-Jun) in response to TNF α stimulation was monitored over the course of 2 h. Preliminary data is shown for cells treated with siRNA against VRK1 as difficulties with the detection of pc-Jun occurred (Figure 5-6B, marked by ‘#’). As anticipated, phosphorylation of c-Jun was detected as early as 15 min post-stimulation in control siRNA transfected cells. In contrast, in cells with reduced VRK1 levels pc-Jun appeared at 30 min post-stimulation. Cells stably transfected with an inducible B12 expression plasmid (see 4.2.1.1) were used to monitor pc-Jun (Figure 5-6C). In absence of CdCl₂, pc-Jun was readily detected at 15 min post-stimulation. In contrast, but similar to cells treated with siRNA against VRK1, in presence of CdCl₂ phosphorylation of c-Jun was greatly reduced and delayed. Although the effect of CdCl₂ on pc-Jun needs to be addressed in empty vector transfected cells, this result may indicate that B12 can impair the role of VRK1 in MAPK signalling.

5.2.2.4. Study of VRK1 effects on type I IFN production and VSV replication

B12 appeared to reduce production of type I IFNs in spite of the effect of CdCl₂ itself (Figure 4-9E). In an attempt to evaluate the effect of VRK1 on type I IFN signalling, production of type I IFN in response to transfection of TRIF and MAVS expression constructs was examined. There was no alteration of the amount of type I IFN (Figure 5-7A).

Replication of a VSV mutant virus expressing GFP was evaluated in A549 cells treated with control siRNA or siRNA against VRK1. Cells were infected at MOI 0.1 and 1 and GFP expression was evaluated by fluorescent microscopy and in protein lysates at 24 h post-infection. A greater cytopathic effect was observed in cells infected at higher MOI, however knockdown of VRK1 did not appear to impact replication of this virus as measured GFP levels were similar to control cells (Figure 5-7B,C).

Overall, these results suggest that B12 is able to impair activation of NF κ B and AP1 activation. The latter may explain how B12 targets VRK1. A role of VRK1 in NF κ B signalling was hinted by the subtle increase in reporter activity by ectopic expression of VRK1 (Figure 5-3A), however this observation requires further investigation.

5.3. Discussion

5.3.1. Identifying host interaction partners of VACV candidates

In an attempt to identify possible host targets of previously characterised inhibitor candidates, HEK 293T cells stably transfected with CdCl₂-inducible VV-WR expression constructs were generated (see 4.2.1.1). Mammalian proteins bound to immunoprecipitated VV-WR proteins were analysed by mass spectrometry by our collaborators Dr. Pichlmair and Dr. Haas. According to this analysis, none of the viral candidates appeared to associate with known components of the IRF and/or NFκB signalling pathways (Figure 5-1). Host proteins detected in B2-, and B13-expressing cells did not appear to be involved in signal transduction or gene transcription. Whilst CPSF3 was identified as an interacting partner of A47, this protein has no reported function in NFκB activation, and studies examining its role in apoptosis are conflicting (Calzado et al. 2004; Zhu et al. 2009). It is therefore unlikely that the effect of A47 exerted on IFNβ-promoter- and NFκB-dependent reporter activation was mediated through interaction with CPSF3 (Figure 4-12). In an attempt to identify VV-WR 204 in cell lysates, significance thresholds were reduced, which resulted in detection of a large number of possible host targets. This viral protein was still not observed, which may indicate that identified host proteins are false positives. For this reason and the reported lack of expression of VV-WR 204 during viral infection (Yang et al. 2015), interactions between VV-WR 204 and identified mammalian proteins were not further characterised. In contrast, host proteins identified in A57-, F17- and B12-expressing cells may have a role in transcriptional gene regulation, and are described in the following sections (see 5.3.1.1 - 5.3.2). B12 was the most potent inhibitor candidate of these three VACV proteins, it is almost exclusively localised to the nucleus, and VRK1 was the only protein identified in this analysis (Figure 4-15). Hence, B12 was the most promising inhibitor candidate of this screen, and its interaction with VRK1 as well as the role of VRK1 in innate immune signalling was examined (see 5.3.2).

5.3.1.1. F17 associates with cyclin-dependent kinase 1

F17 was shown to interact with CDK1, which may support the observation that CDK1, along with ERK and JNK, were reported to phosphorylate F17 in *in vitro* kinase assays (Wickramasekera & Traktman 2010), however recently roles in inflammation have emerged for several members of the CDK family (Schmitz & Kracht 2015). In addition, NFκB activation is characterised by the sequential activation of ubiquitin ligases and protein kinases, wherefore CDK1 could affect signal transduction directly through phosphorylation (see 1.2.1.2.2). Screens aiming to identify possible targets of CDK1 phosphorylation confirmed its role in DNA replication, transcription and metabolism, yet did not detect apparent components of innate immune signalling pathways (Ubersax et al. 2003; Blethrow et al. 2008). Noteworthy proteins are limited to the HIV-restriction factor SAMHD1, the architectural protein of the IFNβ enhanceosome HMGI/Y, RNA polymerase II, the DNA damage checkpoint protein Rad9 and the PP2A regulatory subunit Rts1. Interestingly, recent reports demonstrated that VACV (Hollenbaugh et al. 2013), as well as HSV-1 (Hollenbaugh et al. 2013; Kim, White, et al. 2013), and *Hepatitis B virus* replication was restricted by SAMHD1 (Chen et al. 2014). Furthermore, the cyclin A2-CDK1/CDK2 complex was shown to negatively regulate SAMHD1 activity through phosphorylation of Thr592 during HIV-1 infection, yet proteins levels remain unaltered (Cribier et al. 2013; Yan et al. 2015). This finding

would explain why VACV infection did not alter SAMHD1 protein levels (Hollenbaugh et al. 2013). Although this event is not directly involved in innate immune signalling and does not account for the observed inhibition of PRR and pro-inflammatory cytokine signalling mediated by F17, it may be another function of F17, which ensures replication of the VACV genome.

Whilst CDK1 has not been shown to have a role in type I IFN and pro-inflammatory cytokine signalling pathways, other members of this protein family have been reported to affect NF κ B activity (Schmitz & Kracht 2015). A siRNA screen of 636 human kinases in A549 cells revealed that CDK2, CDK5 and CDK7 play a role in TNF α -driven NF κ B activation (Choudhary et al. 2011).

CDK2 has also been identified as an NF κ B-activating protein in a screen for cellular proteins interacting with the IKK complex subunit NEMO (Fenner et al. 2010). NF κ B-dependent reporter gene expression was activated by transient expression of CDK2 and synergistic effects on TNF α -mediated reporter activation were observed. Similarly to CDK2, CDK4 appears to act prior to nuclear translocation of p65 (Thoms et al. 2007). Impairment of CDK4 activity using a specific inhibitor, resulted in degradation of the p65 repressor I κ B α , which allowed p65 to translocate to the nucleus and subsequently into the nucleoli, hereby preventing induction of NF κ B-dependent gene expression. In the same study, inhibition of CDK1, CDK2 and CDK5 indicated that these CDK family members were not involved in regulating nucleolar translocation of p65.

CDK6 was demonstrated to phosphorylate p65 at Ser536 leading to activation and nuclear translocation (Buss et al. 2012). Interestingly, downregulation of CDK6 did not abolish p65 phosphorylation at Ser536, but resulted in a reduced activation and delayed nuclear translocation which is reminiscent of the effect observed in TNF α -stimulated HEK 293T expressing F17 (Figure 4-4B).

Phosphorylation of p65 at Ser276 recruits CDK9 to the promoter of some NF κ B-dependent genes such as IL-8, but not I κ B α (Nowak et al. 2008). In turn CDK9 phosphorylates RNA pol II promoting transcription elongation of these genes.

A growing body of evidence has suggested potential roles of CDKs in innate immune signalling, and association of F17 with CDK1 may account for the impairment of induction of type I IFN and of pro-inflammatory cytokine signalling mediated by F17 (Figure 4-4B, Figure 4-11). However, this association may merely reflect the phosphorylation of F17 by CDK1 (Wickramasekera & Traktman 2010). Interestingly the regulatory subunit of PP2A, which has been shown to dephosphorylate p65 (Yang et al. 2001; Sakurai et al. 2003), was identified as a potential phosphorylation target of CDK1 (Ubersax et al. 2003). To address these possibilities, interaction of F17 and CDK1 during viral infection needs to be confirmed, and the role of CDK1 in innate immunity could be initially characterised through RGAs.

5.3.1.2. A57 – Nucleosome remodelling and histone deacetylation complex

A large number of host proteins involved in gene regulation were identified as possible interaction partners of A57. Notably, the PP2A inhibitor SET may explain the observed reduction of NF κ B activation mediated by A57 (Figure 4-13), as PP2A has been reported to dephosphorylate p65 (Li et al. 1996; Yang et al. 2001; Sakurai et al. 2003). Other noteworthy proteins are ANP32A and NUCKS1, which have been recently shown to be involved in replication of IAV and HIV-1, respectively (Kim et al. 2014; Long et al. 2016).

The most promising finding, however, was the interaction of A57 with several members of the NuRD complex (Torchy et al. 2015), which is composed of: the ATP-dependent chromatin remodelling enzymes CHD3 or CHD4; the histone deacetylases HDAC1 or HDAC2; MTA1/2/3; the methylated CpG-DNA binding protein MBD3; the structural proteins RbAp46 and RbAp48; GATAD2a and GATAD2b; and deleted in oral cancer 1 (DOC-1). With the exception of CHD3, MTA3 and DOC-1, all of the members of the NuRD complex were detected in cell lysates expressing A57 (Figure 5-1D). Whilst the large number of NuRD components suggests that A57 does indeed target this protein complex, it may still be a possibility that the interaction with one NuRD complex component caused other NuRD subunits to be identified as proteins interacting with A57.

Several recent reports have suggested a role of the NuRD complex in innate immunity (Pakala et al. 2010; Aguilera et al. 2011; Bode & Dalpke 2011), and it may antagonise gene expression induced by the nucleosome remodelling complex SWI/SNF (Ramirez-Carrozzi et al. 2006). In an early study (Ramirez-Carrozzi et al. 2006), Ramirez-Carrozzi and co-authors observed an increased production of IL-12, RANTES, IL-6, and IFN β mRNA in response to LPS in macrophages treated with siRNA against the NuRD subunit CHD4, all of which are genes which are expressed with delayed kinetics compared to early response genes. A similar observation was made in a more recent report (Roger et al. 2011), which also showed that TNF α production was unaltered by reduced CHD4 protein levels.

Interestingly, MTA1, a NF κ B-dependent gene, can act as a repressor and activator of gene regulation (Pakala et al. 2010). It was reported to enhance LPS-stimulated activation of IL-1 β and TNF α , whereas basal levels of these pro-inflammatory cytokines were elevated in mice deficient in MTA1. Furthermore, MTA1 was shown to associate with HDAC2 at the promoter of IL1 β and TNF α , an interaction which is disrupted by LPS stimulation (Pakala et al. 2010).

A role similar to MTA1 was also suggested for MBD3 (Aguilera et al. 2011), which was found to directly bind the TAD of c-Jun. This interaction was abolished through phosphorylation of c-Jun by JNK. These authors hypothesised that c-Jun recruits the NuRD complex to AP1-dependent genes in unstimulated cells. Thereby repressing their expression. Upon activation of the MAPK signalling pathway, JNK phosphorylates c-Jun, which reduces its affinity for MBD3 and the NuRD complex, which dissociates from AP1 target genes enabling their transcription.

Taken together, these reports provide good evidence that the NuRD complex is involved in innate immune signalling. Interestingly, the NuRD subunits MBD3, GATAD2A and GATAD2B have been shown to colocalise in the nucleus in a speckled pattern (Brackertz et al. 2002; Feng et al. 2002), which is reminiscent of the cellular localisation observed for A57 (Figure 4-13F). The impairment of PRR signalling mediated by A57 may therefore be explained by recruitment and/or stabilisation of the NuRD complex to the promoter of IRF3/7- and NF κ B-dependent genes, which leads to repression of gene induction. Interestingly, vv811 Δ 49, which still encodes A57, was able to potently reduce the expression of RANTES, MCP1, and intercellular adhesion molecule 1 (ICAM-1) (Sumner et al. 2014), all of which are late primary and secondary response proteins.

The interaction and a possible co-localisation of A57 with components of the NuRD complex need to be addressed in order to support the role of this complex in antiviral responses and to establish a mechanism of inhibition by A57.

5.3.2. VRK1 is a possible target of B12

The nuclear kinase VRK1 was the only protein detected as an interaction partner of B12 (Figure 5-1F). It also shares 30 % sequence identity with VRK1 (Figure 5-2A). For these reasons and its ability to potentially impair PRR- and pro-inflammatory cytokine-mediated reporter activation (Figure 4-15), B12 was selected for more detailed studies.

VRK1 is ubiquitously expressed, in particular in highly proliferating tissues (Nezu et al. 1997; Valbuena et al. 2007). The 396 a.a. kinase is composed of a N-terminal Ser/Thr kinase domain and a regulatory C-terminal domain (Nezu et al. 1997; Lopez-Borges & Lazo 2000). The latter contains a NLS (Lopez-Borges & Lazo 2000), and the residue Thr355 which is autophosphorylated during activation (Barcia et al. 2002). NMR studies of the active kinase have shown that the C-terminal tail (341-361) interacts with the catalytic site and is required for structure, activation and autophosphorylation (Shin et al. 2011). In addition to autophosphorylation, VRK1 has been reported to phosphorylate various host proteins including histone H3, BAF and transcription factors such as p53, c-Jun, ATF2 and CREB (Lopez-Borges & Lazo 2000; Sevilla, Santos, Barcia, et al. 2004; Sevilla, Santos, Vega, et al. 2004; Nichols et al. 2006; Kang et al. 2007; Kang et al. 2008).

The vaccinia-related kinase family, named after the VACV protein B1 due to their sequence similarity (Nezu et al. 1997), has three members i.e. VRK1, VRK2 and VRK3, which share 20 – 40 % sequence identity (Nichols & Traktman 2004). It is worth mentioning that only VRK1 and not VRK2 or VRK3 were identified as potential interaction partners of B12. VRK orthologs were found in mice, *Xenopus laevis*, *Caenorhabditis elegans* and *Drosophila melanogaster* (Aihara et al. 2004). Loss of VRK1 orthologs in *D.melanogaster* and *C.elegans* resulted in sterility (Ivanovska et al. 2005), embryonic lethality and impaired growth in adults (Wormbase.org 2016), respectively. Murine homologues for each human VRK protein were identified in mice and loss of VRK1 also resulted in male and female sterility (Wiebe et al. 2010; Schober et al. 2011). A point mutation in the NLS of human VRK1 is associated with rare infantile spinal muscular atrophy (Renbaum et al. 2009).

An increasing body of data suggests the involvement of VRK1 in cell cycle regulation (Valbuena et al. 2011), yet a role in innate immune signalling has not been reported to date. The predominantly cytoplasmic VACV protein B1 has been shown to phosphorylate VRK1 target proteins (Banham & Smith 1992; Santos et al. 2004; Nichols et al. 2006; Santos et al. 2006), but B1 did not interact directly with VRK1. VRK1 is predominantly localised to the nucleus outside nucleoli (Nichols & Traktman 2004; Vega et al. 2004), however it has also been observed at the Golgi apparatus in some cell types (Valbuena et al. 2007). Similar to VRK1, B12 is a nuclear protein (Figure 4-15F). Surprisingly, both proteins have been shown to interact directly and to co-localise (Figure 5-2C,D), whereas no other VRK protein has been reported to form homo- or heterodimers.

B12 demonstrated a potent impairment of NF κ B signalling and type I IFN induction (Figure 4-15), which may be mediated through targeting of VRK1. To address this possibility the role of VRK1 in NF κ B activation was studied (Figure 5-3), but contrasting results were obtained. VRK1 may activate NF κ B-dependent reporter gene expression by itself as it was shown for other transcription factors (Figure 5-3A) (Sevilla, Santos, Barcia, et al. 2004; Sevilla, Santos, Vega, et al. 2004; Vega et al. 2004). In contrast, B12-mediated reduction of reporter activity was increased by co-expression of VRK1 (Figure 5-3D,E). Additional experiments are required to clearly demonstrate involvement of VRK1 in NF κ B activation.

Published targets of VRK1 phosphorylation include p53 and c-Jun (Lopez-Borges & Lazo 2000; Sevilla, Santos, Barcia, et al. 2004). VRK1 has been demonstrated to phosphorylate p53 at Thr18 (Lopez-Borges & Lazo 2000; Vega et al. 2004), thereby preventing mdm2-mediated degradation of p53. In turn, p53 was shown to downregulate VRK1 protein levels in a mdm2- and proteasome-independent manner (Valbuena et al. 2006). Interestingly, B12 caused a reduction in both basal and p53-driven p53-dependent reporter activity (Figure 5-4), suggesting that B12 may be able to target multiple host signalling pathways. VRK1 has also been found to phosphorylate the transcription factors c-Jun at Ser63 and Ser73 (Sevilla, Santos, Barcia, et al. 2004), and ATF2 at Thr73 and Ser62 (Sevilla, Santos, Vega, et al. 2004). Although VRK1 did not directly associate with JNK (Sevilla, Santos, Barcia, et al. 2004), an additive effect was observed in transactivation reporter assays for c-Jun and ATF2 (Sevilla, Santos, Barcia, et al. 2004; Sevilla, Santos, Vega, et al. 2004). Given the published role of VRK1 in AP1 activation, the effect of B12 on this signalling pathway was studied. To this end AP1-dependent reporter genes were driven by VRK1 as anticipated, and this activation was reduced by co-transfection of B12 (Figure 5-5). In addition, phosphorylation of c-Jun in response to TNF α was determined in cells transfected with siRNA against VRK1 and in cells expressing B12. Preliminary data suggests that B12 can indeed reduce and delay the phosphorylation of c-Jun (Figure 5-6C), similar to the observations made in siRNA treated cells (Figure 5-6B).

VRK2 is able to activate p53 and it forms a stable complex with TAK1, c-Jun-amino-terminal-interacting protein 1 (JIP1) and MKK7 preventing IL-1 induced JNK activation (Blanco et al. 2006; Blanco et al. 2007; Blanco et al. 2008). Hitherto, VRK3 was assumed to be a pseudokinase, however a recent study demonstrated its ability to bind and phosphorylate BAF (Park et al. 2015). VRK3 also interacts with Vaccinia-H1 related kinase (VHR), whereby it enhances VHR-mediated dephosphorylation of the MAP kinase ERK (Kang & Kim 2006).

Taken together, these results indicate that B12 is able to impair PRR- and pro-inflammatory cytokine-mediated activation of NF κ B and c-Jun, which may be achieved through targeting of VRK1. However, it may also be possible that either of these inhibitory roles are not related to its association with VRK1.

5.3.3. Summary of potential host targets identified for VACV immunomodulators

Eight VACV ORFs were identified and characterised as potential PRR- and pro-inflammatory cytokine signalling inhibitors from a library of 42 genes encoded in the VACV genome. In attempt to identify possible host targets host proteins bound to viral immunomodulators were analysed by mass spectrometry by our collaborators Dr. Haas and Dr. Pichlmair. No clear targets were found for A47, B2, B13 and VV-WR 204, which might explain the inhibition of innate immune signalling. In contrast, F17, A57 and B12 interacted with host proteins, i.e. CDK1, components of the NuRD complex, and VRK1, which could play a role in subverting PRR- and pro-inflammatory cytokine pathway activation. Whilst the role of VRK1 in NF κ B activation requires further investigation, it appeared that B12 is able to impair VRK1-mediated activation of the transcription factor AP1.

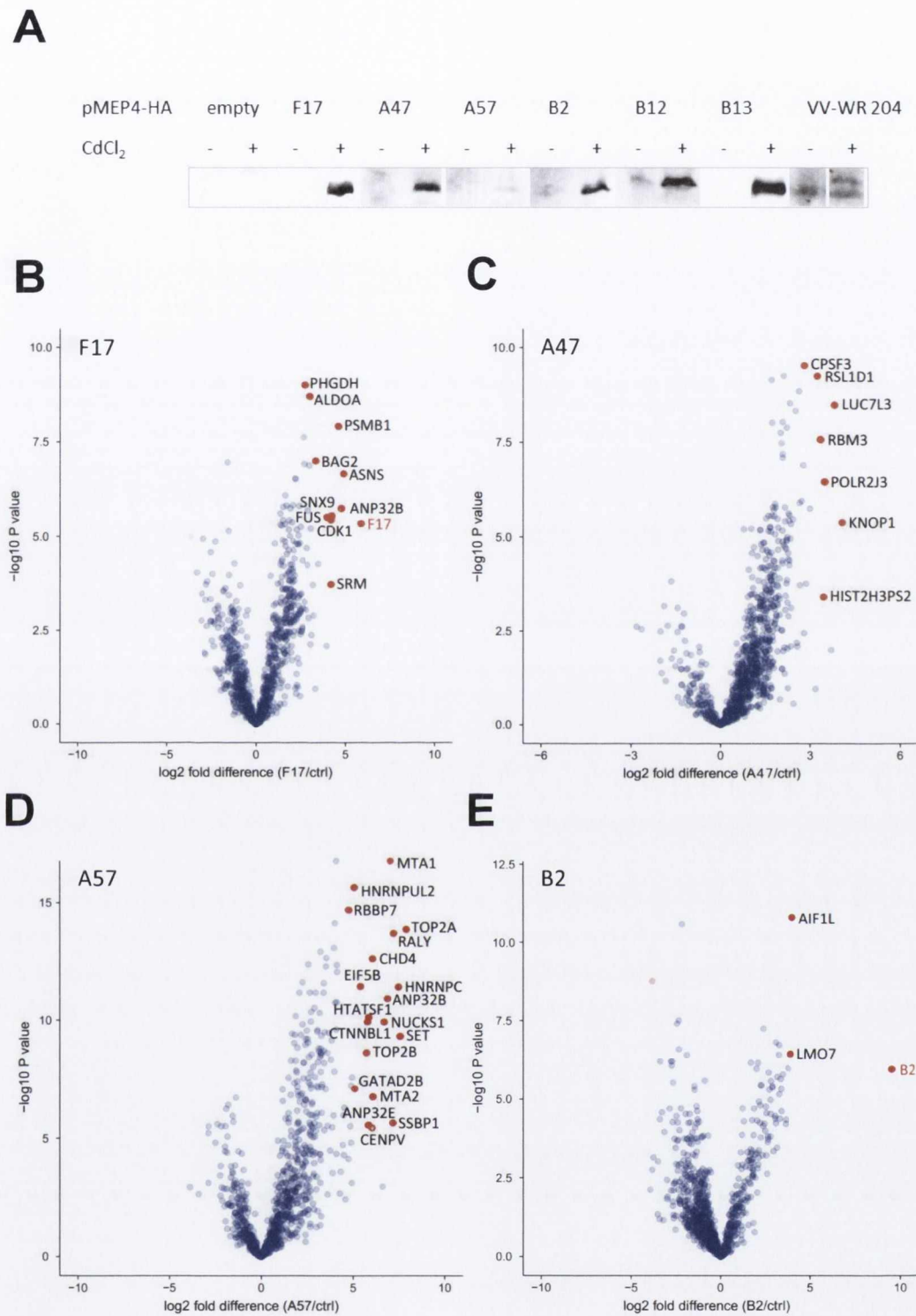


Figure 5-1 Interactome analysis of VV-WR inhibitors (see next page for legend)

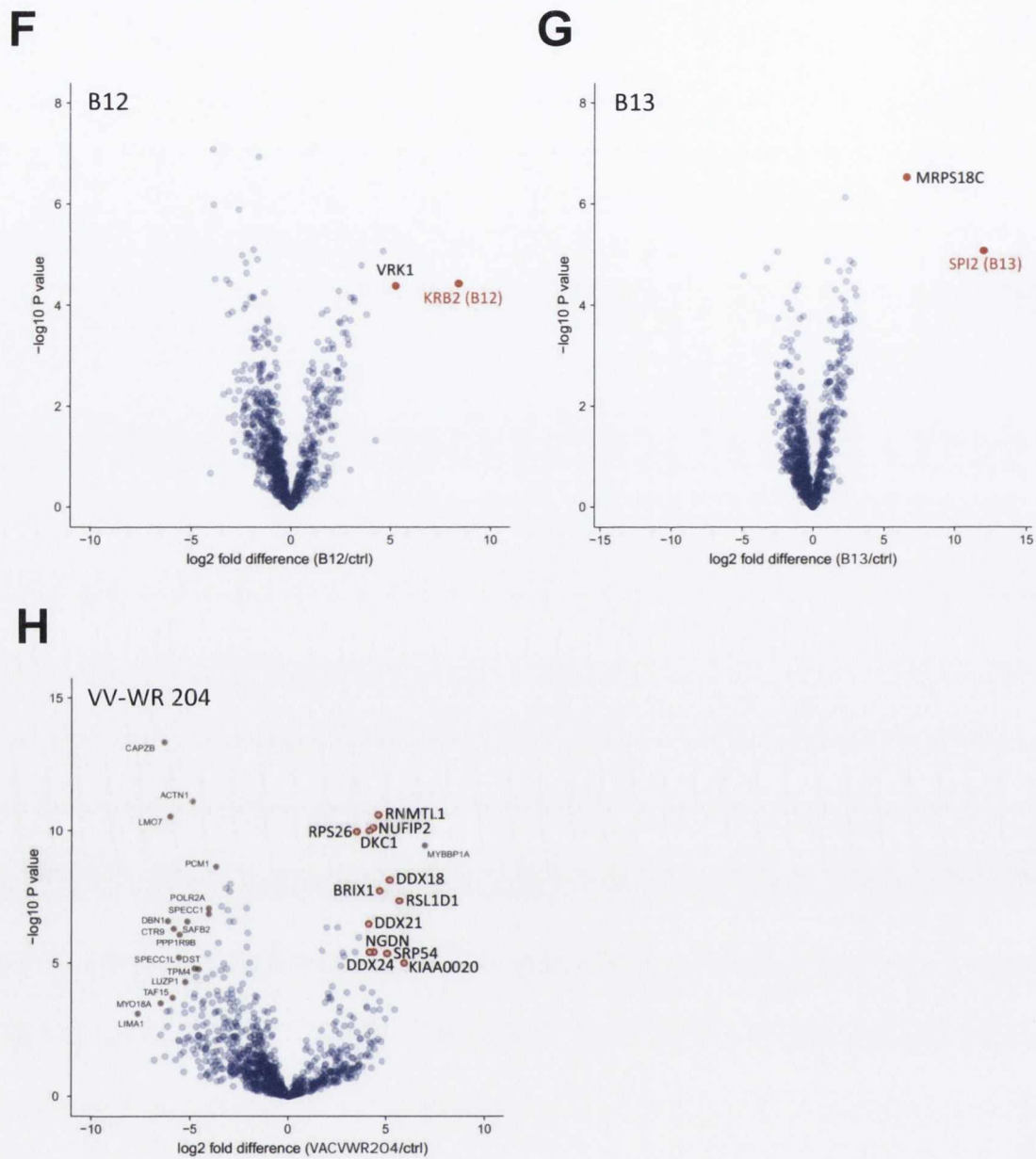
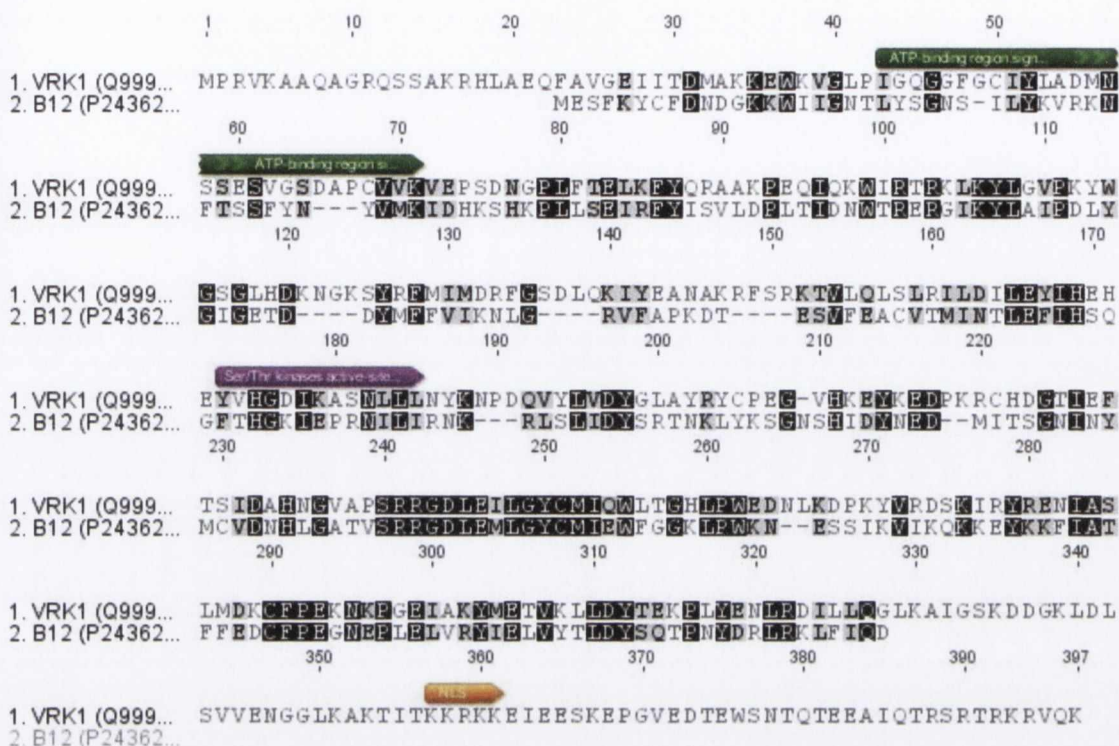


Figure 5-1 Interactome analysis of VV-WR inhibitors

Previously described stably transformed HEK 293T cells were grown in T-175 flasks and induced with 1 μ M CdCl₂. Cells were snap frozen 24 hpi and analysed by mass spectrometry. Cells transfected with empty pMEP4 vector served as negative controls. (A) To ensure protein expression, protein samples from cell lysates were separated by SDS-PAGE and immunoblotted with anti-HA and anti- β -actin antibodies. (B-H) Volcano blots of the individual VV-WR proteins are given. Significant hits are highlighted by red dots. VV-WR proteins, with which the HEK 293T cells were stably transformed are marked in red. A single experiment was carried out in quadruplicate.

A



B

C

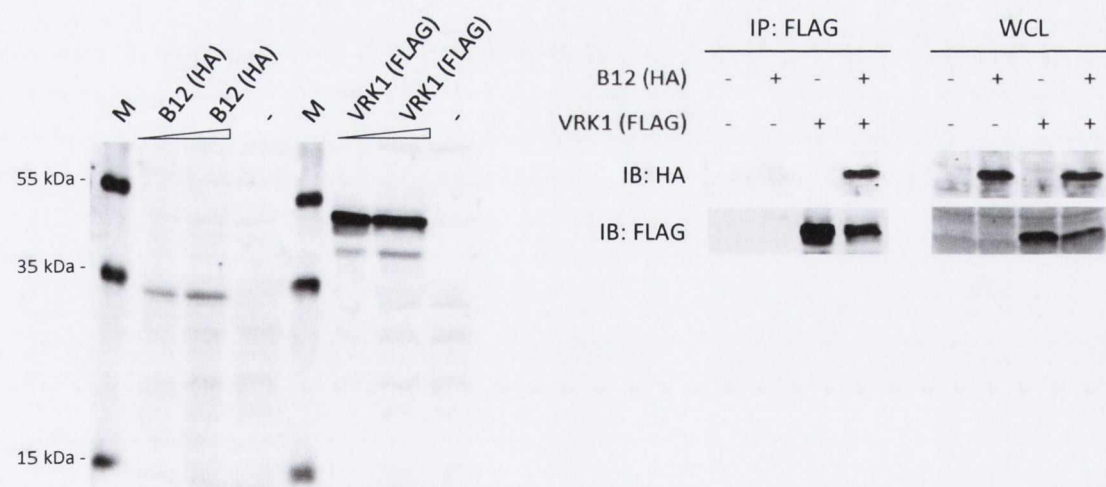


Figure 5-2 VRK1 is a potential target of *Vaccinia virus* B12 (see next page for legend)

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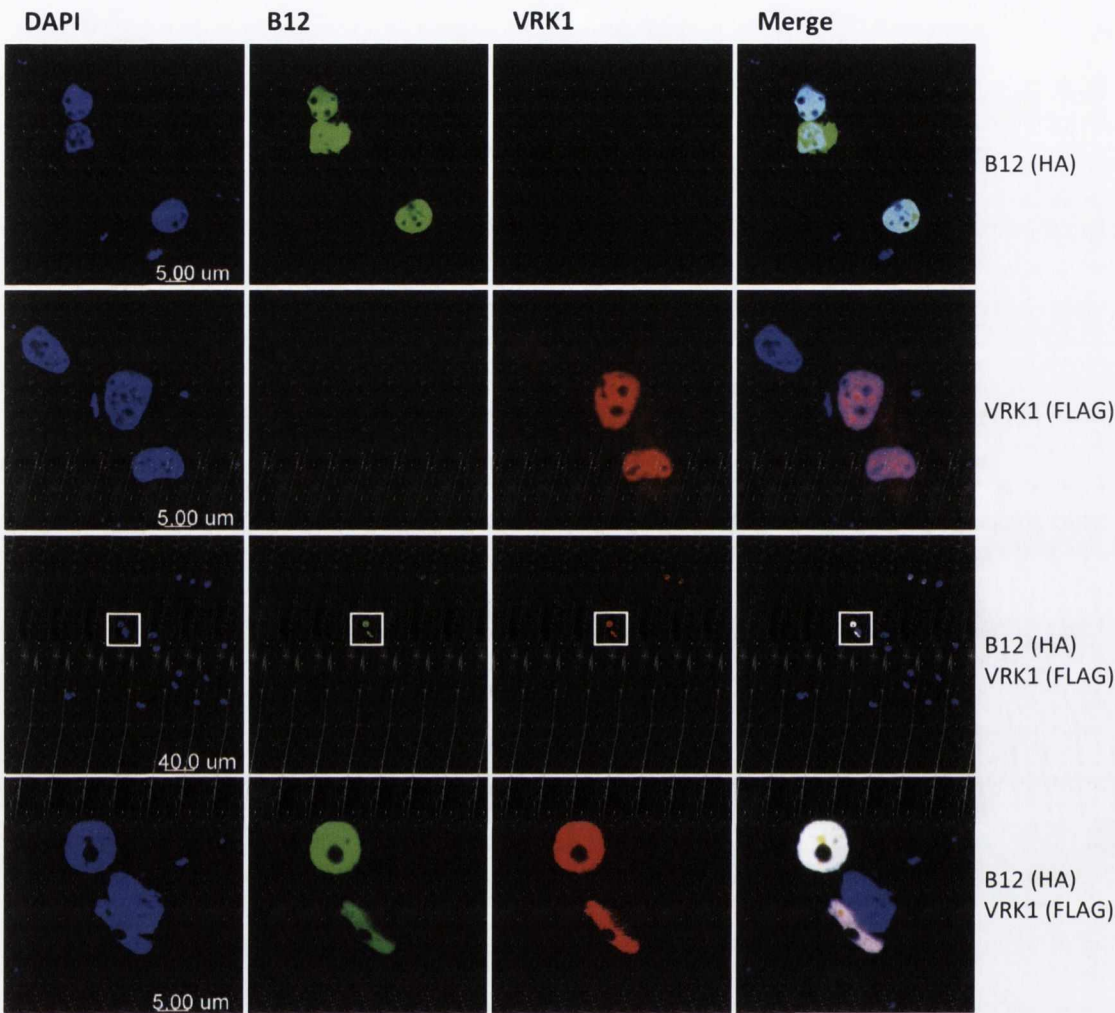


Figure 5-2 VRK1 is a potential target of *Vaccinia virus* B12

(A) Amino acid sequence alignment of full-length human VRK1 and VACV B12. Identical and similar residues are shown in black and grey, respectively (B) HEK 293T cells were transfected with 3 μ g of either HA-tagged B12 expression plasmid or 3 μ g of FLAG-tagged VRK1 expression construct. Cells were harvested 24 hpt and proteins from cell extracts were separated by SDS-PAGE and immunoblotted using anti-HA or anti-FLAG antibodies. Two volumes (5 μ l, 15 μ l) of protein lysates were loaded, as indicated by wedges. Untransfected cells, indicated by '-' served as negative control to evaluate antibody background staining. Nonspecific bands are labelled 'NS'. (C) HEK 293T cells were transfected with 3 μ g of each plasmid encoding HA-tagged B12, FLAG-tagged VRK1, and HA-tagged empty vector as indicated. Untransfected cells served as negative controls. Whole cell lysates (WCL) were subjected to immunoprecipitation (IP) with anti-FLAG M2 agarose beads 24 hpt. Proteins, eluted by FLAG-peptides, were separated by SDS-PAGE and detected by anti-HA and anti-flag immunoblotting (IB). A representative of two independent experiments is shown. (D) HEK 293T cells were grown on coverslips and transfected with either 0.5 μ g of plasmid encoding HA-tagged B12 (upper panel), 0.5 μ g of plasmid encoding FLAG-tagged VRK1 (second panel), or 0.25 μ g of each expression construct (third and lower panel). Cells were fixed and stained with anti-HA (red) and anti-FLAG (green) antibodies 24 hpt. Nuclear DNA was stained with DAPI (blue). White rectangle in third panel indicates positions of magnified section shown in lower panel. A representative of two independent experiment is shown.

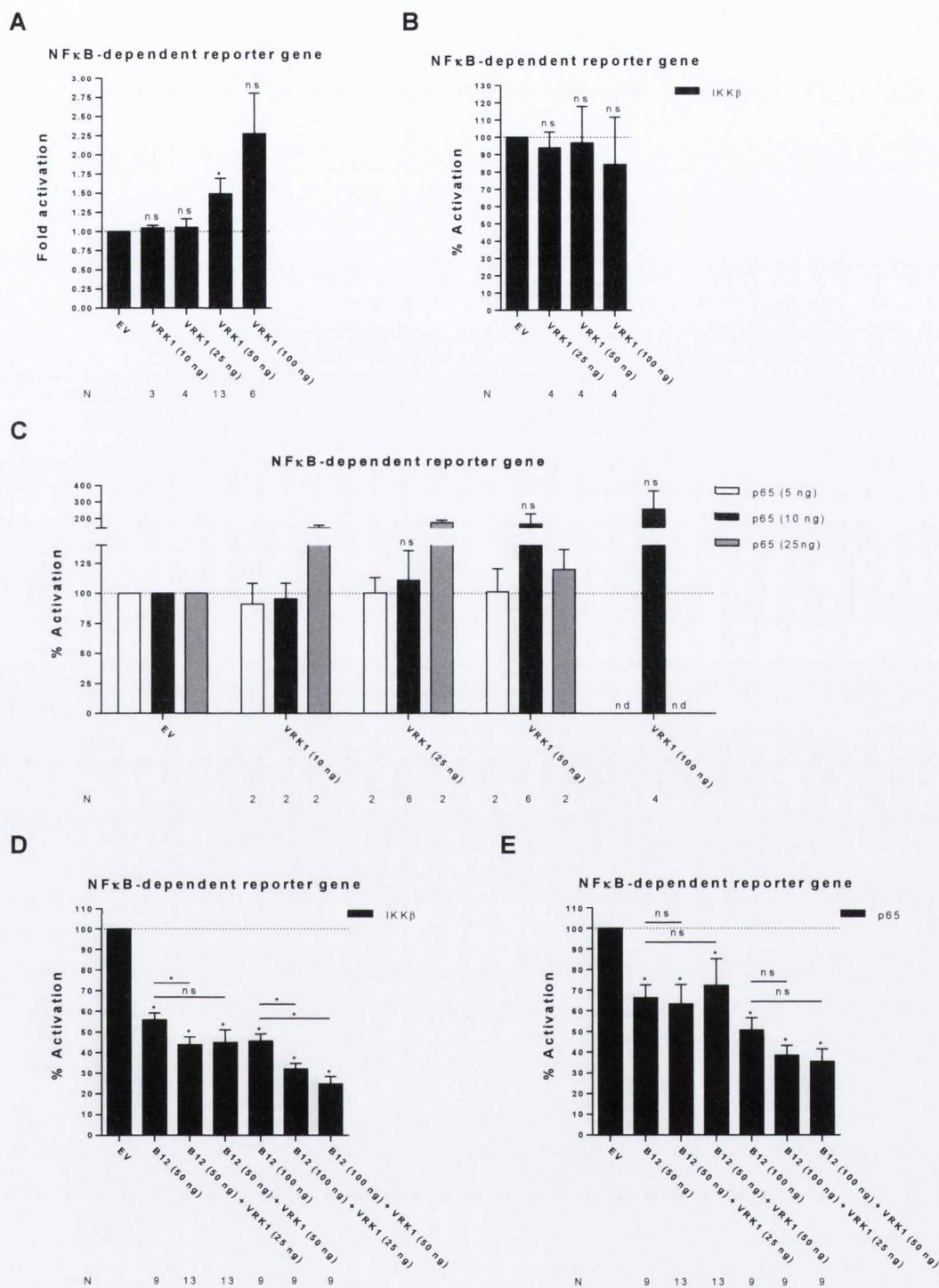


Figure 5-3 Effect of VRK1 and B12 on NFκB activation

HEK 293T cells were co-transfected with a renilla luciferase control and a firefly luciferase reporter plasmid under the control of NFκB-elements. (A) Cells were also transfected with increasing amounts of hVRK1 expression construct. (B, D) To drive reporter gene IKKβ and (C, E) p65 expression plasmids were transfected into these cells along with hVRK1, B12 expression constructs or empty vector control (EV). Cells were harvested 24 hpt, firefly luciferase activity was measured and normalised to renilla luciferase activity. Individual or combined assay values for B12 and VRK1 were scaled relative to EV, which was set to 1 fold activation, or 100 % activation (black dotted line). Means of at least three independent experiments with triplicate determination are shown with their SEMs. Exact number of independent experiments (N) is given below each graph. *p<0.05, ns (not significant).

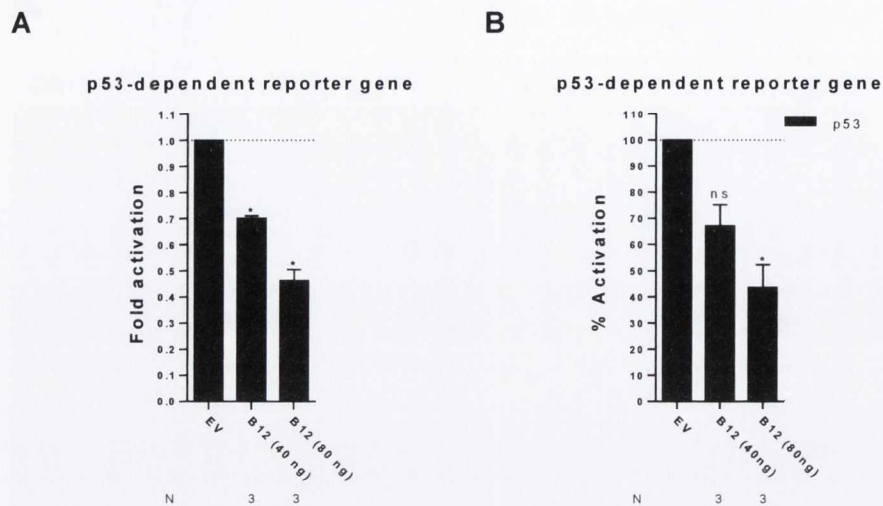


Figure 5-4 Effect of B12 on p53 activity

HEK 293T cells were co-transfected with a renilla luciferase control and a firefly luciferase reporter plasmid under the control of p53-elements. (A) Cells were also transfected with increasing amounts of B12 expression construct. (B) To drive the reporter gene, p53 expression plasmid was transfected into these cells along with B12 expression construct or empty vector control (EV). Cells were harvested 24 hpt, firefly luciferase activity was measured and normalised to renilla luciferase activity. B12 assay values were scaled relative to EV, which was set to 1 fold activation, or 100 % activation (black dotted line). Means of at least three independent experiments with triplicate determination are shown with their SEMs. Exact number of independent experiments (N) is given below each graph.*p<0.05, ns (not significant).

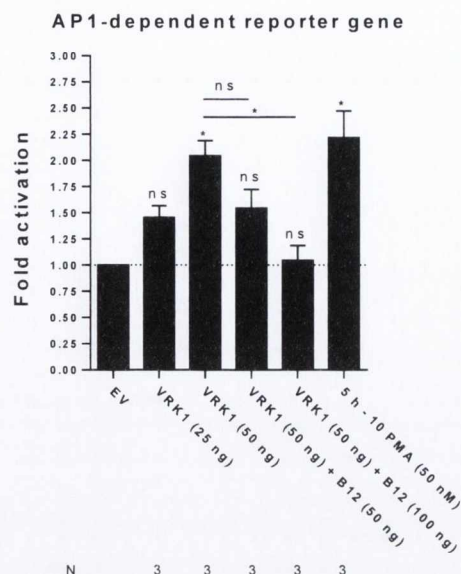


Figure 5-5 VRK1 and B12 effect on AP1 activity

HEK 293T cells were co-transfected with a renilla luciferase control and a firefly luciferase reporter plasmid under the control of an AP1-promoter. Cells were also transfected with increasing amounts of hVRK1 and B12 expression constructs. Twenty-one hours after transfection cells were treated with 50 nM PMA to serve as a positive control for AP1-promoter activation. Cells were harvested 24 hpt, firefly luciferase activity was measured and normalised to renilla luciferase activity. PMA, as well as individual and combined assay values for VRK1 and B12 were scaled relative to EV, which was set to 1 fold activation (black dotted line). Means of at least three independent experiments with triplicate determination are shown with their SEMs. Exact number of independent experiments (N) is given below each graph.*p<0.05, ns (not significant).

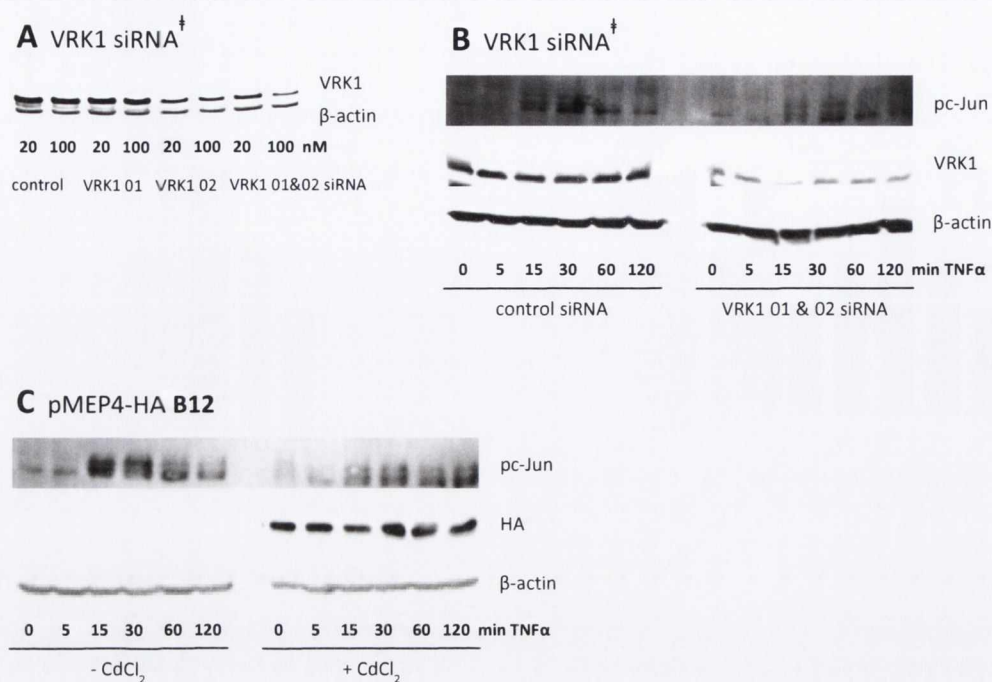


Figure 5-6 Effect of VRK1 and B12 on c-Jun signalling

(A) HEK 293T cells were transfected with 20 nM or 100 nM of negative control siRNA, or hVRK1 01 siRNA, hVRK1 02 siRNA or both for 48 h. (B) HEK293T cells were transfected with 100 nM of negative control siRNA, or hVRK1 01 siRNA and hVRK1 02 siRNA for 48 h. (C) HEK 293T cells were transfected with 8 µg of a CdCl₂ inducible B12 expression construct (pMEP4HA-VV-WR B12), and selected and maintained in 300 µg/ml Hygromycin. (B,C) Forty-eight hours after (B) transfection of siRNA or (C) induction with 1 µM CdCl₂, cells were stimulated with 50 ng/ml TNFα at 0, 5, 15, 30, 60 and 120 min. Proteins from cell extracts were separated by SDS-PAGE and analysed by immunoblotting with anti-pc-Jun, anti-HA or anti-hVRK1, and anti-β-actin antibodies. Cells that were not induced with CdCl₂ served as internal negative controls. Unless indicated by '†', a representative of at least 3 independent experiments is shown.

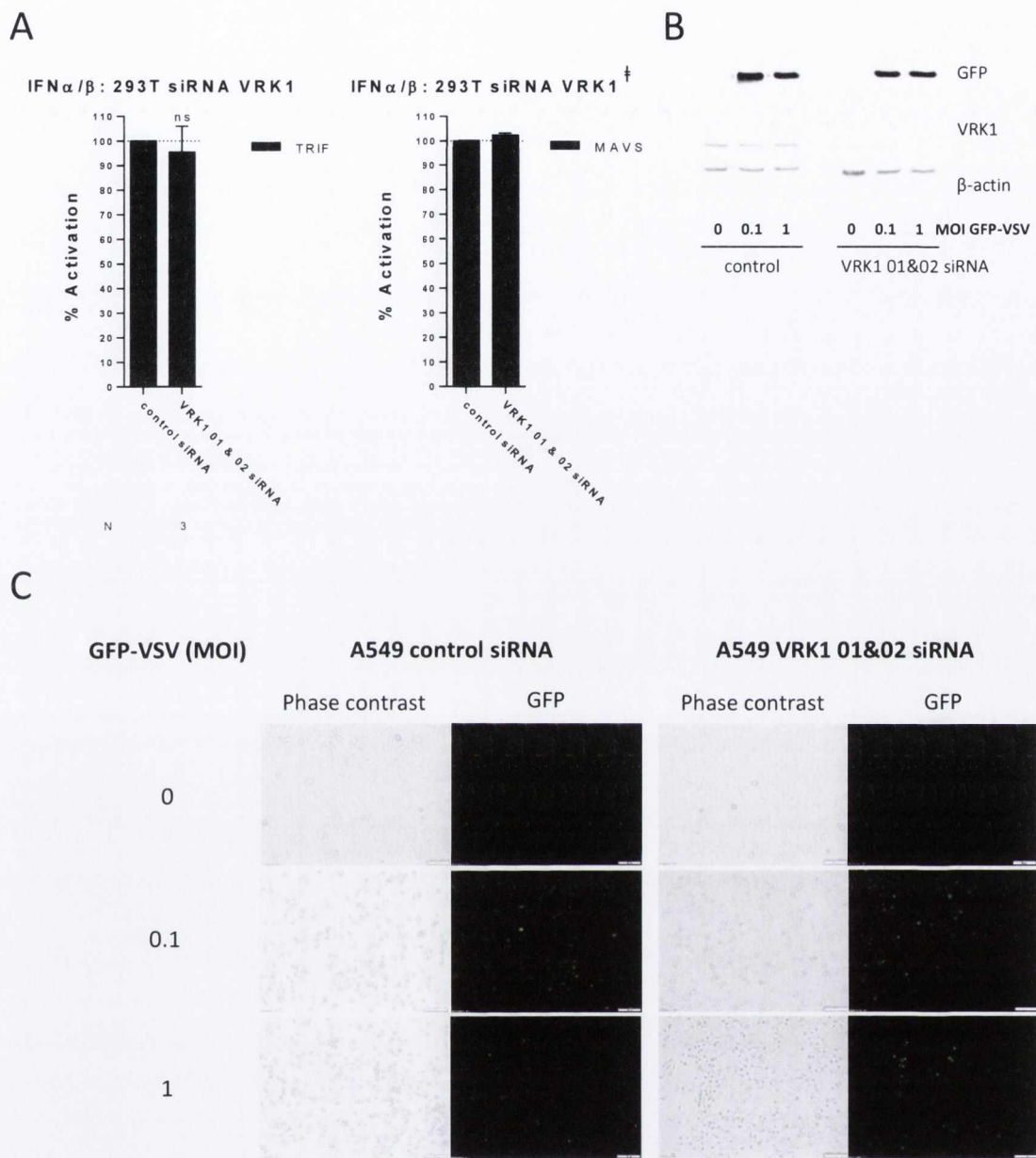


Figure 5-7 Evaluation of physiological effects of VRK1

(A) HEK293T cells were transfected with 100 nM of negative control siRNA, or hVRK1 01 siRNA and hVRK1 02 siRNA. Forty-eight hours later cells were transfected with TRIF or MAVS expression construct. Type I IFN bioactivity in the supernatants was measured using HEK-Blue™ IFN α / β cells 24 hpt. 'VRK1 01 & 02 siRNA' assay values were scaled relative to 'control siRNA', which was set to 100 % activation (black dotted line). Unless marked by '†', means of at least three independent experiments with triplicate determination are shown with their SEMs. *p<0.05, ns (not significant). If marked with '†', mean of two independent experiments with triplicate determination is shown with SD. (B,C) A549 cells transfected with 100 nM of negative control siRNA or hVRK1 01 and hVRK1 02 siRNA were infected with GFP-VSV at MOI 0, 0.1 and 1 for 24 h. GFP levels were analysed (B) in cell lysates by immunoblotting with anti-GFP, anti-hVRK1 and anti- β -actin, and (C) by microscopy. Scale bar, 100 μ m.

Chapter 6 : Final Discussion

Vaccinia virus is a large enveloped dsDNA virus, which was used as the vaccine strain in the eradication of smallpox (Fenner et al. 1988). Extensive research on VACV and other poxviruses is still ongoing (Jacobs et al. 2009; Verardi et al. 2012) due to their potential as vaccines for emerging poxviruses (e.g. MPXV, CPXV), as vaccine vectors against other pathogens (e.g. rabies, HIV), and their application in oncolytic virotherapy. An advantage of VACV as a vaccine vector is that its efficacy in eliciting an adaptive immune response and its adverse effects were extensively studied during the smallpox eradication program (Fenner et al. 1988). Viruses selectively target well-connected host proteins, which play central roles in many cellular processes (Pichlmair et al. 2012). Thus, characterisation of viruses such as VACV has improved our understanding of both basic immunology and cell biology. Recently, cGAS, a DNA sensor, which plays a key role in sensing poxviral DNA during infection (Schoggins et al. 2014), was identified, though no poxviral antagonist of cGAS has yet been found. To this end, the present study examined the potential of 42 intracellular VV-WR proteins to suppress cGAS-STING signalling at the level of the receptor and/or the adaptor protein. Seven novel inhibitors (i.e. M1, F17, A47, A57, B2, B12, B13) proximal to NFκB activation were found instead (Figure 6-1, Table 6-1), of which B2 had the greatest inhibitory potential. Although nine VACV immunomodulators targeting events directly involved in NFκB activation have been published to date (Brady & Bowie 2014), the discovery of additional inhibitors is anticipated, since the VACV-derived mutant virus vv811ΔA49 lacking these proteins is able to impair NFκB activity (Sumner et al. 2014). Examination of host interaction partners of the VV-WR proteins identified in this study led to the discovery of cytoplasmic and nuclear host proteins with established roles in cell cycle regulation (i.e. CDK1, VRK1) (Klerkx et al. 2009; Schmitz & Kracht 2015), and transcription (i.e. NuRD complex) (Torchy et al. 2015). Some of these proteins have been recently associated with innate immune signalling (Pakala et al. 2010). Detailed characterisation of the interaction of VV-WR B12 and host kinase VRK1 suggested that B12 can impair VRK1-mediated AP1 activation, which may be a separate function of B12 in addition to its ability to suppress NFκB activity. A role of VRK1 in NFκB regulation has not been reported to date and further investigations are required.

During this study, reporter gene assays have proven to be rapid and effective tools in the identification and characterisation of immunomodulators encoded in the VACV genome. These initial results were followed up by further functional experiments for selected VV-WR proteins. The approach of examining individual ORFs in isolation allows for the examination of the mechanism of inhibition of individual viral proteins independent of other viral immunoregulators, which otherwise would be present during infection. These results should be complemented by studies using VACV *in vitro* and *in vivo* to assess the contribution of individual viral proteins to suppression of innate immune signalling and pathogenesis. For example, VV-WR 204 was not detected during infection of HeLa cells (Yang et al. 2015), which reduces the likelihood that this protein plays a role in innate immune subversion. Mouse infection models are used to evaluate the virulence of viruses encoding or lacking individual immunoregulators, the observed virulence has been shown to correlate with ability to induce adaptive immune responses and the overall safety of viruses as vaccine vectors (García-Arriaza et al. 2011; García-Arriaza et al. 2014).

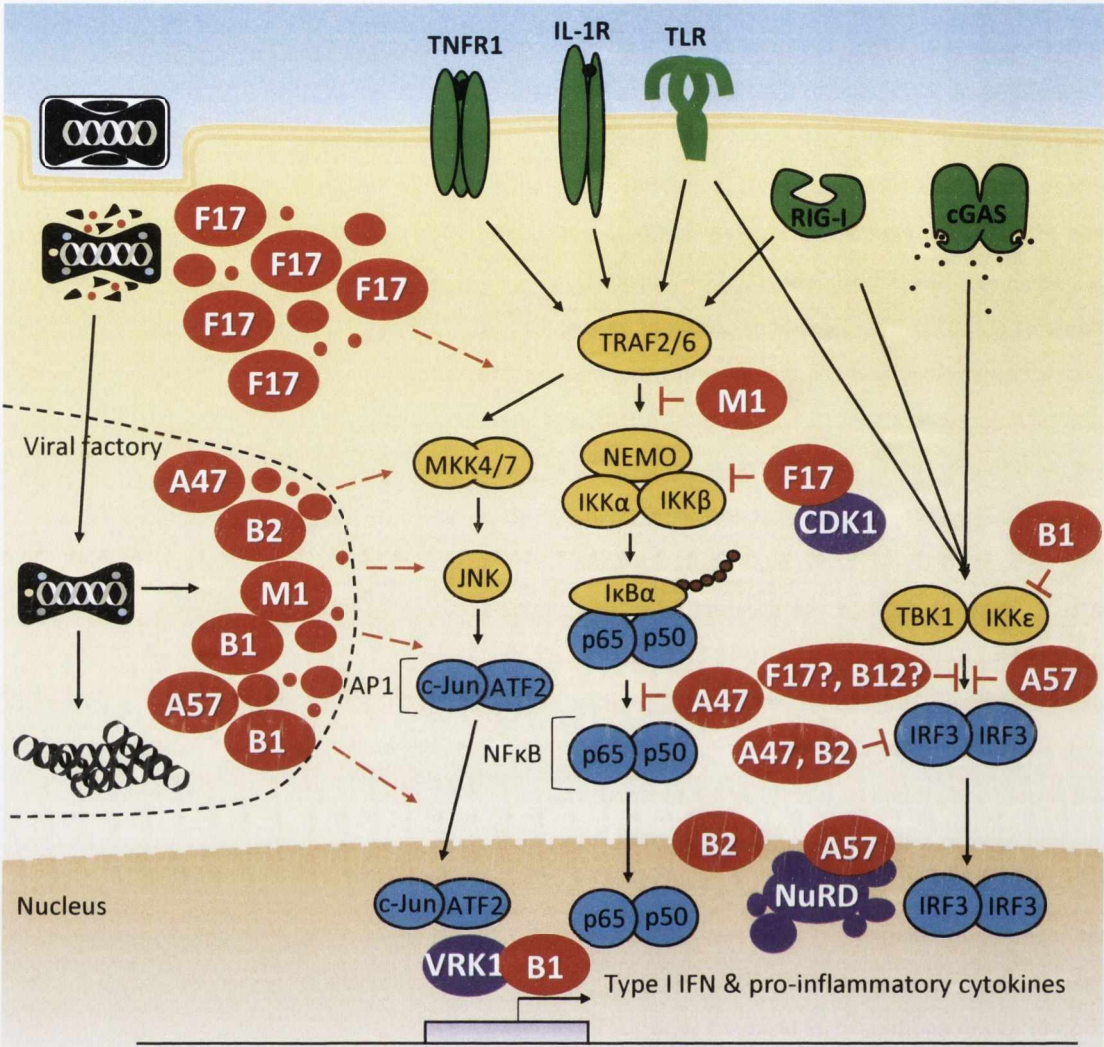


Figure 6-1 Overview of identified novel VACV immunomodulators

Seven immunomodulators of PRR and pro-inflammatory cytokine signalling pathways were identified, all of which act downstream of studied PRRs and cytokine receptors. M1 appears to act upstream of the IKK complex, whereas F17 and B13 inhibit signal transduction at the level of TBK1/IKKε and/or the IKK complex. A47, A57 and B2 act proximal to IRF3 and NFκB. B12 is the only predominantly nuclear protein, and is able to impair both NFκB and IRF signalling.

VV ORF name		Targeted pathway	Proposed points of inhibition	Identified host targets
WR	COP			
030	M1L	NFκB	Adaptor proteins – IKK complex	
056	F17R	NFκB, IRF3/7	IKK complex, TBK1 – IRF3/7	CDK1
173	A47L	NFκB, IRF3/7	IKK complex – p65, IRF3/7?	CPSF3
182	A57R	NFκB, IRF3/7	p65, TBK1/IKKε	NuRD complex
184	B2R	NFκB, IRF3/7	p65, IRF3/7	
194	B12R	NFκB, IRF3/7, MAPK	p65, TBK1 – IRF3/7, cJun, p53	VRK1
195	B13R	NFκB, IRF3/7	IKKε, TBK1?	

Table 6-1 Summary of identified VACV immunomodulators and their potential host targets

Overview of identified VACV immunomodulators (WR, COP nomenclature) is given including the potential host signalling events and host proteins that are targeted.

Seven VV-WR proteins able to interfere with IRF and/or NF κ B signalling were identified and characterised (M1, F17, A47, A57, B2, B12, B13), all of which appeared to target transcription factor-proximal components of the studied signalling pathways (Figure 6-1, Table 6-1). Surprisingly, all of these proteins, with the exception of M1 and F17, localise at least partially to the nucleus. To date only one VACV protein, N2, has been reported to suppress IRF and NF κ B activity in the nucleus, whilst the remaining published immunomodulators have been shown to predominantly reside and target host proteins in the cytoplasm (Brady & Bowie 2014). Poxviruses encode multiple proteins interfering with the signal transduction pathways leading to activation of transcription factors. However comparatively few inhibitors appear to directly target NF κ B activity, as it was demonstrated for the recently discovered MCV protein MC132 (Brady et al. 2015). The deletion virus vv811 Δ A49 demonstrated the existence of additional NF κ B inhibitors (Sumner et al. 2014), and this virus encodes the following VV-WR of the present study: F17, A47, A57, B2 and B12. It is tempting to speculate that these viral proteins may alter NF κ B activity prior or following its nuclear translocation by means of post-translational modifications, or by interfering with co-factors required for transcription of NF κ B-dependent genes. Post-translational modifications of NF κ B subunits in addition to phosphorylation of p65 at Ser536 have crucial roles in regulating the activity of this transcription factor by affecting cellular localisation, affinity for κ B sites and protein stability (Huang et al. 2010). The presence of the VV-WR ORFs identified in this study in the vv811 Δ A49 genome may account for the inhibition of NF κ B activity, and therefore the study of deletion mutant viruses could provide information about the contribution of F17, A47, A57, B2 and B12 to suppression of NF κ B signalling. A very promising potential inhibitor identified in this study was B2. B2, a viral gene expressed early during infection following the establishment of viral factories in the cytoplasm, greatly reduced PRR-, IL-1R-, and TNFR-mediated activation of IRFs and NF κ B. however, no host protein was identified as significantly interacting with B2. Study of B2 deletion mutant viruses may provide information as to whether B2 contributes to immune evasion exerted by VACV, and thereby indicate whether the mechanism of inhibition should be further investigated.

Interestingly, the kinase CDK1 was identified as an interaction partner of F17. Whilst this observation may simply confirm the suggested phosphorylation of F17 by CDK1 (Wickramasekera & Traktman 2010), it may also indicate that F17 acts as a decoy substrate for CDK1, or otherwise affects CDK1 functioning. CDKs are foremost studied for their role in cell cycle progression, and VACV has been found to increase the number of cycling cells during infection by upregulation of specific CDKs (Wali & Strayer 1999). The association of F17 with CDK1 and the increased elk-transactivation might therefore represent a manipulation of cell cycle regulation to facilitate viral replication. However recently CDKs have been increasingly implicated in regulation of immune responses (Schmitz & Kracht 2015). Cyclin-dependent kinases have been reported to interact with NEMO and to phosphorylate p65 (Fenner et al. 2010; Buss et al. 2012). The ability of F17 to suppress TNF α - and PRR-mediated NF κ B activation may therefore be a consequence of its interaction with CDK1. In addition, SAMHD1 has been described as a target of CDK1, which negatively regulates the activity of SAMHD1 through direct phosphorylation (Cribier et al. 2013). SAMHD1 hydrolyses deoxynucleotides (Ballana & Est   2015), thereby reducing host and viral DNA replication. However, VACV requires active DNA replication for transcription of intermediate and late genes, and encodes proteins synthesising dNTPs (Moss 2013). F17 is produced late during infection and packaged into nascent virions allowing it to exert its potential

function(s) immediately after cell entry and before the onset of early gene transcription in a similar fashion to the immunoregulator H1 (Najarro et al. 2001). Recently, a study suggested that F17 was degraded upon cell entry (Schmidt et al. 2013). However, F17 is the most abundant component of the lateral bodies (Schmidt et al. 2013), which are associated with the virion core at cell entry. The same authors proposed that lateral bodies are a means of delivering immunoregulatory proteins into a cell (Bidgood & Mercer 2015), and hinted that F17 may have a role early during infection. F17 may therefore target CDK1 to interfere with SAMHD1 and p65 regulation. To address these hypotheses it will be crucial to confirm the interaction of F17 and with endogenous CDK1 during infection. F17 is an essential and conserved VACV protein, deletion of which resulted aberrant virion formation during late stages of infection (Zhang & Moss 1991), which impedes the examination of its role in innate immune signalling. However, the effect of CDK1 on p65 can be addressed by means of reporter gene assays, measurement of p65 phosphorylation and examination of the effect of CDK1 on induction of NF κ B-dependent genes in the presence or absence of F17. Similarly, the phosphorylation of SAMHD1, and ability to hydrolyse dNTPs by SAMHD1 can be assessed in the presence or absence of F17. Mapping of the residues required for the interaction of F17 with its targets may allow the construction of viable point mutation viruses, which could be used to address the contribution of F17 to suppression of innate immune signalling early during infection.

The previously uncharacterised protein VV-WR protein A57, which is expressed early during infection, suppressed both IRF and NF κ B signalling proximal to activation of these transcription factors. Interestingly, several members of the nuclear chromatin-remodelling complex NuRD were identified as interaction partners of A57, some of which have been reported to form speckles in the nucleus (Brackertz et al. 2002; Feng et al. 2002), similar to the nuclear and cytoplasmic foci observed for ectopic A57. This complex represses gene transcription by removal of acetyl groups from histones (Torchy et al. 2015), retaining genomic DNA in a dense form inaccessible to the transcription machinery. A57 may therefore stabilise and/or recruit NuRD to the site of certain IRF- and NF κ B-dependent genes, thereby preventing their induction by these transcription factors. Previously, the NuRD complex has been shown to negatively regulate late primary and secondary response genes in response to innate immune stimulation (Pakala et al. 2010; Aguilera et al. 2011; Bode & Dalpke 2011). Amongst the cytokines which were suppressed by vv811 Δ A49 were several late primary and secondary response genes such as MCP1, RANTES, and ICAM-1 (Sumner et al. 2014). Deletion of A57 from this virus may therefore reduce the ability of vv811 Δ A49 to impair the production of these cytokines, because of a lack of recruitment of the NuRD complex. In order to verify this hypothesis, the interaction of A57 with members of the NuRD complex needs to be confirmed during infection, and a virus lacking A57 should be characterised *in vitro* and *in vivo*.

The early expressed, predicted Ser/Thr kinase B12 was one of the most potent inhibitors of PRR-, IL-1R- and TNFR-mediated IRF and NF κ B activation. This nuclear protein shares approximately 30 % amino acid sequence identity with the mammalian kinase Vaccinia-related kinase 1. In contrast to B1, early *in vivo* studies of B12 deletion mutant viruses have shown that it was a non-essential pseudokinase (Howard & Smith 1989; Banham & Smith 1993; Tschärke et al. 2002). The presence of B1, with which B12 shares 36 % a.a. sequence identity (Howard & Smith 1989), in the deletion mutant virus may account for the lack of effect of B12 deletion, since

B1 affects some of VRK1's substrates (Santos et al. 2004; Nichols et al. 2006; Santos et al. 2006). VRK1 was identified as the only host protein interacting with B12, which was surprising since no other viral or eukaryotic VRK homologue has been reported to form dimers to date. In addition, neither human VRK2 nor VRK3 were identified by the analysis as B12 interacting proteins. To consolidate their interaction, the association of B12 and endogenous VRK1 should be confirmed during VACV infections. In the initial study of B12, the possibility of an interaction, or even a regulation of B1 by B12 was proposed by the authors, due to the high similarity of their C-terminal regions (Howard & Smith 1989). This observation was not investigated in further detail, but may now be addressed given the interaction between B12 and VRK1 shown in the present study.

VRK1 is an essential regulator of cell cycle progression and deletion of its homologues in other species such as *D.melanogaster* and *C.elegans* resulted in sterility or death (Ivanovska et al. 2005; Wormbase.org 2016). This host protein can affect cell viability through phosphorylation of p53, resulting in cell cycle arrest (Lopez-Borges & Lazo 2000). VACV has been shown to augment the percentage of cycling cells during infection (Wali & Strayer 1999), and its genome encodes proteins, which promote cell cycle progression (McFadden 2005). For example, B1 hyperphosphorylates p53, leading to the degradation of p53 (Santos et al. 2004). Targeting of VRK1 by B12 might therefore point to a manipulation of cell cycle progression to enhance viral replication. This hypothesis is supported by the observations that B12 can reduce basal and activated levels of p53 and it can impair other functions of VRK1. VRK1 has an established role in the activation of AP1 through direct phosphorylation of c-Jun and ATF2 in the nucleus (Sevilla, Santos, Barcia, et al. 2004; Sevilla, Santos, Vega, et al. 2004). B12 is able to suppress VRK1-mediated activation of AP1, and TNF α -driven phosphorylation of c-Jun was delayed and diminished. These results demonstrated that, in addition to NF κ B, B12 can affect AP1, which is also required for the induction of type I IFNs.

Since initial experiments did not demonstrate a clear role for VRK1 in NF κ B activation, the role of VRK1 in this signalling cascade remains to be further investigated. To this end, further RGAs, co-localisation and co-immunoprecipitations of VRK1 and p65, as well as analysis of NF κ B-dependent gene expression in cells depleted of VRK1 will be undertaken.

VACV encodes a large variety of immunomodulators to subvert and evade the immune system, the study of which resulted in the identification of 7 novel VV-WR proteins able to suppress NF κ B activation, namely M1, F17, A47, A57, B2 and B12. Their characterisation revealed roles downstream of pattern recognition and pro-inflammatory cytokine receptors proximal to the activation of transcription factors. Furthermore, potential host targets were identified and indicated roles for CDK1, VRK1 and the NuRD complex in antiviral defences. The present study demonstrated that the characterisation of poxviral ORFs still has the potential to increase our understanding not only of the virus, but also of cell biology and immunology.

References

- Abe & Barber, 2014. Cytosolic-DNA-Mediated, STING-Dependent Proinflammatory Gene Induction Necessitates Canonical NF- κ B Activation through TBK1. *Journal of virology*, 88(10), pp.5328–41. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24600004>.
- Ablasser, Bauernfeind, Hartmann, Latz, Fitzgerald & Hornung, 2009. RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. *Nature immunology*, 10(10), pp.1065–1072. Available at: <http://dx.doi.org/10.1038/ni.1779>.
- Ablasser, Hemmerling, Schmid-Burgk, Behrendt, Roers & Hornung, 2014. TREX1 Deficiency Triggers Cell-Autonomous Immunity in a cGAS-Dependent Manner. *Journal of immunology (Baltimore, Md. : 1950)*, 192(12), pp.5993–7. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24813208>.
- Ablasser, Schmid-Burgk, Hemmerling, Horvath, Schmidt, Latz & Hornung, 2013. Cell intrinsic immunity spreads to bystander cells via the intercellular transfer of cGAMP. *Nature*, 503(7477), pp.530–4. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4142317&tool=pmcentrez&rendertype=abstract>.
- Abrahão, Campos, de Souza Trindade, da Fonseca, Peregrino Ferreira & Kroon, 2015. Outbreak of severe zoonotic vaccinia virus infection, Southeastern Brazil. *Emerging Infectious Diseases*, 21(4), pp.695–698.
- Abramovich, Shulman, Ratovitski, Harroch, Tovey, Eid & Revel, 1994. Differential tyrosine phosphorylation of the IFNAR chain of the type I interferon receptor and of an associated surface protein in response to IFN-alpha and IFN-beta. *EMBO Journal*, 13(24), pp.5871–5877.
- Aggarwal, Gupta & Kim, 2012. Historical perspectives on tumor necrosis factor and its superfamily : 25 years later , a golden journey. *Hematology*, 119(3), pp.651–665.
- Aguilera, Nakagawa, Sancho, Chakraborty, Hendrich & Behrens, 2011. c-Jun N-terminal phosphorylation antagonises recruitment of the Mbd3/NuRD repressor complex. *Nature*, 469(7329), pp.231–235. Available at: <http://dx.doi.org/10.1038/nature09607>.
- Aguirre, Maestre, Pagni, Patel, Savage, Gutman, Maringer, Bernal-Rubio, et al., 2012. DENV Inhibits Type I IFN Production in Infected Cells by Cleaving Human STING. *PLoS Pathogens*, 8(10).
- Aihara, Nakagawa, Yasui, Ohta, Hirose, Dhomae, Takio, Kaneko, et al., 2004. Nucleosomal histone kinase-1 phosphorylates H2A Thr 119 during mitosis in the early Drosophila embryo. *Genes and Development*, 18(8), pp.877–888.
- Alcami & Koszinowski, 2000. Viral mechanisms of immune evasion. *Trends in Microbiology*, 8(9), pp.410–418.
- Alcami & Smith, 1992. A Soluble Receptor for Interleukin-1B Encoded by Vaccinia Virus : A Novel mechanism of virus modulation of the host response to infection. *Cell*, 71, pp.153–167.
- Alcami, Symons, Khanna & Smith, 1998. Poxviruses: Capturing cytokines and chemokines. *Semin Virol*, 8(5), pp.419–427.
- Alexopoulou, Holt, Medzhitov & Flavell, 2001. Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature*, 413(6857), pp.732–738.
- Alkalay, Yaron, Hatzubai, Orian, Ciechanover & Ben-Neriah, 1995. Stimulation-dependent I kappa B alpha phosphorylation marks the NF-kappa B inhibitor for degradation via the ubiquitin-proteasome pathway. *Proceedings of the National Academy of Sciences of the United States of America*, 92(23), pp.10599–10603. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=40659&tool=pmcentrez&rendertype=abstract>.
- Amara & Mercer, 2015. Viral apoptotic mimicry. *Nature Reviews Microbiology*, 13(8), pp.461–469. Available at: <http://www.nature.com/doifinder/10.1038/nrmicro3469>.
- Andrejeva, Childs, Young, Carlos, Stock, Goodbourn & Randall, 2004. The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. *Proceedings of the National Academy of Sciences of the United States of America*, 101(49), pp.17264–17269.
- Ansari, Dutta, Veettil, Dutta, Iqbal, Kumar, Roy, Chikoti, et al., 2015. Herpesvirus Genome Recognition Induced Acetylation of Nuclear IFI16 Is Essential for Its Cytoplasmic Translocation, Inflammasome and IFN- β Responses. *PLoS Pathogens*, 11(7), p.e1005019. Available at: <http://dx.plos.org/10.1371/journal.ppat.1005019>.
- Antoine, Scheiflinger, Dorner & Falkner, 1998. The complete genomic sequence of the modified vaccinia Ankara strain: comparison with other orthopoxviruses. *Virology*, 244(2), pp.365–396.
- Arakawa, Cordeiro & Way, 2007. F11L-Mediated Inhibition of RhoA-mDia Signaling Stimulates Microtubule Dynamics during Vaccinia Virus Infection. *Cell Host and Microbe*, 1(3), pp.213–226.
- Assarsson, Greenbaum, Sundström, Schaffer, Hammond, Pasquetto, Oseroff, Hendrickson, et al., 2008. Kinetic analysis of a complete poxvirus transcriptome reveals an immediate-early class of genes. *Proceedings of the National Academy of Sciences of the United States of America*, 105(6), pp.2140–

2145.

- Backes, Sperling, Zwilling, Gasteiger, Ludwig, Kremmer, Schwantes, Staib, et al., 2010. Viral host-range factor C7 or K1 is essential for modified Vaccinia virus Ankara late gene expression in human and murine cells, irrespective of their capacity to inhibit protein kinase R-mediated phosphorylation of eukaryotic translation initiation factor. *Journal of General Virology*, 91(2), pp.470–482.
- Baeuerle & Baltimore, 1988a. Activation of DNA-binding activity in an apparently cytoplasmic precursor of the NF-kappa B transcription factor. *Cell*, 53(2), pp.211–217.
- Baeuerle & Baltimore, 1988b. IκB: a specific inhibitor of the NF-κB transcription factor. *Science (Washington, DC, United States)*, 242(4878), pp.540–546.
- Ballana & Esté, 2015. SAMHD1: At the Crossroads of Cell Proliferation, Immune Responses, and Virus Restriction. *Trends in Microbiology*, 23(11), pp.680–692. Available at: <http://dx.doi.org/10.1016/j.tim.2015.08.002>.
- Banham, Leader & Smith, 1993. Phosphorylation of ribosomal proteins by the vaccinia virus B1R protein kinase. *FEBS Letters*, 321(1), pp.27–31.
- Banham & Smith, 1993. Characterization of vaccinia virus gene B12R. *The Journal of general virology*, 74(12), pp.2807–2812. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/8277291>.
- Banham & Smith, 1992. Vaccinia Virus Gene B1 R Encodes a 34-kDa Serine/Threonine Kinase That Localizes in Cytoplasmic Factories and Is Packaged into Virions. *virology*, 191, pp.803–812.
- Banninger & Reich, 2004. STAT2 nuclear trafficking. *Journal of Biological Chemistry*, 279(38), pp.39199–39206.
- Barbalat, Lau, Locksley & Barton, 2009. Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands. *Nature immunology*, 10(11), pp.1200–1207.
- Barcia, López-Borges, Vega & Lazo, 2002. Kinetic properties of p53 phosphorylation by the human vaccinia-related kinase 1. *Archives of biochemistry and biophysics*, 399(1), pp.1–5. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/11883897>.
- Baroudy, *Additional tandem repeats and compilation of the entire terminal sequence*,
- Baroudy & Moss, 1982. Sequence homologies of diverse length tandem repetitions near ends of vaccinia virus genome suggest unequal crossing over. *Nucleic Acids Research*, 10(18), pp.5673–5679.
- Baroudy, Venkatesan & Moss, 1982. Incompletely base-paired flip-flop terminal loops link the two DNA strands of the vaccinia virus genome into one uninterrupted polynucleotide chain. *Cell*, 28(2), pp.315–324.
- Di Bartolo, Cannon, Liu, Renne, Chadburn, Boshoff & Cesarman, 2008. KSHV LANA inhibits TGF-beta signaling through epigenetic silencing of the TGF-beta type II receptor. *Blood*, 111(9), pp.4731–4740.
- Bates, Nicol, Wilson, Jacobs, Bourdon, Wardrop, Gregory, Lane, et al., 2005. The DEAD box protein p68: a novel transcriptional coactivator of the p53 tumour suppressor. *The EMBO journal*, 24(3), pp.543–53. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=548656&tool=pmcentrez&rendertype=abstract>.
- Baum, Sachidanandam & García-Sastre, 2010. Preference of RIG-I for short viral RNA molecules in infected cells revealed by next-generation sequencing. *Proceedings of the National Academy of Sciences of the United States of America*, 107(37), pp.16303–8.
- Beard, Froggatt & Smith, 2006. Vaccinia virus kelch protein A55 is a 64 kDa intracellular factor that affects virus-induced cytopathic effect and the outcome of infection in a murine intradermal model. *Journal of General Virology*, 87(6), pp.1521–1529.
- Beaud, Beaud & Leader, 1995. Vaccinia virus gene H5R encodes a protein that is phosphorylated by the multisubstrate vaccinia virus B1R protein kinase. *Journal of Virology*, 69(0022-538X SB - M SB - X), pp.1819–1826.
- Beaud, Sharif, Topa-Masse & Leader, 1994. Ribosomal protein S2/Sa kinase purified from HeLa cells infected with vaccinia virus corresponds to the B1R protein kinase and phosphorylates in vitro the viral ssDNA-binding protein. *Journal of General Virology*, 75(2), pp.283–293.
- Benhnia, McCausland, Moyron, Laudenslager, Granger, Rickert, Koriazova, Kubo, et al., 2009. Vaccinia virus extracellular enveloped virion neutralization in vitro and protection in vivo depend on complement. *Journal of virology*, 83(3), pp.1201–15. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2620895&tool=pmcentrez&rendertype=abstract>.
- Bera, Shanmugasundaram, Barua, Venkatesan, Virmani, Riyesh, Gulati, Bhanuprakash, et al., 2011. Zoonotic cases of camelpox infection in India. *Veterinary Microbiology*, 152(1-2), pp.29–38. Available at: <http://dx.doi.org/10.1016/j.vetmic.2011.04.010>.

- Betakova, Wolffe & Moss, 1999. Regulation of vaccinia virus morphogenesis: phosphorylation of the A14L and A17L membrane proteins and C-terminal truncation of the A17L protein are dependent on the F10L kinase. *Journal of virology*, 73(5), pp.3534–3543.
- Bhattacharyya, Zagórska, Lew, Shrestha, Rothlin, Naughton, Diamond, Lemke, et al., 2013. Enveloped viruses disable innate immune responses in dendritic cells by direct activation of TAM receptors. *Cell Host and Microbe*, 14(2), pp.136–147.
- Bidgood & Mercer, 2015. Cloak and dagger: Alternative immune evasion and modulation strategies of poxviruses. *Viruses*, 7(8), pp.4800–4825.
- Bieback, Lien, Klagge, Avota, Schneider-schaulies, Duprex, Kirschning, Meulen, et al., 2002. Hemagglutinin Protein of Wild-Type Measles Virus Activates Toll-Like Receptor Hemagglutinin Protein of Wild-Type Measles Virus Activates Toll-Like Receptor 2 Signaling. *Journal of Virology*, 76(17), pp.8729–8736.
- Black, Rauch, Kozlosky, Peschon, Slack, Wolfson, Castner, Stocking, et al., 1997. A metalloproteinase disintegrin that releases tumour-necrosis factor- α from cells. *Nature*, 385(6618), pp.729–733.
- Blake, Kettle, Law, Gould, Bastin, Townsend & Smith, 1995. Vaccinia virus serpins B13R and B22R do not inhibit antigen presentation to class I-restricted cytotoxic T lymphocytes. *Journal of General Virology*, 76(9), pp.2393–2398.
- Blanco, Klimcakova, Vega & Lazo, 2006. The subcellular localization of vaccinia-related kinase-2 (VRK2) isoforms determines their different effect on p53 stability in tumour cell lines. *FEBS Journal*, 273(11), pp.2487–2504.
- Blanco, Santos & Lazo, 2007. Vaccinia-related kinase 2 modulates the stress response to hypoxia mediated by TAK1. *Molecular and cellular biology*, 27(20), pp.7273–7283.
- Blanco, Sanz-García, Santos & Lazo, 2008. Modulation of interleukin-1 transcriptional response by the interaction between VRK2 and the JIP1 scaffold protein. *PLoS ONE*, 3(2).
- Blethrow, Glavy, Morgan & Shokat, 2008. Covalent capture of kinase-specific phosphopeptides reveals Cdk1-cyclin B substrates. *Proceedings of the National Academy of Sciences of the United States of America*, 105(5), pp.1442–1447.
- Bode & Dalpke, 2011. HDAC inhibitors block innate immunity. *Blood*, 117(4), pp.1102–1103.
- Bohuslav, Chen, Kwon, Mu & Greene, 2004. p53 induces NF- κ B activation by an I κ B kinase-independent mechanism involving phosphorylation of p65 by ribosomal S6 kinase 1. *Journal of Biological Chemistry*, 279(25), pp.26115–26125.
- Bonham, Orzalli, Hayashi, Wolf, Glanemann, Weninger, Iwasaki, Knipe, et al., 2014. A promiscuous lipid-binding protein diversifies the subcellular sites of toll-like receptor signal transduction. *Cell*, 156(4), pp.705–716. Available at: <http://dx.doi.org/10.1016/j.cell.2014.01.019>.
- Boraschi & Tagliabue, 2013. The interleukin-1 receptor family. *Seminars in Immunology*, 25(6), pp.394–407. Available at: <http://dx.doi.org/10.1016/j.smim.2013.10.023>.
- Boritz, Gerlach, Johnson & Rose, 1999. Replication-Competent Rhabdoviruses with Human Immunodeficiency Virus Type 1 Coats and Green Fluorescent Protein: Entry by a pH-Independent Pathway. *J Virol*, 73(8), pp.6937–6945. Available at: <http://jvi.asm.org/content/73/8/6937.full\http://www.ncbi.nlm.nih.gov/pmc/articles/PMC112779/pdf/jv006937.pdf>.
- Bowie, Kiss-Toth, Symons, Smith, Dower & O'Neill, 2000. A46R and A52R from vaccinia virus are antagonists of host IL-1 and toll-like receptor signaling. *Proceedings of the National Academy of Sciences of the United States of America*, 97(18), pp.10162–7. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=27775&tool=pmcentrez&rendertype=abstract>.
- Boyle & Traktman, 2004. Members of a novel family of mammalian protein kinases complement the DNA-negative phenotype of a vaccinia virus ts mutant defective in the B1 kinase. *Journal of Virology*, 78(4), pp.1992–2005.
- Brackertz, Boeke, Zhang & Renkawitz, 2002. Two highly related p66 proteins comprise a new family of potent transcriptional repressors interacting with MBD2 and MBD3. *Journal of Biological Chemistry*, 277(43), pp.40958–40966.
- Brady & Bowie, 2014. Innate immune activation of NF κ B and its antagonism by poxviruses. *Cytokine & growth factor reviews*, 25(5), pp.611–620. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/25081317> [Accessed January 6, 2015].
- Brady, Haas, Farrell, Pichlmair & Bowie, 2015. Poxvirus Protein MC132 from Mollusum Contagiosum Virus Inhibits NF- κ B Activation by Targeting p65 for Degradation. *Journal of Virology*, 89(16), pp.8406–8415. Available at: <http://jvi.asm.org/lookup/doi/10.1128/JVI.00799-15>.
- Brázda, Coufal, Liao & Arrowsmith, 2012. Preferential binding of IFI16 protein to cruciform structure and

- superhelical DNA. *Biochemical and Biophysical Research Communications*, 422(4), pp.716–720.
- Breitbach, Moon, Burke, Hwang & Kirn, 2015. A Phase 2, Open-Label, Randomized Study of Pexa-Vec (JX-594) Administered by Intratumoral Injection in Patients with Unresectable Primary Hepatocellular Carcinoma. *Methods in Molecular Biology*, 1317, p.387 pp. Available at: <http://www.springer.com/gb/book/9781493927265>.
- Bridgeman, Maelfait, Davenne, Partridge, Peng, Mayer, Dong, Kaeffer, et al., 2015. Viruses transfer the antiviral second messenger cGAMP between cells. *Science*, 349(6253), pp.1228–1232.
- Brikos, Wait, Begum, O'Neill & Saklatvala, 2007. Mass spectrometric analysis of the endogenous type I interleukin-1 (IL-1) receptor signaling complex formed after IL-1 binding identifies IL-1RAcP, MyD88, and IRAK-4 as the stable components. *Molecular & cellular proteomics : MCP*, 6(9), pp.1551–1559.
- Brockhaus, Schoenfeld, Schlaeger, Hunziker, Lesslauer & Loetscher, 1990. Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies. *Proceedings of the National Academy of Sciences of the United States of America*, 87(8), pp.3127–3131.
- Brown, Nick Morrice D, Beaud, Hardie & Leader, 2000. Identification of sites phosphorylated by the vaccinia virus B1R kinase in viral protein H5R. *BMC biochemistry*, 1, p.2.
- Burdette, Monroe, Sotelo-Troha, Iwig, Eckert, Hyodo, Hayakawa & Vance, 2011. STING is a direct innate immune sensor of cyclic di-GMP. *Nature*, 478(7370), pp.515–518. Available at: <http://dx.doi.org/10.1038/nature10429>.
- Burgess & Mohr, 2015. Cellular 5'-3' mRNA Exonuclease Xrn1 Controls Double-Stranded RNA Accumulation and Anti-Viral Responses. *Cell Host & Microbe*, 17(3), pp.332–344. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S1931312815000608>.
- Burles, Irwin, Burton, Schriewer, Evans, Buller & Barry, 2014. Initial characterization of vaccinia virus B4 suggests a role in virus spread. *Virology*, 456-457, pp.108–20. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24889230> [Accessed November 24, 2014].
- Burns, Janssens, Brissoni, Olivos, Beyaert & Tschopp, 2003. Inhibition of interleukin 1 receptor/Toll-like receptor signaling through the alternatively spliced, short form of MyD88 is due to its failure to recruit IRAK-4. *The Journal of experimental medicine*, 197(2), pp.263–268.
- Buss, Dörrie, Schmitz, Hoffmann, Resch & Kracht, 2004. Constitutive and interleukin-1-inducible phosphorylation of p65 NF- κ B at serine 536 is mediated by multiple protein kinases including I κ B kinase (IKK)- α , IKK β , IKK ϵ , TRAF family member-associated (TANK)-binding kinase 1 (TBK). *Journal of Biological Chemistry*, 279(53), pp.55633–55643.
- Buss, Handschick, Jurrmann, Pekkonen, Beuerlein, Müller, Wait, Saklatvala, et al., 2012. Cyclin-dependent kinase 6 phosphorylates NF- κ B P65 at serine 536 and contributes to the regulation of inflammatory gene expression. *PloS one*, 7(12), p.e51847. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3530474&tool=pmcentrez&rendertype=abstract> [Accessed November 18, 2014].
- Calzado, Sancho & Muñoz, 2004. Human immunodeficiency virus type 1 Tat increases the expression of cleavage and polyadenylation specificity factor 73-kilodalton subunit modulating cellular and viral expression. *Journal of virology*, 78(13), pp.6846–54. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=421638&tool=pmcentrez&rendertype=abstract>.
- Cao, Dai, Wang, Li, Yuan, Wang, Fang, Pitha, et al., 2012. Innate immune response of human Plasmacytoid dendritic cells to poxvirus infection is subverted by vaccinia E3 via its Z-DNA/RNA binding domain. *PLoS ONE*, 7(5).
- Cao, Xiong, Takeuchi, Kurama & Goeddel, 1996. TRAF6 is a signal transducer for interleukin-1. *Nature*, 383(6599), pp.443–446.
- Carpentier, Gao, Ewles, Morgan & Smith, 2015. Vaccinia Virus Protein Complex F12/E2 Interacts with Kinesin Light Chain Isoform 2 to Engage the Kinesin-1 Motor Complex. *PLoS Pathogens*, 11(3), pp.1–27.
- Carswell, Old, Kassel, Green, Fiore & Williamson, 1975. An endotoxin-induced serum factor that causes necrosis of tumors. *Proceedings of the National Academy of Sciences of the United States of America*, 72(9), pp.3666–3670. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=433057&tool=pmcentrez&rendertype=abstract>.
- Cayley, Davies, McCullagh & Kerr, 1984. Activation of the ppp(A2'p)nA system in interferon-treated, herpes simplex virus-infected cells and evidence for novel inhibitors of the ppp(A2'p)nA-dependent RNase. *Eur J Biochem*, 143(1), pp.165–174.
- Chang, Watson & Jacobs, 1992. The E3L gene of vaccinia virus encodes an inhibitor of the interferon-induced, double-stranded RNA-dependent protein kinase. *Proceedings of the National Academy of Sciences of*

- the United States of America, 89(11), pp.4825–4829.
- Chen, Jacobs & Smith, 2006. Vaccinia virus strain Western Reserve protein B14 is an intracellular virulence factor. *Journal of General Virology*, 87(6), pp.1451–1458.
- Chen, Ryzhakov, Cooray, Randow & Smith, 2008. Inhibition of IkappaB kinase by vaccinia virus virulence factor B14. *PLoS pathogens*, 4(2), p.e22. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2233672&tool=pmcentrez&rendertype=abstract> [Accessed December 11, 2014].
- Chen, Williams, Mu, Duerr, Buckbinder, Greene, Nakano & Greene, 2005. NF-kappaB RelA phosphorylation regulates RelA acetylation. *Molecular and cellular biology*, 25(18), pp.7966–7975.
- Chen, Zhu, Pan, Zhu, Yan, Jiang, Shen, Dong, et al., 2014. Inhibition of Hepatitis B virus replication by SAMHD1. *Biochemical and Biophysical Research Communications*, 450(4), pp.1462–1468. Available at: <http://dx.doi.org/10.1016/j.bbrc.2014.07.023>.
- Chiu, MacMillan & Chen, 2009. RNA Polymerase III Detects Cytosolic DNA and Induces Type I Interferons through the RIG-I Pathway. *Cell*, 138(3), pp.576–591. Available at: <http://dx.doi.org/10.1016/j.cell.2009.06.015>.
- Choudhary, Rosenblatt, Fang, Tian, Wu & Brasier, 2011. High throughput short interfering RNA (siRNA) screening of the human kinome identifies novel kinases controlling the canonical nuclear factor- κ B (NF- κ B) activation pathway. *Journal of Biological Chemistry*, 286(43), pp.37187–37195.
- Chung, Chen, Ho, Huang, Liao & Chang, 2006. Vaccinia Virus Proteome: Identification of Proteins in Vaccinia Virus Intracellular Mature Virion Particles. *Journal of Virology*, 80(5), pp.2127–2140. Available at: <http://jvi.asm.org/cgi/doi/10.1128/JVI.80.5.2127-2140.2006>.
- Lo Cigno, De Andrea, Borgogna, Albertini, Landini, Peretti, Johnson, Chandran, et al., 2015. The Nuclear DNA Sensor IFI16 Acts as a Restriction Factor for Human Papillomavirus Replication through Epigenetic Modifications of the Viral Promoters. *Journal of virology*, 89(15), pp.7506–20. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/25972554>.
- Cohen, Novick, Barak & Rubinstein, 1995. Ligand-induced association of the type I interferon receptor components. *Molecular and cellular biology*, 15(8), pp.4208–4214.
- Condit, Moussatche & Traktman, 2006. In A Nutshell: Structure and Assembly of the Vaccinia Virion. *Advances in Virus Research*, 65(06), pp.31–124.
- Connolly & Bowie, 2014. The emerging role of human PYHIN proteins in innate immunity: Implications for health and disease. *Biochemical Pharmacology*, 92(3), pp.405–414. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0006295214005218>.
- Conrady, Zheng, Fitzgerald, Liu & Carr, 2012. Resistance to HSV-1 infection in the epithelium resides with the novel innate sensor, IFI-16. *Mucosal Immunology*, 5(2), pp.173–183. Available at: <http://dx.doi.org/10.1038/mi.2011.63>.
- Conze, Wu, Thomas, Landstrom & Ashwell, 2008. Lys63-linked polyubiquitination of IRAK-1 is required for interleukin-1 receptor- and toll-like receptor-mediated NF-kappaB activation. *Molecular and cellular biology*, 28(10), pp.3538–3547. Available at: <http://mcb.asm.org/cgi/doi/10.1128/MCB.02098-07>.
- Cooray, Bahar, Abrescia, McVey, Bartlett, Chen, Stuart, Grimes, et al., 2007. Functional and structural studies of the vaccinia virus virulence factor N1 reveal a Bcl-2-like anti-apoptotic protein. *Journal of General Virology*, 88(6), pp.1656–1666.
- Cordeiro, Guerra, Arakawa, Dodding, Esteban & Way, 2009. F11-mediated inhibition of RhoA signalling enhances the spread of vaccinia virus in vitro and in vivo in an intranasal mouse model of infection. *PLoS ONE*, 4(12).
- Cribier, Descours, Valadão, Laguet & Benkirane, 2013. Phosphorylation of SAMHD1 by Cyclin A2/CDK1 Regulates Its Restriction Activity toward HIV-1. *Cell Reports*, 3(4), pp.1036–1043.
- Cui, Eisenächer, Kirchhofer, Brzózka, Lammens, Lammens, Fujita, Conzelmann, et al., 2008. The C-Terminal Regulatory Domain Is the RNA 5'-Triphosphate Sensor of RIG-I. *Molecular Cell*, 29(2), pp.169–179.
- Cui, Li, Walton, Sun, Hanover, Furth & Hennighausen, 2001. The Stat3/5 locus encodes novel endoplasmic reticulum and helicase-like proteins that are preferentially expressed in normal and neoplastic mammary tissue. *Genomics*, 78(3), pp.129–134.
- Cyrklaff, Risco, Fernández, Jiménez, Estéban, Baumeister & Carrascosa, 2005. Cryo-electron tomography of vaccinia virus. *Proceedings of the National Academy of Sciences of the United States of America*, 102(8), pp.2772–2777.
- Daffis, Szretter, Schriewer, Li, Youn, Errett, Lin, Schneller, et al., 2010. 2'-O methylation of the viral mRNA cap evades host restriction by IFIT family members. *Nature*, 468(7322), pp.452–6. Available at: <http://dx.doi.org/10.1038/nature09489>.
- van Dam, Wilhelm, Herr, Steffen, Herrlich & Angel, 1995. ATF-2 is preferentially activated by stress-activated

- protein kinases to mediate c-jun induction in response to genotoxic agents. *The EMBO journal*, 14(8), pp.1798–1811.
- Damon, Roth & Chowdhary, 2006. Discovery of monkeypox in Sudan. *The New England journal of medicine*, 355(9), pp.962–3. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16943415>.
- Dar & Sicheri, 2002. X-ray crystal structure and functional analysis of vaccinia virus K3L reveals molecular determinants for PKR subversion and substrate recognition. *Molecular Cell*, 10(2), pp.295–305.
- Daugherty & Malik, 2012. Rules of engagement: molecular insights from host-virus arms races. *Annual review of genetics*, 46, pp.677–700. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23145935> [Accessed June 1, 2013].
- Davies, Sei, Siciliano, Xu, Roscoe, Sigal, Eisenlohr & Norbury, 2014. MyD88-Dependent Immunity to a Natural Model of Vaccinia Virus Infection Does Not Involve Toll-Like Receptor 2. *Journal of virology*, 88(January), pp.3557–3567. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24403581>.
- Deak, Clifton, Lucocq & Alessi, 1998. Mitogen- and stress-activated protein kinase-1 (MSK1) is directly activated by MAPK and SAPK2/p38, and may mediate activation of CREB. *EMBO Journal*, 17(15), pp.4426–4441.
- DeFilippes, 1982. Restriction enzyme mapping of vaccinia virus DNA. *Journal of virology*, 43(1), pp.136–149.
- Dempsey & Bowie, 2015. Innate immune recognition of DNA: A recent history. *Virology*, 479–480, pp.146–152. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S004268221500149X>.
- Deng, Wang, Spencer, Yang, Braun, You, Slaughter, Pickart, et al., 2000. Activation of the I κ B kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. *Cell*, 103(2), pp.351–361.
- Dériard, Hibi, Wu, Barrett, Su, Deng, Karin & Davis, 1994. JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell*, 76(6), pp.1025–37.
- Dériard, Raingeaud, Barrett, Wu, Han, Ulevitch & Davis, 1995. Independent human MAP-kinase signal transduction pathways defined by MEK and MKK isoforms. *Science (New York, N.Y.)*, 267(5198), pp.682–685.
- Derrien, Punjabi, Khanna, Grubisha & Traktman, 1999. Tyrosine Phosphorylation of A17 during Vaccinia Virus Infection: Involvement of the H1 Phosphatase and the F10 Kinase. *J Virol*, 73(9), pp.7287–7296. Available at: <http://jvi.asm.org/content/73/9/7287.long>.
- Devarkar, Wang, Miller, Ramanathan, Jiang, Khan, Patel & Marcotrigiano, 2016. Structural basis for m7G recognition and 2'-O-methyl discrimination in capped RNAs by the innate immune receptor RIG-I. *Pnas*, p.1515152113-. Available at: <http://www.pnas.org/content/early/2016/01/04/1515152113.abstract>.
- Devin, Cook, Lin, Rodriguez, Kelliher & Liu, 2000. The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNF-R1 while RIP mediates IKK activation. *Immunity*, 12(4), pp.419–429. Available at: <http://www.sciencedirect.com/science/article/pii/S1074761300801946>.
- Diebold, Kaisho, Hemmi, Akira & Reis e Sousa, 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science (New York, N.Y.)*, 303(5663), pp.1529–1531.
- Dinarello, 2010. IL-1: Discoveries, controversies and future directions. *European Journal of Immunology*, 40(3), pp.599–606.
- Dinarello, 2009. Immunological and inflammatory functions of the interleukin-1 family. *Annual review of immunology*, 27, pp.519–550.
- Dinarello, 2015. The history of fever, leukocytic pyrogen and interleukin-1. *Temperature*, 2(1), pp.8–16. Available at: <http://www.tandfonline.com/doi/full/10.1080/23328940.2015.1017086>.
- DiPerna, Stack, Bowie, Boyd, Kotwal, Zhang, Arvikar, Latz, et al., 2004. Poxvirus protein N1L targets the I κ B kinase complex, inhibits signaling to NF- κ B by the tumor necrosis factor superfamily of receptors, and inhibits NF- κ B and IRF3 signaling by toll-like receptors. *The Journal of biological chemistry*, 279(35), pp.36570–8. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15215253> [Accessed December 11, 2014].
- Dobbelstein & Shenk, 1996. Protection against apoptosis by the vaccinia virus SPI-2 (B13R) gene product. *Journal of virology*, 70(9), pp.6479–85. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=190684&tool=pmcentrez&rendertype=abstract>.
- Dobson, Procter, Hollett, Fleisch, Newsome & Tschärke, 2014. Vaccinia virus F5 is required for normal plaque morphology in multiple cell lines but not replication in culture or virulence in mice. *Virology*, 456–457, pp.145–56. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24889234> [Accessed December 5, 2014].
- Doceul, Hollinshead, van der Linden & Smith, 2010. Repulsion of superinfecting virions: a mechanism for rapid virus spread. *Science (New York, N.Y.)*, 327(5967), pp.873–876.

- Dodding, Newsome, Collinson, Edwards & Way, 2009. An E2-F12 complex is required for intracellular enveloped virus morphogenesis during vaccinia infection. *Cellular microbiology*, 11(5), pp.808–24. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2688674&tool=pmcentrez&rendertype=abstract> [Accessed December 1, 2014].
- Doglio, De Marco, Schleich, Roos & Krijnse Locker, 2002. The Vaccinia virus E8R gene product: a viral membrane protein that is made early in infection and packaged into the virions' core. *Journal of virology*, 76(19), pp.9773–9786.
- Domi & Beaud, 2000. The punctate sites of accumulation of vaccinia virus early proteins are precursors of sites of viral DNA synthesis. *Journal of General Virology*, 81(5), pp.1231–1235.
- Domi, Weisberg & Moss, 2008. Vaccinia virus E2L null mutants exhibit a major reduction in extracellular virion formation and virus spread. *Journal of virology*, 82(9), pp.4215–26. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2293024&tool=pmcentrez&rendertype=abstract> [Accessed November 7, 2013].
- Drappier & Michiels, 2015. Inhibition of the OAS/RNase L pathway by viruses. *Current Opinion in Virology*, 15, pp.19–26. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S1879625715001005>.
- Duesberg & Colby, 1969. On the biosynthesis and structure of double-stranded RNA in vaccinia virus-infected cells. *Proceedings of the National Academy of Sciences of the United States of America*, 64(1), pp.396–403.
- Ea, Deng, Xia, Pineda & Chen, 2006. Activation of IKK by TNF α requires site-specific ubiquitination of RIP1 and polyubiquitin binding by NEMO. *Molecular Cell*, 22(2), pp.245–257.
- Elde, Child, Eickbush, Kitman, Rogers, Shendure, Geballe & Malik, 2012. Poxviruses deploy genomic accordions to adapt rapidly against host antiviral defenses. *Cell*, 150(4), pp.831–41. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3499626&tool=pmcentrez&rendertype=abstract> [Accessed June 1, 2013].
- Elde & Malik, 2009. The evolutionary conundrum of pathogen mimicry. *Nature reviews. Microbiology*, 7(11), pp.787–797. Available at: <http://dx.doi.org/10.1038/nrmicro2222>.
- Elsendoorn, Agius, Le Moal, Aajaji, Favier, Wierzbicka-Hainault, Béraud, Flusin, et al., 2011. Severe ear chondritis due to cowpox virus transmitted by a pet rat. *Journal of Infection*, 63(5), pp.391–393.
- Ember, Ren, Ferguson & Smith, 2012. Vaccinia virus protein C4 inhibits NF- κ B activation and promotes virus virulence. *The Journal of general virology*, 93(Pt 10), pp.2098–108. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3541790&tool=pmcentrez&rendertype=abstract> [Accessed November 7, 2013].
- Emmerich, Ordureau, Strickson, Arthur, Pedrioli, Komander & Cohen, 2013. Activation of the canonical IKK complex by K63/M1-linked hybrid ubiquitin chains. *Proceedings of the National Academy of Sciences of the United States of America*, 110(38), pp.15247–52. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3780889&tool=pmcentrez&rendertype=abstract>.
- Esposito, Frace, Sammons, Olsen-Rasmussen, Osborne & Wohlhueter, *Sequencing of the coding region of Vaccinia-WR to an average 9-fold redundancy and an error rate of 0.16/10kb*.
- Esposito, Moss, Sammons, Frace, Olsen-Rasmussen, M., Osborne, Baroudy & Wohlhueter, 2003. *Direct Submission*.
- Essbauer, Pfeffer & Meyer, 2010. Zoonotic poxviruses. *Veterinary Microbiology*, 140(3-4), pp.229–236.
- Evans, Stuart & McFadden, 1988. High levels of genetic recombination among cotransfected plasmid DNAs in poxvirus-infected mammalian cells. *Journal of virology*, 62(2), pp.367–375.
- Fagan-Garcia & Barry, 2011. A vaccinia virus deletion mutant reveals the presence of additional inhibitors of NF- κ B. *Journal of virology*, 85(2), pp.883–894.
- Favier, Flusin, Lepreux, Fleury, Labrèze, Georges, Crance & Boralevi, 2011. Necrotic ulcerated lesion in a young boy caused by cowpox virus infection. *Case Reports in Dermatology*, 3(3), pp.186–194.
- Feng, Cao, Xia, Erdjument-bromage, Tempst & Zhang, 2002. Identification and Functional Characterization of the p66 / p68 Components of the MeCP1. *Molecular and Cellular Biology*, 22(2), pp.536–546.
- Fenner, Henderson, Arita, Jezek & Ladnyj, 1988. Smallpox and Its Eradication. *World Health Organisation: History of international public health*, 6(1), p.92. Available at: <http://apps.who.int/iris/handle/10665/39485>.
- Fenner, Scannell & Prehn, 2010. Expanding the substantial interactome of NEMO using protein microarrays. *PLoS ONE*, 5(1).
- Fensterl & Sen, 2015. Interferon-Induced Ifit Proteins: Their Role in Viral Pathogenesis. *Journal of Virology*, 89(5), pp.2462–2468. Available at: <http://jvi.asm.org/lookup/doi/10.1128/JVI.02744-14>.

- Ferguson, Benfield, Ren, Lee, Frazer, Strnadova, Sumner & Smith, 2013. Vaccinia virus protein N2 is a nuclear IRF3 inhibitor that promotes virulence. *The Journal of general virology*, 94(PART9), pp.2070–2081. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3749055&tool=pmcentrez&rendertype=abstract> [Accessed November 7, 2013].
- Ferguson, Mansur, Peters, Ren & Smith, 2012. DNA-PK is a DNA sensor for IRF-3-dependent innate immunity. *eLife*, 2012(1), pp.1–17.
- Fine, Jezek, Grab & Dixon, 1988. The transmission potential of monkeypox virus in human populations. *International journal of epidemiology*, 17(3), pp.643–50. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/2850277>.
- Fitzgerald, Katherine, McWhirter, Faia, Rowe, Latz, Golenbock, Coyle, Liao, et al., 2003. IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nature immunology*, 4(5), pp.491–496. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/12692549>.
- Fitzgerald, Palsson-McDermott, Bowie, Jefferies, Mansell, Brady, Brint, Dunne, et al., 2001. Mal (MyD88-adaptor-like) is required for Toll-like receptor-4 signal transduction. *Nature*, 413(6851), pp.78–83.
- Fitzgerald, Katherine a, Rowe, Barnes, Caffrey, Visintin, Latz, Monks, Pitha, et al., 2003. LPS-TLR4 signaling to IRF-3/7 and NF-kappaB involves the toll adapters TRAM and TRIF. *The Journal of experimental medicine*, 198(7), pp.1043–55. Available at: <http://jem.rupress.org/content/198/7/1043.abstract>.
- Fitzgibbon, Katsanis, Wells, Delhanty, Vallins & Hunt, 1996. Human guanylate kinase (GUK1): cDNA sequence, expression and chromosomal localisation. *FEBS Letters*, 385(3), pp.185–188.
- Flannery & Bowie, 2010. The interleukin-1 receptor-associated kinases: Critical regulators of innate immune signalling. *Biochemical Pharmacology*, 80(12), pp.1981–1991. Available at: <http://dx.doi.org/10.1016/j.bcp.2010.06.020>.
- Fonseca, Thillainadesan, Yousef, Ablack, Mossman, Torchia & Mymryk, 2012. Adenovirus evasion of interferon-mediated innate immunity by direct antagonism of a cellular histone posttranslational modification. *Cell Host and Microbe*, 11(6), pp.597–606. Available at: <http://dx.doi.org/10.1016/j.chom.2012.05.005>.
- Froggatt, Smith & Beard, 2007. Vaccinia virus gene F3L encodes an intracellular protein that affects the innate immune response. *The Journal of general virology*, 88(7), pp.1917–21. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2884953&tool=pmcentrez&rendertype=abstract> [Accessed November 7, 2013].
- Fu, Schindler, Improta, Aebersold & Darnell, 1992. The proteins of ISGF-3, the interferon alpha-induced transcriptional activator, define a gene family involved in signal transduction. *Proceedings of the National Academy of Sciences of the United States of America*, 89(16), pp.7840–7843.
- Gack, Shin, Joo, Urano, Liang, Sun, Takeuchi, Akira, et al., 2007. TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. *Nature*, 446(7138), pp.916–920.
- Gall, Treuting, Elkon, Loo, Gale, Barber & Stetson, 2012. Autoimmunity Initiates in Nonhematopoietic Cells and Progresses via Lymphocytes in an Interferon-Dependent Autoimmune Disease. *Immunity*, 36(1), pp.120–131. Available at: <http://dx.doi.org/10.1016/j.immuni.2011.11.018>.
- Gammon, Duraffour, Rozelle, Hehnly, Sharma, Sparks, West, Chen, et al., 2014. A single vertebrate DNA virus protein disarms invertebrate immunity to RNA virus infection. *eLife*, 2014(3), pp.1–29.
- Gao, Ascano, Wu, Barchet, Gaffney, Zillinger, Serganov, Liu, et al., 2013. Cyclic [G(2',5')pA(3',5')p] is the metazoan second messenger produced by DNA-activated cyclic GMP-AMP synthase. *Cell*, 153(5), pp.1094–1107.
- Gao, Li, Li, Chen, Li, Wight-Carter & Chen, 2015. Activation of cyclic GMP-AMP synthase by self-DNA causes autoimmune diseases. *Proceedings of the National Academy of Sciences of the United States of America*, 112(42), pp.E5699–705. Available at: <http://www.pnas.org/content/112/42/E5699.abstract>.
- García-Arriaza, Gómez, Sorzano & Esteban, 2014. Deletion of the vaccinia virus N2L gene encoding an inhibitor of IRF3 improves the immunogenicity of modified vaccinia virus Ankara expressing HIV-1 antigens. *Journal of virology*, 88(6), pp.3392–3410. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24390336>.
- García-Arriaza, Nájera, Gómez, Tewabe, Sorzano, Calandra, Roger & Esteban, 2011. A candidate HIV/AIDS vaccine (MVA-B) lacking vaccinia virus gene C6L enhances memory HIV-1-specific T-cell responses. *PLoS ONE*, 6(8).
- Gardner, Tsharke, Reading & Smith, 2001. Vaccinia virus semaphorin A39R is a 50-55 kDa secreted glycoprotein that affects the outcome of infection in a murine intradermal model. *The Journal of general virology*, 82(9), pp.2083–2093. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/11514717>.
- Gariano, Dell'Oste, Bronzini, Gatti, Luganini, de Andrea, Gribaudo, Gariglio, et al., 2012. The intracellular DNA

- sensor IFI16 gene acts as restriction factor for human Cytomegalovirus replication. *PLoS Pathogens*, 8(1).
- Gedey, Jin, Hinthong & Shisler, 2006. Poxviral regulation of the host NF-kappaB response: the vaccinia virus M2L protein inhibits induction of NF-kappaB activation via an ERK2 pathway in virus-infected human embryonic kidney cells. *Journal of virology*, 80(17), pp.8676–85. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1563854&tool=pmcentrez&rendertype=abstract> [Accessed November 7, 2013].
- Gentili, Kowal, Tkach, Satoh, Lahaye, Conrad, Boyron, Lombard, et al., 2015. Transmission of innate immune signaling by packaging of cGAMP in viral particles. *Science (New York, N.Y.)*, 349(6253), pp.1232–1236. Available at: <http://www.sciencemag.org/content/349/6253/1232.long>.
- Gilmore, 2016. NF-kB Target Genes. <http://www.bu.edu/nf-kb/gene-resources/target-genes/>. Available at: <http://www.bu.edu/nf-kb/gene-resources/target-genes/>.
- Gitlin, Barchet, Gilfillan, Cella, Beutler, Flavell, Diamond & Colonna, 2006. Essential role of mda-5 in type I IFN responses to polyriboinosinic:polyribocytidylic acid and encephalomyocarditis picornavirus. *Proceedings of the National Academy of Sciences of the United States of America*, 103(22), pp.8459–8464.
- Glasgow & Habel, 1962. The role of interferon in vaccinia virus infection of mouse embryo tissue culture. *The Journal of Experimental Medicine*, 115, pp.503–12. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2137503&tool=pmcentrez&rendertype=abstract>.
- Glatz, Richter, Ginter-Hanselmayer, Aberer & M??llegger, 2010. Human cowpox in a veterinary student. *The Lancet Infectious Diseases*, 10(4), p.288.
- Goenka & Weaver, 2008. The p26 gene of the Autographa californica nucleopolyhedrovirus: timing of transcription, and cellular localization and dimerization of product. *Virus research*, 131(2), pp.136–44. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17935816> [Accessed December 10, 2014].
- Goepfert, Elizaga, Seaton, Tomaras, Montefiori, Sato, Hural, DeRosa, et al., 2014. Specificity and 6-month durability of immune responses induced by DNA and recombinant modified vaccinia ankara vaccines expressing HIV-1 virus-like particles. *Journal of Infectious Diseases*, 210(1), pp.99–110.
- Goubau, Schlee, Deddouche, Pruijssers, Zillinger, Goldeck, Schuberth, Van der Veen, et al., 2014. Antiviral immunity via RIG-I-mediated recognition of RNA bearing 5'-diphosphates. *Nature*, 514(7522), pp.372–375. Available at: <http://www.nature.com/doi/10.1038/nature13590>.
- Gray, Treuting, Woodward & Stetson, 2015. Cutting Edge: cGAS Is Required for Lethal Autoimmune Disease in the Trex1-Deficient Mouse Model of Aicardi-Goutières Syndrome. *Journal of Immunology (Baltimore, Md. : 1950)*, 195(5), pp.1939–43. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/26223655>.
- Greenbaum, Levine, Bhanot & Rabadan, 2008. Patterns of evolution and host gene mimicry in influenza and other RNA viruses. *PLoS Pathogens*, 4(6), pp.1–9.
- Greenfeder, Nunes, Kwee, Labow, Chizzonite & Ju, 1995. Molecular cloning and characterization of a second subunit of the interleukin 1 receptor complex. *Journal of Biological Chemistry*, 270(23), pp.13757–13765.
- Gubser, Hué, Kellam & Smith, 2004. Poxvirus genomes: A phylogenetic analysis. *Journal of General Virology*, 85(1), pp.105–117.
- Gubser & Smith, 2002. The sequence of camelpox virus shows it is most closely related to variola virus, the cause of smallpox. *Journal of General Virology*, 83(4), pp.855–872.
- Guma, Ronacher, Liu-Bryan, Takai, Karin & Corr, 2009. Caspase 1-independent activation of interleukin-1beta in neutrophil-predominant inflammation. *Arthritis and Rheumatism*, 60(12), pp.3642–3650.
- Guo, Naik, O'Malley, Popovic, Demarco, Hu, Yin, Yang, et al., 2005. The enhanced tumor selectivity of an oncolytic vaccinia lacking the host range and antiapoptosis genes SPI-1 and SPI-2. *Cancer Research*, 65(21), pp.9991–9998.
- Guo, Thorne & Bartlett, 2008. Oncolytic virotherapy: Molecular targets in tumor-selective replication and carrier cell-mediated delivery of oncolytic viruses. *Biochimica et Biophysica Acta - Reviews on Cancer*, 1785(2), pp.217–231.
- Gupta, Campbell, Dérijard & Davis, 1995. Factor ATF2 Regulation by the Transcription JNK Signal Transduction Pathway. *Science*, 267(5196), pp.389–393.
- Hai & Curran, 1991. Cross family dimerization of transcriptional factors Fos / Jun and ATF / CREB alters DNA binding specificity. *Proc.Natl.Acad.Sci.U.S.A.*, 88, pp.3720–3724.
- Haller, Peng, McFadden & Rothenburg, 2014. Poxviruses and the evolution of host range and virulence. *Infection, Genetics and Evolution*, 21, pp.15–40. Available at: <http://dx.doi.org/10.1016/j.meegid.2013.10.014>.

References

- Hammarlund, Lewis, Hansen, Strelow, Nelson, Sexton, Hanifin & Slifka, 2003. Duration of antiviral immunity after smallpox vaccination. *Nature medicine*, 9(9), pp.1131–1137.
- Handa, Durkin, Dodding & Way, 2013. Vaccinia virus F11 promotes viral spread by acting as a PDZ-containing scaffolding protein to bind myosin-9A and inhibit RhoA signaling. *Cell host & microbe*, 14(1), pp.51–62. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23870313> [Accessed November 24, 2014].
- Hansen, Prabakaran, Laustsen, Jørgensen, Rahbæk, Jensen, Nielsen, Leber, et al., 2014. Listeria monocytogenes induces IFN β expression through an IFI16-, cGAS- and STING-dependent pathway. *The EMBO journal*, 33(15), pp.1654–66. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24970844>.
- Hansen, Vojtech & Laing, 2011. Sensing disease and danger: A survey of vertebrate PRRs and their origins. *Developmental and Comparative Immunology*, 35(9), pp.886–897. Available at: <http://dx.doi.org/10.1016/j.dci.2011.01.008>.
- Harte, Haga, Maloney, Gray, Reading, Bartlett, Smith, Bowie, et al., 2003. The Poxvirus Protein A52R Targets Toll-like Receptor Signaling Complexes to Suppress Host Defense. *The Journal of experimental medicine*, 197(3), pp.343–351. Available at: <http://www.jem.org/cgi/doi/10.1084/jem.20021652>.
- Hayden & Ghosh, 2008. Shared principles in NF-kappaB signaling. *Cell*, 132(3), pp.344–62. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/18267068> [Accessed July 12, 2014].
- Heil, Hemmi, Hochrein, Ampenberger, Kirschning, Akira, Lipford, Wagner, et al., 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science (New York, N.Y.)*, 303(5663), pp.1526–1529.
- Hemmi, Takeuchi, Sato, Yamamoto, Kaisho, Sanjo, Kawai, Hoshino, et al., 2004. The roles of two IkappaB kinase-related kinases in lipopolysaccharide and double stranded RNA signaling and viral infection. *The Journal of experimental medicine*, 199(12), pp.1641–50. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2212809&tool=pmcentrez&rendertype=abstract>.
- Henkel, Machleidt, Alkalay, Krönke, Ben-Neriah & Baeuerle, 1993. Rapid proteolysis of I κ B- α is necessary for activation of transcription factor NF- κ B. *Nature*, 365(6442), pp.182–185.
- Herzner, Hagmann, Goldeck, Wolter, Kübler, Wittmann, Gramberg, Andreeva, et al., 2015. Sequence-specific activation of the DNA sensor cGAS by Y-form DNA structures as found in primary HIV-1 cDNA. *Nature Immunology*, 16(10), pp.1025–1033. Available at: <http://dx.doi.org/10.1038/ni.3267>.
- Hinthong, Jin & Shisler, 2008. Characterization of wild-type and mutant vaccinia virus M2L proteins' abilities to localize to the endoplasmic reticulum and to inhibit NF-kappaB activation during infection. *Virology*, 373(2), pp.248–262. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2679263&tool=pmcentrez&rendertype=abstract> [Accessed November 7, 2013].
- Hochrainer, Racchumi & Anrather, 2013. Site-specific Phosphorylation of the p65 Protein Subunit Mediates Selective Gene Expression by Differential NF- κ B and RNA Polymerase II Promoter Recruitment. *Journal of Biological Chemistry*, 288(1), pp.285–293.
- Hodgson, Ewer, Bliss, Edwards, Rampling, Anagnostou, De Barra, Havelock, et al., 2015. Evaluation of the efficacy of ChAd63-MVA vectored vaccines expressing circumsporozoite protein and ME-TRAP against controlled human malaria infection in malaria-naïve individuals. *Journal of Infectious Diseases*, 211(7), pp.1076–1086.
- Hollenbaugh, Gee, Baker, Daly, Amie, Tate, Kasai, Kanemura, et al., 2013. Host Factor SAMHD1 Restricts DNA Viruses in Non-Dividing Myeloid Cells. *PLoS Pathogens*, 9(6).
- Honda, Ohba, Yanai, Negishi, Mizutani, Takaoka, Taya & Taniguchi, 2005. Spatiotemporal regulation of MyD88-IRF-7 signalling for robust type-I interferon induction. *Nature*, 434(7036), pp.1035–1040.
- Honda, Yanai, Negishi, Asagiri, Sato, Mizutani, Shimada, Ohba, et al., 2005. IRF-7 is the master regulator of type-I interferon-dependent immune responses. *Nature*, 434(April), pp.772–777. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15800576>.
- Horan, Hansen, Jakobsen, Holm, Søby, Unterholzner, Thompson, West, et al., 2013. Proteasomal degradation of herpes simplex virus capsids in macrophages releases DNA to the cytosol for recognition by DNA sensors. *Journal of immunology (Baltimore, Md. : 1950)*, 190(5), pp.2311–9. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23345332> [Accessed February 27, 2013].
- Hornung, Ellegast, Kim, Brzózka, Jung, Kato, Poeck, Akira, et al., 2006. 5'-Triphosphate RNA is the ligand for RIG-I. *Science*, 314(5801), pp.994–997.
- Hou, Sun, Zheng, Skaug, Jiang & Chen, 2011. MAVS forms functional prion-like aggregates to activate and propagate antiviral innate immune response. *Cell*, 146(3), pp.448–461. Available at: <http://dx.doi.org/10.1016/j.cell.2011.06.041>.
- Howard & Smith, 1989. Two early vaccinia virus genes encode polypeptides related to protein kinases. *The*

- Journal of general virology*, 70(Pt 12), pp.3187–3201. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/2607336>.
- Hsu, Huang, Shu, Baichwal & Goeddel, 1996. TNF-dependent recruitment of the protein kinase RIP to the TNF receptor-1 signaling complex. *Immunity*, 4(4), pp.387–396.
- Hsu, Shu, Pan & Goeddel, 1996. TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. *Cell*, 84(2), pp.299–308.
- Hsu, Xiong & Goeddel, 1995. The TNF receptor 1-associated protein TRADD signals cell death and NF-kappa B activation. *Cell*, 81(4), pp.495–504.
- Huang, Yang, Lamb & Chen, 2010. Posttranslational modifications of NF-kappaB: another layer of regulation for NF-kappaB signaling pathway. *Cellular signalling*, 22(9), pp.1282–1290.
- Hultmark, 1994. Macrophage differentiation marker MyD88 is a member of the Toll/IL-1 receptor family. *Biochemical and Biophysical Research Communications*, 199(1), pp.144–146. Available at: http://ac.elsa-cdn.com/S0006291X8471206X/1-s2.0-S0006291X8471206X-main.pdf?_tid=838225b2-4c55-11e3-99c0-00000aacb35e&acdnat=1384341771_c67e32d549aaf76c93cb4e2a5c11e2af.
- Hutchens, Luker, Sonstein, Núñez, Curtis & Luker, 2008. Protective effect of toll-like receptor 4 in pulmonary vaccinia infection. *PLoS Pathogens*, 4(9).
- Hutchens, Luker, Sottile, Sonstein, Lukacs, Núñez, Curtis & Luker, 2008. TLR3 increases disease morbidity and mortality from vaccinia infection. *Journal of immunology (Baltimore, Md. : 1950)*, 180(1), pp.483–491.
- Ibrahim, Wicklund, Jamin & Wiebe, 2013. Barrier to autointegration factor (BAF) inhibits vaccinia virus intermediate transcription in the absence of the viral B1 kinase. *Virology*, 444(1-2), pp.363–373. Available at: <http://dx.doi.org/10.1016/j.virol.2013.07.002>.
- Improta, Schindler, Horvath, Kerr, Stark & Darnell, 1994. Transcription factor ISGF-3 formation requires phosphorylated Stat91 protein, but Stat113 protein is phosphorylated independently of Stat91 protein. *Proceedings of the National Academy of Sciences of the United States of America*, 91(11), pp.4776–4780.
- Isaacs, Kotwal & Moss, 1992. Vaccinia Virus Complement-Control Protein Prevents Antibody-Dependent Complement-Enhanced Neutralization of Infectivity and Contributes to Virulence. *Proceedings of the National Academy of Sciences of the United States of America*, 89(2), pp.628–632.
- Isaacs & Lindenmann, 1957. Virus interference. I. The interferon. *Proceedings of the Royal Society of London B: Biological Sciences*, 147(927), pp.258–67. Available at: <http://rspb.royalsocietypublishing.org/content/147/927/258>.
- Ishikawa & Barber, 2008. STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling. *Nature*, 455(7213), pp.674–678.
- Ishikawa, Ma & Barber, 2009. STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. *Nature*, 461(7265), pp.788–92. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/19776740>.
- Ivanovska, Khandan, Ito & Orr-Weaver, 2005. A histone code in meiosis: The histone kinase, NHK-1, is required for proper chromosomal architecture in Drosophila oocytes. *Genes and Development*, 19(21), pp.2571–2582.
- Iwasaki & Medzhitov, 2015. Regulation of adaptive immunity by the innate immune system. *Science (New York, N.Y.)*, 327(5963), pp.291–295.
- Izaguirre, Barnes, Amrute, Yeow, Megjugorac, Dai, Feng, Chung, et al., 2003. Comparative analysis of IRF and IFN-alpha expression in human plasmacytoid and monocyte-derived dendritic cells. *Journal of leukocyte biology*, 74(6), pp.1125–38. Available at: http://www.jleukbio.org/content/74/6/1125.abstract?ijkey=8644e043cc9b068a21e25317594608515381ad04&keytype=tf_ipsecsha.
- Izquierdo, Leever, Marshall & Cantrell, 1993. p21ras couples the T cell antigen receptor to extracellular signal-regulated kinase 2 in T lymphocytes. *Journal of experimental medicine*, 178(October), pp.1109–1208.
- Jacobs, Langland, Kibler, Denzler, White, Holechek, Wong, Huynh, et al., 2009. Vaccinia virus vaccines: Past, present and future. *Antiviral Research*, 84(1), pp.1–13.
- Jakobsen, Bak, Andersen, Berg, Jensen, Jin, Laustsen, Hansen, et al., 2013. IFI16 senses DNA forms of the lentiviral replication cycle and controls HIV-1 replication. *Proceedings of the National Academy of Sciences*, 110(48), pp.E4571–80. Available at: <http://www.pnas.org/cgi/doi/10.1073/pnas.1319465110>.
- Jakobsen & Paludan, 2014. IFI16: At the interphase between innate DNA sensing and genome regulation. *Cytokine and Growth Factor Reviews*, 25(6), pp.649–655. Available at: <http://dx.doi.org/10.1016/j.cytogfr.2014.06.004>.

- Jamin, Ibrahim, Wicklund, Weskamp & Wiebe, 2015. Vaccinia Virus B1 Kinase Is Required for Postreplicative Stages of the Viral Life Cycle in a BAF-Independent Manner in U2OS Cells. *Journal of virology*, 89(20), pp.10247–10259.
- Janeway, 1989. Approaching the Asymptote? Evolution and Revolution in Immunology. *Cold Spring Harb Symp Quant Biol*, LIV, pp.1–13.
- Jeremiah, Neven, Gentili, Callebaut, Maschalidi, Stolzenberg, Goudin, Frémond, et al., 2014. Inherited STING-activating mutation underlies a familial inflammatory syndrome with lupus-like manifestations. *Journal of Clinical Investigation*, 124(12), pp.5516–5520.
- Jiang, Mak, Sen & Li, 2004. Toll-like receptor 3-mediated activation of NF-kappaB and IRF3 diverges at Toll-IL-1 receptor domain-containing adapter inducing IFN-beta. *Proceedings of the National Academy of Sciences of the United States of America*, 101(10), pp.3533–3538.
- Jin, Kim, Heo, Lee, Kim, Paik, Lee & Lee, 2007. Crystal Structure of the TLR1-TLR2 Heterodimer Induced by Binding of a Tri-Acylated Lipopeptide. *Cell*, 130(6), pp.1071–1082.
- Jin, Perry, Jiang, Smith, Curry, Unterholzner, Jiang, Horvath, et al., 2012. Structures of the HIN Domain: DNA Complexes Reveal Ligand Binding and Activation Mechanisms of the AIM2 Inflammasome and IFI16 Receptor. *Immunity*, 36(4), pp.561–571. Available at: <http://dx.doi.org/10.1016/j.immuni.2012.02.014> [Accessed March 11, 2013].
- Jin, Waterman, Jonscher, Short, Reisdorph & Cambier, 2008. MPYS, a novel membrane tetraspanner, is associated with major histocompatibility complex class II and mediates transduction of apoptotic signals. *Molecular and cellular biology*, 28(16), pp.5014–5026.
- Joklik & Becker, 1964. The replication and coating of vaccinia DNA. *Journal of Molecular Biology*, 10(3), pp.452–474. Available at: [http://dx.doi.org/10.1016/S0022-2836\(64\)80066-8](http://dx.doi.org/10.1016/S0022-2836(64)80066-8).
- Joosten, Netea, Fantuzzi, Koenders, Helsen, Sparrer, Pham, Van Der Meer, et al., 2009. Inflammatory arthritis in caspase 1 gene-deficient mice: Contribution of proteinase 3 to caspase 1-independent production of bioactive interleukin-1 β . *Arthritis and Rheumatism*, 60(12), pp.3651–3662.
- Kagan & Medzhitov, 2006. Phosphoinositide-Mediated Adaptor Recruitment Controls Toll-like Receptor Signaling. *Cell*, 125(5), pp.943–955.
- Kagan, Su, Horng, Chow, Akira & Medzhitov, 2008. TRAM couples endocytosis of Toll-like receptor 4 to the induction of interferon-beta. *Nature immunology*, 9(4), pp.361–368.
- Kalamvoki, Du & Roizman, 2014. Cells infected with herpes simplex virus 1 export to uninfected cells exosomes containing STING, viral mRNAs, and microRNAs. *Proceedings of the National Academy of Sciences of the United States of America*, 111(46), pp.E4991–6. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4246290&tool=pmcentrez&rendertype=abstract>.
- Kanayama, Seth, Sun, Ea, Hong, Shaito, Chiu, Deng, et al., 2004. TAB2 and TAB3 activate the NF-kB pathway through binding to polyubiquitin chains. *Molecular Cell*, 15(4), pp.535–548.
- Kang, Gopalkrishnan, Wu, Jankowsky, Pyle & Fisher, 2002. mda-5: An interferon-inducible putative RNA helicase with double-stranded RNA-dependent ATPase activity and melanoma growth-suppressive properties. *Proceedings of the National Academy of Sciences of the United States of America*, 99(2), pp.637–642.
- Kang & Kim, 2006. Negative regulation of ERK activity by VRK3-mediated activation of VHR phosphatase. *Nature cell biology*, 8(8), pp.863–869.
- Kang, Nan, Jin, Youn, Ryu, Mah, Han, Lee, et al., 2009. Recognition of Lipopeptide Patterns by Toll-like Receptor 2-Toll-like Receptor 6 Heterodimer. *Immunity*, 31(6), pp.873–884. Available at: <http://dx.doi.org/10.1016/j.immuni.2009.09.018>.
- Kang, Park, Choi, Kim, Yoon & Kim, 2007. Mitotic histone H3 phosphorylation by vaccinia-related kinase 1 in mammalian cells. *Molecular and cellular biology*, 27(24), pp.8533–8546.
- Kang, Park, Kim & Kim, 2008. VRK1 phosphorylates CREB and mediates CCND1 expression. *Journal of cell science*, 121(Pt 18), pp.3035–3041.
- Kao & Bauer, 1987. Biosynthesis and Phosphorylation of Vaccinia Virus Structural Protein VP1 1. *Virology*, 159, pp.399–407.
- Kao, Rensner, Kates & Bauer, 1981. Purification and characterization of a superhelix binding protein from vaccinia virus. *Virology*, 111, pp.500–508.
- Kato, Sato, Yoneyama, Yamamoto, Uematsu, Matsui, Tsujimura, Takeda, et al., 2005. Cell type-specific involvement of RIG-I in antiviral response. *Immunity*, 23(1), pp.19–28.
- Kato, Takeuchi, Mikamo-Satoh, Hirai, Kawai, Matsushita, Hiiragi, Dermody, et al., 2008. Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. *The Journal of experimental medicine*, 205(7), pp.1601–1610.

- Available at:
<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2442638&tool=pmcentrez&rendertype=abstract>.
- Kato, Takeuchi, Sato, Yoneyama, Yamamoto, Matsui, Uematsu, Jung, et al., 2006. Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature*, 441(7089), pp.101–105.
- Kawagoe, Sato, Matsushita, Kato, Matsui, Kumagai, Saitoh, Kawai, et al., 2008. Sequential control of Toll-like receptor-dependent responses by IRAK1 and IRAK2. *Nature immunology*, 9(6), pp.684–691.
- Kawahara, Michishita, Adler, Damian, Berber, Lin, McCord, Ongaigui, et al., 2009. SIRT6 links histone H3 lysine 9 deacetylation to NF-kappaB-dependent gene expression and organismal life span. *Cell*, 136(1), pp.62–74. Available at: <http://dx.doi.org/10.1016/j.cell.2008.10.052>.
- Kawai, Sato, Ishii, Coban, Hemmi, Yamamoto, Terai, Matsuda, et al., 2004. Interferon-alpha induction through Toll-like receptors involves a direct interaction of IRF7 with MyD88 and TRAF6. *Nature immunology*, 5(10), pp.1061–1068.
- Kawai, Takahashi, Sato, Coban, Kumar, Kato, Ishii, Takeuchi, et al., 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nature immunology*, 6(10), pp.981–988.
- Kearse, Moir, Wilson, Stones-Havas, Cheung, Sturrock, Buxton, Cooper, et al., 2012. Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics (Oxford, England)*, 28(12), pp.1647–9. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3371832&tool=pmcentrez&rendertype=abstract> [Accessed July 10, 2014].
- Keating, Maloney, Moran & Bowie, 2007. IRAK-2 participates in multiple Toll-like receptor signaling pathways to NFkB via activation of TRAF6 ubiquitination. *Journal of Biological Chemistry*, 282(46), pp.33435–33443.
- Kessler, Levy & Darnell, 1988. Two interferon-induced nuclear factors bind a single promoter element in interferon-stimulated genes. *Proceedings of the National Academy of Sciences of the United States of America*, 85(November), pp.8521–8525.
- Kessler, Veals, Fu & Levy, 1990. Interferon-α regulates nuclear translocation and DNA-binding affinity of ISGF3, a multimeric transcriptional activator. *Genes and Development*, 4(10), pp.1753–1765.
- Kettle, Alcamí, Khanna, Ehret, Jassoy & Smith, 1997. Vaccinia virus serpin B13R (SPI-2) inhibits interleukin-1β-converting enzyme and protects virus-infected cells from TNF- and Fas-mediated apoptosis, but does not prevent IL-1β-induced fever. *The Journal of general virology*, 78 (Pt 3)(1997), pp.677–685.
- Kettle, Blake, Katherine & Smith, 1995. Vaccinia virus serpins B13R (SPI-2) and B22R (SPI-1) encode M(r) 38.5 and 40K, intracellular polypeptides that do not affect virus virulence in a murine intranasal model. *Virology*, 147, pp.136–147.
- Kim, Choi, Kim, Roh, Park & Yoon, 2014. NUCKS1, a novel Tat coactivator, plays a crucial role in HIV-1 replication by increasing Tat-mediated viral transcription on the HIV-1 LTR promoter. *Retrovirology*, 11, p.67. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4181878&tool=pmcentrez&rendertype=abstract> [Accessed December 17, 2014].
- Kim, Lee, Kim, Kwak, Ryoo, Bae, Azam, Kim, et al., 2013. The interleukin-1α precursor is biologically active and is likely a key alarmin in the IL-1 family of cytokines. *Frontiers in Immunology*, 4(NOV), pp.1–9.
- Kim, White, Brandariz-Núñez, Diaz-Griffero & Weitzman, 2013. SAMHD1 restricts herpes simplex virus 1 in macrophages by limiting DNA replication. *Journal of virology*, 87(23), pp.12949–56. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3838123&tool=pmcentrez&rendertype=abstract>.
- Kitchen, Müller, Zobl, Windisch, Romani & Huemer, 2014. Orf virus infection in a hunter in Western Austria, presumably transmitted by game. *Acta Dermato-Venereologica*, 94(2), pp.212–214.
- Klerkx, Lazo & Askjaer, 2009. Emerging biological functions of the Vaccinia-related Kinase (VRK) family. *Histology and Histopathology*, 24(6), pp.749–759.
- Kollewe, Mackensen, Neumann, Knop, Cao, Li, Wesche & Martin, 2004. Sequential Autophosphorylation Steps in the Interleukin-1 Receptor-associated Kinase-1 Regulate its Availability as an Adapter in Interleukin-1 Signaling. *Journal of Biological Chemistry*, 279(7), pp.5227–5236.
- Kovacs, Vasilakis & Moss, 2001. Regulation of Viral Intermediate Gene Expression by the Vaccinia Virus B1 Protein Kinase Regulation of Viral Intermediate Gene Expression by the Vaccinia Virus B1 Protein Kinase †. *Journal of virology*, 75(9), pp.4048–4055.
- Kowalinski, Lunardi, McCarthy, Loubser, Brunel, Grigorov, Gerlier & Cusack, 2011. Structural basis for the activation of innate immune pattern-recognition receptor RIG-I by viral RNA. *Cell*, 147(2), pp.423–435. Available at: <http://dx.doi.org/10.1016/j.cell.2011.09.039>.

References

- Kreijtz, Goeijenbier, Moesker, van den Dries, Goeijenbier, De Gruyter, Lehmann, de Mutsert, et al., 2014. Safety and immunogenicity of a modified-vaccinia-virus-Ankara-based influenza A H5N1 vaccine: A randomised, double-blind phase 1/2a clinical trial. *The Lancet Infectious Diseases*, 14(12), pp.1196–1207. Available at: [http://dx.doi.org/10.1016/S1473-3099\(14\)70963-6](http://dx.doi.org/10.1016/S1473-3099(14)70963-6).
- Kumar, Sweeney, Skabkin, Skabkina, Hellen & Pestova, 2014. Inhibition of translation by IFIT family members is determined by their ability to interact selectively with the 5'-terminal regions of cap0-, cap1- and 5'ppp- mRNAs. *Nucleic acids research*, 42(5), pp.3228–45. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3950709&tool=pmcentrez&rendertype=abstract>.
- Kunsch, Ruben & Rosen, 1992. Selection of optimal kappa B/Rel DNA-binding motifs: interaction of both subunits of NF-kappa B with DNA is required for transcriptional activation. *Molecular and cellular biology*, 12(10), pp.4412–4421.
- Kurt-Jones, Popova, Kwinn, Haynes, Jones, Tripp, Walsh, Freeman, et al., 2000. Pattern recognition receptors TLR4 and CD14 mediate response to respiratory syncytial virus. *Nature immunology*, 1(5), pp.398–401.
- Ladnyj, Ziegler & Kima, 1972. A human infection caused by monkeypox virus in Basankusu Territory, Democratic Republic of the Congo. *Bulletin of the World Health Organization*, 46(5), pp.593–597.
- Lamothe, Besse, Campos, Webster, Wu & Darnay, 2007. Site-specific Lys-63-linked tumor necrosis factor receptor-associated factor 6 auto-ubiquitination is a critical determinant of I κ B kinase activation. *Journal of Biological Chemistry*, 282(6), pp.4102–4112.
- Learned, Reynolds, Wasswa Wasswa, Li, Olson, Karem, Stempora, Braden, et al., 2005. Extended interhuman transmission of monkeypox in a hospital community in the Republic of the Congo, 2003. *American Journal of Tropical Medicine and Hygiene*, 73(2), pp.428–434.
- Lee, Shank, Cusson & Kelliher, 2004. The kinase activity of Rip1 is not required for tumor necrosis factor- α -induced I κ B kinase or p38 MAP kinase activation or for the ubiquitination of Rip1 by Traf2. *The Journal of biological chemistry*, 279(32), pp.33185–33191.
- Lefkowitz, Wang & Upton, 2006. Poxviruses: past, present and future. *Virus research*, 117(1), pp.105–18. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/16503070> [Accessed June 12, 2013].
- Légrand, Verardi, Jones, Chan, Peng & Yilma, 2004. Induction of potent humoral and cell-mediated immune responses by attenuated vaccinia virus vectors with deleted serpin genes. *Journal of virology*, 78(6), pp.2770–2779.
- Leonard, Ghirlando, Askins, Bell, Margulies, Davies & Segal, 2008. The TLR3 signaling complex forms by cooperative receptor dimerization. *Proceedings of the National Academy of Sciences of the United States of America*, 105(1), pp.258–263.
- Lester & Li, 2014. Toll-like receptors in antiviral innate immunity. *Journal of Molecular Biology*, 426(6), pp.1246–1264. Available at: <http://dx.doi.org/10.1016/j.jmb.2013.11.024>.
- Li & Beg, 2000. Induction of necrotic-like cell death by tumor necrosis factor α and caspase inhibitors: novel mechanism for killing virus-infected cells. *Journal of virology*, 74(16), pp.7470–7. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=112267&tool=pmcentrez&rendertype=abstract>.
- Li, Diner, Chen & Cristea, 2012. Acetylation modulates cellular distribution and DNA sensing ability of interferon-inducible protein IFI16. *Proceedings of the National Academy of Sciences*, 109(26), pp.10558–10563.
- Li, Leung, Kerr & Stark, 1997. Functional subdomains of STAT2 required for preassociation with the α interferon receptor and for signaling. *Molecular and cellular biology*, 17(4), pp.2048–2056.
- Li, Makkinje & Damuni, 1996. The Myeloid Leukemia- associated Protein SET Is a Potent Inhibitor of Protein Phosphatase 2A. *The Journal of biological chemistry*, 271(19), pp.11059–11062.
- Li, Shu, Yi, Chaton, Shelton, Diao, Zuo, Kao, et al., 2013. Cyclic GMP-AMP Synthase Is Activated by Double-Stranded DNA-Induced Oligomerization. *Immunity*, 39(6), pp.1019–1031. Available at: <http://dx.doi.org/10.1016/j.immuni.2013.10.019>.
- Lin & Broyles, 1994. Vaccinia protein kinase 2: a second essential serine/threonine protein kinase encoded by vaccinia virus. *Proceedings of the National Academy of Sciences of the United States of America*, 91(16), pp.7653–7. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=44460&tool=pmcentrez&rendertype=abstract>.
- Lin, Chen & Broyles, 1992. The vaccinia virus B1R gene product is a serine/threonine protein kinase. *Journal of Virology*, 66(0022-538X SB - M SB - X), pp.2717–2723.
- Lin, Heylbroeck, Pitha & Hiscott, 1998. Virus-dependent phosphorylation of the IRF-3 transcription factor regulates nuclear translocation, transactivation potential, and proteasome-mediated degradation.

- Molecular and cellular biology*, 18(5), pp.2986–2996.
- Lin, Lo & Wu, 2010. Helical assembly in the MyD88-IRAK4-IRAK2 complex in TLR/IL-1R signalling. *Nature*, 465(7300), pp.885–890. Available at: <http://dx.doi.org/10.1038/nature09121>.
- Liu, Botos, Wang, Leonard, Shiloach, Segal & Davies, 2008. Structural basis of toll-like receptor 3 signaling with double-stranded RNA. *Science (New York, N.Y.)*, 320(5874), pp.379–381. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/18420935>.
- Liu, Cai, Wu, Cong, Chen, Li, Du, Ren, et al., 2015. Phosphorylation of innate immune adaptor proteins MAVS, STING, and TRIF induces IRF3 activation. *Science*, 347(6227), p.aaa2630. Available at: <http://www.sciencemag.org/cgi/doi/10.1126/science.aaa2630>.
- Liu, Chen, Cai, Wu, Chen, Wu, Sun & Chen, 2013. MAVS recruits multiple ubiquitin E3 ligases to activate antiviral signaling cascades. *eLife*, 2013(2), pp.1–24.
- Liu, Jesus, Marrero, Yang, Ramsey, Montealegre Sanchez, Tenbrock, Wittkowski, et al., 2014. Activated STING in a Vascular and Pulmonary Syndrome. *New England Journal of Medicine*, 371(6), pp.507–518. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4174543&tool=pmcentrez&rendertype=abstract>.
- Liu, Katsafanas, Liu, Wyatt & Moss, 2015. Poxvirus Decapping Enzymes Enhance Virulence by Preventing the Accumulation of dsRNA and the Induction of Innate Antiviral Responses. *Cell Host & Microbe*, 17(3), pp.320–331. Available at: <http://dx.doi.org/10.1016/j.chom.2015.02.002>.
- Liu, Lemon & Traktman, 1995. The Dual-Specificity Phosphatase Encoded by Vaccinia Virus, VH1, Is Essential for Viral Transcription In Vivo and In Vitro. *J Virol*, 69(12), pp.7823–7834. Available at: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC189726/\nhttp://europepmc.org/articles/PMC189726?sessionid=YAieGxr7duZHamlNBaie.25>.
- Liu & McFadden, 2015. SAMD9 is an innate antiviral host factor with stress response properties that can be antagonized by poxviruses. *Journal of virology*, 89(3), pp.1925–31. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4300762&tool=pmcentrez&rendertype=abstract>.
- Liu, Rothenburg & McFadden, 2012. The poxvirus C7L host range factor superfamily. *Current Opinion in Virology*, 2(6), pp.758–766. Available at: <http://dx.doi.org/10.1016/j.coviro.2012.09.012>.
- Lomvardas & Thanos, 2001. Nucleosome sliding via TBP DNA binding in vivo. *Cell*, 106(6), pp.685–696.
- Long, Giotis, Moncorgé, Frise, Mistry, James, Morisson, Iqbal, et al., 2016. Species difference in ANP32A underlies influenza A virus polymerase host restriction. *Nature*, 529(7584), pp.101–104. Available at: <http://dx.doi.org/10.1038/nature16474\n10.1038/nature16474>.
- Loo, Fornek, Crochet, Bajwa, Perwitasari, Martinez-Sobrido, Akira, Gill, et al., 2008. Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. *Journal of virology*, 82(1), pp.335–345.
- Lopez-Borges & Lazo, 2000. The human vaccinia-related kinase 1 (VRK1) phosphorylates threonine-18 within the mdm-2 binding site of the p53 tumour suppressor protein. *Oncogene*, 19(32), pp.3656–3664.
- Lu, Xu, Ranjith-Kumar, Brooks, Hou, Hu, Herr, Strong, et al., 2010. The structural basis of 5' triphosphate double-stranded RNA recognition by RIG-I C-terminal domain. *Structure*, 18(8), pp.1032–1043. Available at: <http://dx.doi.org/10.1016/j.str.2010.05.007>.
- Lund, Alexopoulou, Sato, Karow, Adams, Gale, Iwasaki & Flavell, 2004. Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proceedings of the National Academy of Sciences of the United States of America*, 101(15), pp.5598–5603.
- Lund, Sato, Akira, Medzhitov & Iwasaki, 2003. Toll-like receptor 9-mediated recognition of Herpes simplex virus-2 by plasmacytoid dendritic cells. *J Exp Med*, 198(3), pp.513–520. Available at: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12900525\nhttp://jem.rupress.org/cgi/reprint/198/3/513.pdf.
- Luo, Kohlway, Vela & Pyle, 2012. Visualizing the determinants of viral RNA recognition by innate immune sensor RIG-I. *Structure*, 20(11), pp.1983–1988. Available at: <http://dx.doi.org/10.1016/j.str.2012.08.029>.
- Lysakova-Devine, Keogh, Harrington, Nagpal, Halle, Golenbock, Monie & Bowie, 2010. Viral inhibitory peptide of TLR4, a peptide derived from vaccinia protein A46, specifically inhibits TLR4 by directly targeting MyD88 adaptor-like and TRIF-related adaptor molecule. *Journal of immunology (Baltimore, Md. : 1950)*, 185(7), pp.4261–4271.
- Ma & Damania, 2016. The cGAS-STING Defense Pathway and Its Counteraction by Viruses. *Cell Host & Microbe*, 19(2), pp.150–158. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S1931312816300099>.
- Ma, Jacobs, West, Stopford, Zhang, Davis, Barber, Glaunsinger, et al., 2015. Modulation of the cGAS-STING

References

- DNA sensing pathway by gammaherpesviruses. *Proceedings of the National Academy of Sciences of the United States of America*, 112(31), pp.E4306–15. Available at: <http://www.pnas.org/lookup/doi/10.1073/pnas.1503831112> \n <http://www.ncbi.nlm.nih.gov/pubmed/26199418> \n <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4534226>.
- Mahalingam, Damon & Lidbury, 2004. 25 years since the eradication of smallpox: why poxvirus research is still relevant. *Trends in Immunology*, 25(12), pp.632–636.
- Malathi, Dong, Gale & Silverman, 2007. Small self-RNA generated by RNase L amplifies antiviral innate immunity. *Nature*, 448(7155), pp.816–9. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3638316&tool=pmcentrez&rendertype=abstract>.
- Mansur, Maluquer de Motes, Unterholzner, Sumner, Ferguson, Ren, Strnadova, Bowie, et al., 2013. Poxvirus Targeting of E3 Ligase β -TrCP by Molecular Mimicry: A Mechanism to Inhibit NF- κ B Activation and Promote Immune Evasion and Virulence. *PLoS Pathogens*, 9(2), p.e1003183. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3585151&tool=pmcentrez&rendertype=abstract> [Accessed November 7, 2013].
- Marazzi, Ho, Kim, Manicassamy, Dewell, Albrecht, Seibert, Schaefer, et al., 2012. Suppression of the antiviral response by an influenza histone mimic. *Nature*, 483(7390), pp.428–433. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3598589&tool=pmcentrez&rendertype=abstract>.
- Martinez, Huang & Yang, 2010a. Direct TLR2 signaling is critical for NK cell activation and function in response to vaccinia viral infection. *PLoS Pathogens*, 6(3).
- Martinez, Huang & Yang, 2010b. Toll-like receptor 8-mediated activation of murine plasmacytoid dendritic cells by vaccinia viral DNA. *Proceedings of the National Academy of Sciences of the United States of America*, 107(14), pp.6442–6447.
- Martinez-Moczygemba, Gutch, French & Reich, 1997. Distinct STAT structure promotes interaction of STAT2 with the p48 subunit of the interferon- α -stimulated transcription factor ISGF3. *Journal of Biological Chemistry*, 272(32), pp.20070–20076.
- McCollum & Damon, 2014. Human monkeypox. *Clinical Infectious Diseases*, 58(2), pp.260–267.
- McFadden, 2005. Poxvirus tropism. *Nature reviews. Microbiology*, 3(3), pp.201–13. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4382915&tool=pmcentrez&rendertype=abstract>.
- Mcnab, Mayer-barber, Sher, Wack & Garra, 2015. Type I interferons in infectious disease. *Nature Publishing Group*, 15(2), pp.87–103. Available at: <http://dx.doi.org/10.1038/nri3787>.
- McWhirter, Fitzgerald, Rosains, Rowe, Golenbock & Maniatis, 2004. IFN-regulatory factor 3-dependent gene expression is defective in Tbk1-deficient mouse embryonic fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America*, 101(1), pp.233–8. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=314168&tool=pmcentrez&rendertype=abstract>.
- Medzhitov, Preston-Hurlburt & Janeway, 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature*, 388(6640), pp.394–397. Available at: <http://www.nature.com/nature/journal/v388/n6640/abs/388394a0.html>.
- Mellacheruvu, Wright, Couzens, Lambert, St-Denis, Li, Miteva, Hauri, et al., 2013. The CRAPome: a contaminant repository for affinity purification-mass spectrometry data. *Nature methods*, 10(8), pp.730–6. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3773500&tool=pmcentrez&rendertype=abstract> [Accessed July 21, 2014].
- Meng, Chao & Xiang, 2008. Identification from diverse mammalian poxviruses of host-range regulatory genes functioning equivalently to vaccinia virus C7L. *Virology*, 372(2), pp.372–383.
- Meng, Krumm, Li, Deng & Xiang, 2015. Structural basis for antagonizing a host restriction factor by C7 family of poxvirus host-range proteins. *Proceedings of the National Academy of Sciences of the United States of America*, 112(48), pp.14858–63. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/26578811>.
- Meng, Schoggins, Rose, Cao, Ploss, Rice & Xiang, 2012. C7L Family of Poxvirus Host Range Genes Inhibits Antiviral Activities Induced by Type I Interferons and Interferon Regulatory Factor 1. *Journal of virology*, 86(8), pp.4538–47. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3318637&tool=pmcentrez&rendertype=abstract> [Accessed November 7, 2013].
- Meng & Xia, 2011. c-Jun, at the crossroad of the signaling network. *Protein and Cell*, 2(11), pp.889–898.
- Mercer & Helenius, 2008. Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells.

- Science* (New York, N.Y.), 320(5875), pp.531–535.
- Mercurio, Zhu, Murray, Shevchenko, Bennett, Li, Young, Barbosa, et al., 1997. IKK-1 And IKK-2 : Cytokine-Activated I κ B Kinases Essential for NF- κ B Activation. *Science*, 923(October).
- Meylan, Burns, Hofmann, Blancheteau, Martinon, Kelliher & Tschopp, 2004. RIP1 is an essential mediator of Toll-like receptor 3-induced NF- κ B activation. *Nature immunology*, 5(5), pp.503–507.
- Meylan, Curran, Hofmann, Moradpour, Binder, Bartenschlager & Tschopp, 2005. Cardif is an adaptor protein in the RIG-I antiviral pathway and is targeted by hepatitis C virus. *Nature*, 437(7062), pp.1167–1172.
- Michallet, Meylan, Ermolaeva, Vazquez, Rebsamen, Curran, Poeck, Bscheider, et al., 2008. TRADD Protein Is an Essential Component of the RIG-like Helicase Antiviral Pathway. *Immunity*, 28(5), pp.651–661.
- Michallet, Rota, Maslowski & Guarda, 2013. Innate receptors for adaptive immunity. *Current Opinion in Microbiology*, 16(3), pp.286–302.
- Monroe, Yang, Johnson, Geng, Doitsh, Krogan & Greene, 2014. IFI16 DNA sensor is required for death of lymphoid CD4 T cells abortively infected with HIV. *Science* (New York, N.Y.), 343, pp.428–432.
- Morales, Carbajal, Bohn, Holzer, Kato, Greco, Moussatché & Locker, 2008. The vaccinia virus F11L gene product facilitates cell detachment and promotes migration. *Traffic*, 9(8), pp.1283–1298.
- Moriguchi, Kuroyanagi, Yamaguchi, Gotoh, Irie, Kano, Shirakabe, Muro, et al., 1996. A Novel Kinase Cascade Mediated by Mitogen-activated Protein Kinase Kinase 6 and MKK3. *Journal of Biological Chemistry*, 271(23), pp.13675–13679.
- Morikawa, Sakiyama, Hasegawa, Saijo, Maeda, Kurane, Maeno, Kimura, et al., 2005. An Attenuated LC16m8 Smallpox Vaccine : Analysis of Full-Genome Sequence and Induction of Immune Protection An Attenuated LC16m8 Smallpox Vaccine : Analysis of Full-Genome Sequence and Induction of Immune Protection §. *Journal of virology*, 79(18), pp.11873–11891.
- Morrone, Wang, Constantoulakis, Hooy, Delannoy & Sohn, 2014. Cooperative assembly of IFI16 filaments on dsDNA provides insights into host defense strategy. *Proceedings of the National Academy of Sciences of the United States of America*, 111(1), pp.E62–71. Available at: <http://eutils.ncbi.nlm.nih.gov/entrez/eutils/elink.fcgi?dbfrom=pubmed&id=24367117&retmode=ref&cmd=prlinks&npapers2://publication/doi/10.1073/pnas.1313577111>.
- Moss, 2013. Poxvirus DNA Replication. *Cold Spring Harb Perspect Biol*, 5(9), pp.775–798.
- Moss, 2011. Smallpox vaccines: Targets of protective immunity. *Immunological Reviews*, 239(1), pp.8–26.
- Motshwene, Moncrieffe, Grossmann, Kao, Ayaluru, Sandercock, Robinson, Latz, et al., 2009. An Oligomeric Signaling Platform formed by the toll-like receptor signal transducers MyD88 and IRAK-4. *Journal of Biological Chemistry*, 284(37), pp.25404–25411.
- Müller, Briscoe, Laxton, Guschin, Ziemiecki, Silvennoinen, Harpur, Barbieri, et al., 1993. The protein tyrosine kinase JAK1 complements defects in interferon- α /beta and - γ signal transduction. *Nature*, 366(6451), pp.814–820. Available at: <http://www.nature.com/nature/journal/v366/n6451/abs/366129a0.html>.
- Munir & Berg, 2013. The multiple faces of protein kinase R in antiviral defense. *Virulence*, 4(1), pp.85–89. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/?term=23314571>.
- Muzio, M, Ni, Feng & Dixit, 1997. IRAK (Pelle) family member IRAK-2 and MyD88 as proximal mediators of IL-1 signaling. *Science* (New York, N.Y.), 278(5343), pp.1612–1615.
- Muzio, Ni, Feng, Dixit & Alerts, 1997. IRAK (Pelle) Family Member IRAK-2 and MyD88 as Proximal Mediators. *Science Signalling*, 278(November), pp.1612–1615.
- Myskiw, Arsenio, Booy, Hammett, Deschambault, Gibson & Cao, 2011. RNA species generated in vaccinia virus infected cells activate cell type-specific MDA5 or RIG-I dependent interferon gene transcription and PKR dependent apoptosis. *Virology*, 413(2), pp.183–193. Available at: <http://dx.doi.org/10.1016/j.virol.2011.01.034>.
- Myskiw, Arsenio, van Bruggen, Deschambault & Cao, 2009. Vaccinia virus E3 suppresses expression of diverse cytokines through inhibition of the PKR, NF- κ B, and IRF3 pathways. *Journal of virology*, 83(13), pp.6757–6768. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2698532&tool=pmcentrez&rendertype=abstract> [Accessed December 11, 2014].
- Najarro, Traktman & Lewis, 2001. Vaccinia virus blocks gamma interferon signal transduction: viral VH1 phosphatase reverses Stat1 activation. *Journal of virology*, 75(7), pp.3185–96. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=114112&tool=pmcentrez&rendertype=abstract>.
- Nájera, Gómez, Domingo-Gil, Gherardi & Esteban, 2006. Cellular and biochemical differences between two attenuated poxvirus vaccine candidates (MVA and NYVAC) and role of the C7L gene. *Journal of virology*, 80(12), pp.6033–6047.

- De Nardo, 2015. Toll-like receptors: Activation, signalling and transcriptional modulation. *Cytokine*, 74(2), pp.181–189. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S1043466615001039>.
- Natoli, Sacconi, Bosio & Marazzi, 2005. Interactions of NF-kappaB with chromatin: the art of being at the right place at the right time. *Nature immunology*, 6(5), pp.439–445. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/15843800>.
- Neidel, Maluquer de Motes, Mansur, Strnadova, Smith & Graham, 2015. Vaccinia Virus Protein A49 Is an Unexpected Member of the B-cell Lymphoma (Bcl)-2 Protein Family. *Journal of Biological Chemistry*, 290(10), pp.5991–6002. Available at: <http://www.jbc.org/lookup/doi/10.1074/jbc.M114.624650>.
- Nezu, Oku, Jones & Shimane, 1997. Identification of two novel human putative serine/threonine kinases, VRK1 and VRK2, with structural similarity to vaccinia virus B1R kinase. *Genomics*, 45(2), pp.327–331. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/9344656>.
- Nichols & Traktman, 2004. Characterization of Three Paralogous Members of the Mammalian Vaccinia Related Kinase Family. *Journal of Biological Chemistry*, 279(9), pp.7934–7946.
- Nichols, Wiebe & Traktman, 2006. The vaccinia-related kinases phosphorylate the N' terminus of BAF, regulating its interaction with DNA and its retention in the nucleus. *Molecular biology of the cell*, 17(2), pp.1018–1032.
- Nilsson, Godoy-Ramirez, Hejdeman, Brave, Gudmundsdotter, Hallengard, Currier, Wiczorek, et al., 2014. Broad and potent cellular and humoral immune responses after a second late HIV-modified vaccinia virus ankara vaccination in HIV-DNA-primed and HIV-modified vaccinia virus Ankara-boosted Swedish vaccinees. *AIDS research and human retroviruses*, 30(3), pp.299–311.
- Ninomiya-Tsuji, Kishimoto, Hiyama, Inoue, Cao & Matsumoto, 1999. The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway. *Nature*, 398(6724), pp.252–256.
- Noguchi, Hirai, Ishiura & Ikegami, 1963. Viral treatment of skin cancers. *The Australasian Journal of Dermatology*, 7, pp.26–30.
- Novick, Cohen & Rubinstein, 1994. The human interferon alpha/beta receptor: characterization and molecular cloning. *Cell*, 77(3), pp.391–400.
- Nowak, Tian, Jamaluddin, Boldogh, Vergara, Choudhary & Brasier, 2008. RelA Ser276 phosphorylation is required for activation of a subset of NF-kappaB-dependent genes by recruiting cyclin-dependent kinase 9/cyclin T1 complexes. *Molecular and cellular biology*, 28(11), pp.3623–38. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2423290&tool=pmcentrez&rendertype=abstract>.
- Oguri, Spehner & Drillien, 1993. Detection of a protein encoded by the vaccinia virus C7L open reading frame and study of its effect on virus multiplication in different cell lines. *Journal of General Virology*, 74(7), pp.1409–1413.
- Ohto, Shibata, Tanji, Ishida, Krayukhina, Uchiyama, Miyake & Shimizu, 2015. Structural basis of CpG and inhibitory DNA recognition by Toll-like receptor 9. *Nature*, 520(7549), pp.702–5. Available at: <http://www.nature.com/doi/10.1038/nature14138>.
- Oliveira, Assis, Ferreira, Bonjardim, de Souza Trindade, Kroon & Abrahao, 2013. Group 1 Vaccinia virus Zoonotic Outbreak in Maranhao State, Brazil. *American Journal of Tropical Medicine and Hygiene*, 89(6), pp.1142–1145. Available at: <http://www.ajtmh.org/cgi/doi/10.4269/ajtmh.13-0369>.
- Oliveira, Fernandes, De Assis, Alves, Luiz, Figueiredo, De Almeida, Travassos, et al., 2014. Short report: Intrafamilial transmission of Vaccinia virus during a bovine vaccinia outbreak in Brazil: A new insight in viral transmission chain. *American Journal of Tropical Medicine and Hygiene*, 90(6), pp.1021–1023.
- Orzalli, Broekema, Diner, Hancks, Elde, Cristea & Knipe, 2015. cGAS-mediated stabilization of IFI16 promotes innate signaling during herpes simplex virus infection. *Proceedings of the National Academy of Sciences*, p.201424637. Available at: <http://www.pnas.org/lookup/doi/10.1073/pnas.1424637112>.
- Orzalli, DeLuca & Knipe, 2012. Nuclear IFI16 induction of IRF-3 signaling during herpesviral infection and degradation of IFI16 by the viral ICP0 protein. *Proceedings of the National Academy of Sciences*, 109(44), pp.E3008–E3017.
- Oseroff, Kos, Bui, Peters, Pasquetto, Glenn, Palmore, Sidney, et al., 2005. HLA class I-restricted responses to vaccinia recognize a broad array of proteins mainly involved in virulence and viral gene regulation. *Proceedings of the National Academy of Sciences of the United States of America*, 102(39), pp.13980–13985.
- Oshimi, Matsumoto, Hatakeyama & Seya, 2009. Riplet/RNF135, a RING finger protein, ubiquitinates RIG-I to promote interferon-beta induction during the early phase of viral infection. *Journal of Biological Chemistry*, 284(2), pp.807–817.
- Pakala, Bui-Nguyen, Reddy, Li, Peng, Rayala, Behringer & Kumar, 2010. Regulation of NF-kappaB circuitry by

- a component of the nucleosome remodeling and deacetylase complex controls inflammatory response homeostasis. *Journal of Biological Chemistry*, 285(31), pp.23590–23597.
- Paludan & Bowie, 2013. Immune Sensing of DNA. *Immunity*, 38(5), pp.870–880. Available at: <http://dx.doi.org/10.1016/j.immuni.2013.05.004>.
- Park, Ryu, Kim, Lee, Song & Kim, 2015. Presumed pseudokinase VRK3 functions as a BAF kinase. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1853(7), pp.1738–1748. Available at: <http://linkinghub.elsevier.com/retrieve/pii/S0167488915001263>.
- Park, Song, Kim, Choi, Lee & Lee, 2009. The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex. *Nature*, 458(7242), pp.1191–1195. Available at: <http://dx.doi.org/10.1038/nature07830>.
- Park, Ye, Hsia, Segal, Rich, Liou, Myszkowski & Wu, 2000. A novel mechanism of TRAF signaling revealed by structural and functional analyses of the TRADD-TRAF2 interaction. *Cell*, 101(7), pp.777–787.
- Pastoret & Brochier, 1996. The development and use of a vaccinia-rabies recombinant oral vaccine for the control of wildlife rabies; a link between Jenner and Pasteur. *Epidemiology and infection*, 116, pp.235–240.
- Paz, Vilasco, Werden, Arguello, Joseph-Pillai, Zhao, Nguyen, Sun, et al., 2011. A functional C-terminal TRAF3-binding site in MAVS participates in positive and negative regulation of the IFN antiviral response. *Cell research*, 21(6), pp.895–910. Available at: <http://dx.doi.org/10.1038/cr.2011.2>.
- Pedersen, Snijder, Schleich, Roos, Griffiths & Locker, 2000. Characterization of vaccinia virus intracellular cores: implications for viral uncoating and core structure. *Journal of virology*, 74(8), pp.3525–3536.
- Peisley, Wu, Xu, Chen & Hur, 2014. Structural basis for ubiquitin-mediated antiviral signal activation by RIG-I. *Nature*, 509(7498), pp.110–4. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24590070>.
- Peisley, Wu, Yao, Walz & Hur, 2013. RIG-I Forms Signaling-Competent Filaments in an ATP-Dependent, Ubiquitin-Independent Manner. *Molecular Cell*, 51(5), pp.573–583. Available at: <http://dx.doi.org/10.1016/j.molcel.2013.07.024>.
- Perkus, Piccini, Lipinskas & Paoletti, 1985. Recombinant vaccinia virus: immunization against multiple pathogens. *Scie*, 229(4717), pp.981–4.
- Peters, Ferguson, Mazzon, Fahy, Kryzstofinska, Arribas-Bosacoma, Pearl, Ren, et al., 2013. A Mechanism for the Inhibition of DNA-PK-Mediated DNA Sensing by a Virus. *PLoS Pathogens*, 9(10).
- Peters, Liao & Maniatis, 2000. IKKε Is Part of a Novel PMA-Inducible IκB Kinase Complex. *Molecular Cell*, 5(3), pp.513–522.
- Pichlmair, Kandasamy, Alvisi, Mulhern, Sacco, Habjan, Binder, Stefanovic, et al., 2012. Viral immune modulators perturb the human molecular network by common and unique strategies. *Nature*, 487(7408), pp.486–90. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22810585> [Accessed March 11, 2013].
- Pichlmair, Lassnig, Eberle, Górna, Baumann, Burkard, Bürckstümmer, Stefanovic, et al., 2011. IFIT1 is an antiviral protein that recognizes 5'-triphosphate RNA. *Nature immunology*, 12(7), pp.624–30. Available at: <http://dx.doi.org/10.1038/ni.2048>.
- Pichlmair, Schulz, Tan, Näslund, Liljeström, Weber & Reis e Sousa, 2006. RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science*, 314(5801), pp.997–1001. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17038589>.
- Pichlmair, Schulz, Tan, Rehwinkel, Kato, Takeuchi, Akira, Way, et al., 2009. Activation of MDA5 requires higher-order RNA structures generated during virus infection. *Journal of virology*, 83(20), pp.10761–10769.
- Plato, Willment & Brown, 2013. C-type lectin-like receptors of the dectin-1 cluster: ligands and signaling pathways. *International reviews of immunology*, 32(2), pp.134–56. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3634610&tool=pmcentrez&rendertype=abstract>.
- Pomerantz & Baltimore, 1999. NF-κB activation by a signaling complex containing TRAF2, TANK and TBK1, a novel IKK-related kinase. *The EMBO journal*, 18(23), pp.6694–6704.
- Price, Tschärke, Hollinshead & Smith, 2000. Vaccinia virus gene B7R encodes an 18-kDa protein that is resident in the endoplasmic reticulum and affects virus virulence. *Virology*, 267(1), pp.65–79. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/10648184> [Accessed January 29, 2015].
- Punjabi & Traktman, 2005. Cell biological and functional characterization of the vaccinia virus F10 kinase: implications for the mechanism of virion morphogenesis. *Journal of virology*, 79(4), pp.2171–90. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=546551&tool=pmcentrez&rendertype=abstract>.

References

- Quigley, Martinez, Huang & Yang, 2009. A critical role for direct TLR2-MyD88 signaling in CD8 T-cell clonal expansion and memory formation following vaccinia viral infection. *Blood*, 113(10), pp.2256–2264.
- Ramanathan, Devarkar, Jiang, Miller, Khan, Marcotrigiano & Patel, 2015. The autoinhibitory CARD2-Hel2i Interface of RIG-I governs RNA selection. *Nucleic acids research*, 44(2), pp.896–909. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/26612866>.
- Ramirez-Carrozzi, Nazarian, Li, Gore, Sridharan, Imbalzano & Smale, 2006. Selective and antagonistic functions of SWI/SNF and Mi-2beta nucleosome remodeling complexes during an inflammatory response. *Genes and Development*, 20(3), pp.282–296.
- Reckmann, Higley & Way, 1997. The vaccinia virus F17R protein interacts with actin. *FEBS Letters*, 409(2), pp.141–146. Available at: [http://dx.doi.org/10.1016/S0014-5793\(97\)00450-X](http://dx.doi.org/10.1016/S0014-5793(97)00450-X) [Accessed November 7, 2013].
- Reed, Melski, Graham, Regnery, Sotir, Wegner, Kazmierczak, Stratman, et al., 2004. The detection of monkeypox in humans in the Western Hemisphere. *The New England journal of medicine*, 350(4), pp.342–350.
- Rehwinkel, Tan, Goubau, Schulz, Pichlmair, Bier, Robb, Vreede, et al., 2010. RIG-I Detects Viral Genomic RNA during Negative-Strand RNA Virus Infection. *Cell*, 140(3), pp.397–408. Available at: <http://dx.doi.org/10.1016/j.cell.2010.01.020>.
- Rempel & Traktman, 1992. Vaccinia virus B1 kinase: phenotypic analysis of temperature-sensitive mutants and enzymatic characterization of recombinant proteins. *Journal of virology*, 66(7), pp.4413–26. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=241249&tool=pmcentrez&rendertype=abstract>.
- Renbaum, Kellerman, Jaron, Geiger, Segel, Lee, King & Levy-Lahad, 2009. Spinal Muscular Atrophy with Pontocerebellar Hypoplasia Is Caused by a Mutation in the VRK1 Gene. *American Journal of Human Genetics*, 85(2), pp.281–289. Available at: <http://dx.doi.org/10.1016/j.ajhg.2009.07.006>.
- Resch, Hixson, Moore, Lipton & Moss, 2007. Protein composition of the vaccinia virus mature virion. *Virology*, 358(1), pp.233–47. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/17005230> [Accessed January 6, 2015].
- Rice, Kerr, Roberts, Brown & Kerr, 1985. Novel 2',5'-oligoadenylates synthesized in interferon-treated, vaccinia virus-infected cells. *Journal of virology*, 56(3), pp.1041–1044.
- Rice, Roberts & Kerr, 1984. 2-5A accumulates to high levels in interferon-treated, vaccinia virus-infected cells in the absence of any inhibition of virus replication. *Journal of virology*, 50(1), pp.220–228.
- Riedel, 2005. Edward Jenner and the history of smallpox and vaccination. *BUMC Proceedings*, 18, pp.21–25. Available at: <http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1200696/pdf/bumc0018-0021.pdf>.
- Rimoin, Mulembakani, Johnston, Lloyd Smith, Kisalu, Kinkela, Blumberg, Thomassen, et al., 2010. Major increase in human monkeypox incidence 30 years after smallpox vaccination campaigns cease in the Democratic Republic of Congo. *Proceedings of the National Academy of Sciences of the United States of America*, 107(37), pp.16262–16267.
- Roberts & Smith, 2008. Vaccinia virus morphogenesis and dissemination. *Trends in Microbiology*, 16(10), pp.472–479.
- Rock, Hardiman, Timans, Kastelein & Bazan, 1998. A family of human receptors structurally related to Drosophila Toll. *Proceedings of the National Academy of Sciences of the United States of America*, 95(2), pp.588–593.
- Roger, Roy, Mombelli, Koessler, Ding, Chanson, Reymond, Miconnet, et al., 2011. Histone deacetylase inhibitors impair innate immune responses to Toll-like receptor agonists and to infection. *Blood*, 117(4), pp.1205–1217.
- Rosenwasser, Dinarello & Rosenthal, 1979. Adherent cell function in murine T-lymphocyte antigen recognition. IV. Enhancement of murine T-cell antigen recognition by human leukocytic pyrogen. *Journal of Experimental Medicine*, 150(3), pp.709–14.
- Rotem, Cox & Isaacs, 1963. Inhibition of virus multiplication by foreign nucleic acid. *Nature*, 197, pp.564–566. Available at: <http://europepmc.org/abstract/MED/13975288>.
- Rothlin, Ghosh, Zuniga, Oldstone & Lemke, 2007. TAM Receptors Are Pleiotropic Inhibitors of the Innate Immune Response. *Cell*, 131(6), pp.1124–1136.
- Rothwarf, Zandi, Natoli & Karin, 1998. IKK-gamma is an essential regulatory subunit of the I κ B kinase complex. *Nature*, 395(6699), pp.297–300.
- Rowe & Cen, 2014. TroVax in colorectal cancer. *Human Vaccines and Immunotherapeutics*, 10(11), pp.3196–3200.
- Rusinova, Forster, Yu, Kannan, Masse, Cumming, Chapman & Hertzog, 2013. INTERFEROME v2.0: An updated

- database of annotated interferon-regulated genes. *Nucleic Acids Research*, 41(D1), pp.1040–1046.
- Saccani, Pantano & Natoli, 2001. Two waves of nuclear factor kappaB recruitment to target promoters. *The Journal of experimental medicine*, 193(12), pp.1351–1359.
- Saha, Pietras, He, Kang, Liu, Oganessian, Shahangian, Zarnegar, et al., 2006. Regulation of antiviral responses by a direct and specific interaction between TRAF3 and Cardif. *The EMBO journal*, 25(14), pp.3257–3263.
- Saito, Hirai, Loo, Owen, Johnson, Sinha, Akira, Fujita, et al., 2007. Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. *Proceedings of the National Academy of Sciences of the United States of America*, 104(2), pp.582–587.
- Saitoh, Fujita, Hayashi, Takahara, Satoh, Lee, Matsunaga, Kageyama, et al., 2009. Atg9a controls dsDNA-driven dynamic translocation of STING and the innate immune response. *Pnas*, 106, pp.20842–46.
- Sakurai, Suzuki, Kawasaki, Nakano, Okazaki, Chino, Doi & Saiki, 2003. Tumor necrosis factor- α -induced IKK phosphorylation of NF- κ B p65 on serine 536 is mediated through the TRAF2, TRAF5, and TAK1 signaling pathway. *Journal of Biological Chemistry*, 278(38), pp.36916–36923.
- Samuelsson, Hausmann, Lauterbach, Schmidt, Akira, Wagner, Chaplin, Suter, et al., 2008. Survival of lethal poxvirus infection in mice depends on TLR9, and therapeutic vaccination provides protection. *Journal of Clinical Investigation*, 118(5), pp.1776–1784.
- Sánchez-Puig, Sánchez, Roy & Blasco, 2004. Susceptibility of different leukocyte cell types to Vaccinia virus infection. *Virology journal*, 1, p.10.
- Sánchez-Sampedro, Perdiguero, Mejías-Pérez, García-Arriaza, Di Pilato & Esteban, 2015. The evolution of poxvirus vaccines. *Viruses*, 7(4), pp.1726–1803.
- Sandgren, Wilkinson, Miranda-Saksena, McInerney, Byth-Wilson, Robinson & Cunningham, 2010. A differential role for macropinocytosis in mediating entry of the two forms of vaccinia virus into dendritic cells. *PLoS pathogens*, 6(4), p.e1000866.
- Santos, Blanco, Sevilla & Lazo, 2006. Vaccinia virus B1R kinase interacts with JIP1 and modulates c-Jun-dependent signaling. *Journal of virology*, 80(15), pp.7667–75. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1563719&tool=pmcentrez&rendertype=abstract> [Accessed November 7, 2013].
- Santos, Vega, Blanco, Barcia & Lazo, 2004. The vaccinia virus B1R kinase induces p53 downregulation by an Mdm2-dependent mechanism. *Virology*, 328(2), pp.254–265.
- Sarov & Joklik, 1972. Studies on the nature and location of the capsid polypeptides of vaccinia virions. *Virology*, 50(2), pp.579–592.
- Sato, Hata, Asagiri, Nakaya, Taniguchi & Tanaka, 1998. Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF-7. *FEBS letters*, 441(1), pp.106–110.
- Sato, Sanjo, Takeda, Ninomiya-Tsuji, Yamamoto, Kawai, Matsumoto, Takeuchi, et al., 2005. Essential function for the kinase TAK1 in innate and adaptive immune responses. *Nature immunology*, 6(11), pp.1087–1095.
- Sato, Sugiyama, Yamamoto, Watanabe, Kawai, Takeda & Akira, 2003. Toll/IL-1 receptor domain-containing adaptor inducing IFN- β (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF- κ B and IFN-regulatory factor-3, in the Toll-like re. *Journal of immunology (Baltimore, Md. : 1950)*, 171(8), pp.4304–4310.
- Schindler, Shuai, Prezioso & Darnell, 1992. Interferon-Dependent Tyrosine Phosphorylation of a latent cytoplasmic transcription factor. *Science*, 257(August), pp.809–813.
- Schlee, Roth, Hornung, Hagmann, Wimmenauer, Barchet, Coch, Janke, et al., 2009. Recognition of 5' Triphosphate by RIG-I Helicase Requires Short Blunt Double-Stranded RNA as Contained in Panhandle of Negative-Strand Virus. *Immunity*, 31(1), pp.25–34. Available at: <http://dx.doi.org/10.1016/j.immuni.2009.05.008>.
- Schmidt, Bleck, Helenius & Mercer, 2011. Vaccinia extracellular virions enter cells by macropinocytosis and acid-activated membrane rupture. *The EMBO Journal*, 30(17), pp.3647–61. Available at: <http://dx.doi.org/10.1038/emboj.2011.245>.
- Schmidt, Bleck, Reh, Novy, Wollscheid, Helenius, Stahlberg & Mercer, 2013. Vaccinia virus entry is followed by core activation and proteasome-mediated release of the immunomodulatory effector VH1 from lateral bodies. *Cell Reports*, 4(3), pp.464–476. Available at: <http://dx.doi.org/10.1016/j.celrep.2013.06.028>.
- Schmidt, Schwerd, Hamm, Hellmuth, Cui, Wenzel, Hoffmann, Michallet, et al., 2009. 5'-triphosphate RNA requires base-paired structures to activate antiviral signaling via RIG-I. *Proceedings of the National Academy of Sciences of the United States of America*, 106(29), pp.12067–12072.
- Schmitz & Kracht, 2015. Cyclin-Dependent Kinases as Coregulators of Inflammatory Gene Expression. *Trends*

References

- in *Pharmacological Sciences*, 37(2), pp.101–113. Available at: <http://dx.doi.org/10.1016/j.tips.2015.10.004>.
- Schneider, Chevillotte & Rice, 2014. Interferon-stimulated genes: a complex web of host defenses. *Annual review of immunology*, 32, pp.513–45. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/24555472>.
- Schober, Aydiner, Booth, Seli & Reinke, 2011. The kinase VRK1 is required for normal meiotic progression in mammalian oogenesis. *Mechanisms of Development*, 128(3-4), pp.178–190. Available at: <http://dx.doi.org/10.1016/j.mod.2011.01.004>.
- Schoggins, 2014. Interferon-stimulated genes: Roles in viral pathogenesis. *Current Opinion in Virology*, 6(1), pp.40–46. Available at: <http://dx.doi.org/10.1016/j.coviro.2014.03.006>.
- Schoggins, MacDuff, Imanaka, Gainey, Shrestha, Eitson, Mar, Richardson, et al., 2014. Pan-viral specificity of IFN-induced genes reveals new roles for cGAS in innate immunity. *Nature*, 505(7485), pp.691–5. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4077721&tool=pmcentrez&rendertype=abstract>.
- Schröder, Baran & Bowie, 2008. Viral targeting of DEAD box protein 3 reveals its role in TBK1/IKKepsilon-mediated IRF activation. *The EMBO journal*, 27(15), pp.2147–57. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2516890&tool=pmcentrez&rendertype=abstract> [Accessed November 19, 2014].
- Schuberth-Wagner, Ludwig, Bruder, Herzner, Zillinger, Goldeck, Schmidt, Schmid-Burgk, et al., 2015. A Conserved Histidine in the RNA Sensor RIG-I Controls Immune Tolerance to N1-2'-O-Methylated Self RNA. *Immunity*, 43(1), pp.41–52.
- Sen & Baltimore, 1986. Inducibility of κ immunoglobulin enhancer-binding protein NF- κ B by a posttranslational mechanism. *Cell*, 47(6), pp.921–928.
- Senkevich, Koonin & Moss, 2011. Vaccinia virus F16 protein, a predicted catalytically inactive member of the prokaryotic serine recombinase superfamily, is targeted to nucleoli. *Virology*, 417(2), pp.334–42. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3163719&tool=pmcentrez&rendertype=abstract> [Accessed November 7, 2013].
- Seth, Sun, Ea & Chen, 2005. Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF- κ B and IRF3. *Cell*, 122(5), pp.669–682.
- Sevilla, Santos, Barcia, Vega & Lazo, 2004. c-Jun phosphorylation by the human vaccinia-related kinase 1 (VRK1) and its cooperation with the N-terminal kinase of c-Jun (JNK). *Oncogene*, 23(55), pp.8950–8958.
- Sevilla, Santos, Vega & Lazo, 2004. Human vaccinia-related kinase 1 (VRK1) activates the ATF2 transcriptional activity by novel phosphorylation on Thr-73 and Ser-62 and cooperates with JNK. *Journal of Biological Chemistry*, 279(26), pp.27458–27465.
- Sharma, TenOever, Grandvaux, Zhou, Lin & Hiscott, 2003. Triggering the Interferon Antiviral Response Through an IKK-Related Pathway. *Science*, 300(5622), pp.1148–1151. Available at: <http://www.sciencemag.org/cgi/doi/10.1126/science.1081315>.
- Shchelkunov, 2013. An Increasing Danger of Zoonotic Orthopoxvirus Infections. *PLoS Pathogens*, 9(12), pp.1–4.
- Shim, Xiao, Paschal, Bailey, Rao, Hayden, Lee, Bussey, et al., 2005. TAK1, but not TAB1 or TAB2, plays an essential role in multiple signaling pathways in vivo. *Genes & Development*, 1, pp.2668–2681.
- Shin, Chakraborty, Bharatham, Kang, Tochio, Koshiba, Kigawa, Kim, et al., 2011. NMR solution structure of human vaccinia-related kinase 1 (VRK1) reveals the C-terminal tail essential for its structural stability and autocatalytic activity. *Journal of Biological Chemistry*, 286(25), pp.22131–22138.
- Shirey, Lai, Patel, Pletneva, Pang, Kurt-Jones, Lipsky, Roger, et al., 2016. Novel strategies for targeting innate immune responses to influenza. *Mucosal Immunology*, (November 2015), pp.1–10. Available at: <http://www.nature.com/doi/10.1038/mi.2015.141>.
- Shisler & Jin, 2004. The vaccinia virus K1L gene product inhibits host NF-kappaB activation by preventing IkappaBalpha degradation. *Journal of virology*, 78(7), pp.3553–3560.
- Sims, Acres, Grubin, McMahan, Wignall, March & Dower, 1989. Cloning the interleukin 1 receptor from human T cells. *Proceedings of the National Academy of Sciences of the United States of America*, 86(22), pp.8946–8950.
- Singh, Balamurugan, Bhanuprakash, Venkatesan & Hosamani, 2012. Emergence and reemergence of vaccinia-like viruses: Global scenario and perspectives. *Indian Journal of Virology*, 23(1), pp.1–11.
- Sivan, Ormanoglu, Buehler, Martin & Moss, 2015. Identification of Restriction Factors by Human Genome-Wide RNA Interference Screening of Viral Host Range Mutants Exemplified by Discovery of SAMD9 and WDR6 as Inhibitors of the Vaccinia Virus. *mBio*, 6(4), pp.01122–15. Available at:

- http://www.ncbi.nlm.nih.gov/nuccore/nc_006998.1 [Accessed December 6, 2014].
- Smith, Benfield, Maluquer de Motes, Mazzon, Ember, Ferguson & Sumner, 2013. Vaccinia virus immune evasion: mechanisms, virulence and immunogenicity. *The Journal of general virology*, 94(Pt 11), pp.2367–92. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23999164> [Accessed November 21, 2014].
- Smith, Howard & Chan, 1989. Vaccinia virus encodes a family of genes with homology to serine proteinase inhibitors. *Journal of General Virology*, 70(9), pp.2333–2343.
- Smith & Moss, 1983. Infectious poxvirus vectors have capacity for at least 25 000 base pairs of foreign DNA. *Gene*, 25(1), pp.21–28.
- Smith & Symons, 1998. Poxviruses: interfering with interferon. *Seminars in virology*, 418, pp.409–418. Available at: <http://www.sciencedirect.com/science/article/pii/S1044577397901451>.
- Spencer, Jiang & Chen, 1999. Signal-induced ubiquitination of I κ B α by the F-box protein Slimb / Beta-TrCP. *Genes & Development*, 13(Ci), pp.284–294.
- Stack & Bowie, 2012. Poxviral protein A46 antagonizes Toll-like receptor 4 signaling by targeting BB loop motifs in Toll-IL-1 receptor adaptor proteins to disrupt receptor:adaptor interactions. *The Journal of biological chemistry*, 287(27), pp.22672–82. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/22593572> [Accessed March 11, 2013].
- Stack, Doyle, Connolly, Reinert, O’Keeffe, McLoughlin, Paludan & Bowie, 2014. TRAM Is Required for TLR2 Endosomal Signaling to Type I IFN Induction. *The Journal of Immunology*, 193(12), pp.6090–6102. Available at: <http://www.jimmunol.org/cgi/doi/10.4049/jimmunol.1401605>.
- Stack, Haga, Schröder, Bartlett, Maloney, Reading, Fitzgerald, Smith, et al., 2005. Vaccinia virus protein A46R targets multiple Toll-like-interleukin-1 receptor adaptors and contributes to virulence. *The Journal of experimental medicine*, 201(6), pp.1007–18. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2213104&tool=pmcentrez&rendertype=abstract>.
- Strahle, Garcin & Kolakofsky, 2006. Sendai virus defective-interfering genomes and the activation of interferon-beta. *Virology*, 351(1), pp.101–111.
- Sumner, Maluquer de Motes, Veyer & Smith, 2014. Vaccinia virus inhibits NF- κ B-dependent gene expression downstream of p65 translocation. *Journal of virology*, 88(6), pp.3092–102. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3957909&tool=pmcentrez&rendertype=abstract>.
- Sumner, Ren & Smith, 2013. Deletion of immunomodulator C6 from vaccinia virus strain Western Reserve enhances virus immunogenicity and vaccine efficacy. *Journal of General Virology*, 94(PART 5), pp.1121–1126.
- Sun, Li, Chen, Chen, You, Zhou, Zhou, Zhai, et al., 2009. ERIS, an endoplasmic reticulum IFN stimulator, activates innate immune signaling through dimerization. *Proceedings of the National Academy of Sciences of the United States of America*, 106(21), pp.8653–8658.
- Sun, Wu, Du, Chen & Chen, 2013. Cyclic GMP-AMP synthase is a cytosolic DNA sensor that activates the type I interferon pathway. *Science*, 339(February), pp.786–91. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3863629&tool=pmcentrez&rendertype=abstract>.
- Sun, Xing, Chen, Zheng, Yang, Nichols, Clementz, Banach, et al., 2012. Coronavirus papain-like proteases negatively regulate antiviral innate immune response through disruption of STING-mediated signaling. *PLoS ONE*, 7(2).
- Sutherland, Ranasinghe, Regner, Phipps, Mattheaei, Day & Ramshaw, 2011. Evaluating vaccinia virus cytokine co-expression in TLR GKO mice. *Immunol Cell Biol*, 89(6), pp.706–715. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/21173782>.
- Szajner, Weisberg & Moss, 2004a. Evidence for an essential catalytic role of the F10 protein kinase in vaccinia virus morphogenesis. *Journal of virology*, 78(1), pp.257–265.
- Szajner, Weisberg & Moss, 2004b. Physical and functional interactions between vaccinia virus F10 protein kinase and virion assembly proteins A30 and G7. *Journal of virology*, 78(1), pp.266–274.
- Takaesu, Surabhi, Park, Ninomiya-Tsuji, Matsumoto & Gaynor, 2003. TAK1 is critical for I κ B kinase-mediated activation of the NF- κ B pathway. *Journal of Molecular Biology*, 326(1), pp.105–115.
- Takeuchi, Kawai, Mühlradt, Morr, Radolf, Zychlinsky, Takeda & Akira, 2001. Discrimination of bacterial lipoproteins by Toll-like receptor 6. *International immunology*, 13(7), pp.933–940.
- Takeuchi, Sato, Horiuchi, Hoshino, Takeda, Dong, Modlin & Akira, 2002. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. *Journal of immunology (Baltimore, Md. : 1950)*, 169(1), pp.10–14.

References

- Tameris, Geldenhuys, Luabeya, Smit, Hughes, Vermaak, Hanekom, Hatherill, et al., 2014. The candidate TB vaccine, MVA85A, induces highly durable Th1 responses. *PLoS ONE*, 9(2).
- Tanaka & Chen, 2012. STING specifies IRF3 phosphorylation by TBK1 in the cytosolic DNA signaling pathway. *Science Signalling*, 5(214), pp.997–1003.
- Tanji, Ohto, Shibata, Miyake & Shimizu, 2013. Structural Reorganization of the Toll-Like Receptor 8 Dimer Induced by Agonistic Ligands. *Science*, 339(6126), pp.1426–1429. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23520111>.
- Tartaglia, Perkus, Taylor, Norton, Audonnet, Cox, Davis, Van Der Hoeven, et al., 1992. NYVAC: A highly attenuated strain of vaccinia virus. *Virology*, 188(1), pp.217–232.
- Tatematsu, Ishii, Oshiumi, Horiuchi, Inagaki, Seya & Matsumoto, 2010. A molecular mechanism for Toll-IL-1 receptor domain-containing adaptor molecule-1-mediated IRF-3 activation. *Journal of Biological Chemistry*, 285(26), pp.20128–20136.
- Taub, Ershler, Janowski, Artz, Key, McKelvey, Muller, Moss, et al., 2008. Immunity from Smallpox Vaccine Persists for Decades: A Longitudinal Study. *American Journal of Medicine*, 121(12), pp.1058–1064. Available at: <http://dx.doi.org/10.1016/j.amjmed.2008.08.019>.
- Terajima, Urban & Leporati, 2013. The N-terminus of vaccinia virus host range protein C7L is essential for function. *Virus genes*, 46(1), pp.20–7. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3546155&tool=pmcentrez&rendertype=abstract> [Accessed December 8, 2014].
- Thoms, Dunlop & Stark, 2007. p38-mediated inactivation of cyclin D1/cyclin-dependent kinase 4 stimulates nucleolar translocation of RelA and apoptosis in colorectal cancer cells. *Cancer Research*, 67(4), pp.1660–1669.
- Tojima, Fujimoto, Delhase, Chen, Hatakeyama, Nakayama, Kaneko, Nimura, et al., 2000. NAK is an IkappaB kinase-activating kinase. *Nature*, 404(6779), pp.778–782.
- Torchy, Hamiche & Klaholz, 2015. Structure and function insights into the NuRD chromatin remodeling complex. *Cellular and Molecular Life Sciences*, 72(13), pp.2491–2507. Available at: ["http://dx.doi.org/10.1007/s00018-015-1880-8"](http://dx.doi.org/10.1007/s00018-015-1880-8).
- Tournier, Dong, Turner, Jones, Flavell & Davis, 2001. MKK7 is an essential component of the JNK signal transduction pathway activated by proinflammatory cytokines. *Genes & Development*, 15(11), pp.1419–1426.
- Traenckner, Pahl, Henkel, Schmidt, Wilk & Baeuerle, 1995. Phosphorylation of human IKB-ox on serines 32 and 36 controls IKB-a proteolysis and NF-KB activation in response to diverse stimuli. *EMBO Journal*, 14(12), pp.2876–2883.
- Traktman, Anderson & Rempel, 1989. Vaccinia virus encodes an essential gene with strong homology to protein kinases. *Journal of Biological Chemistry*, 264(36), pp.21458–21461.
- Traktman, Caligiuri, Jesty, Liu & Sankar, 1995. Temperature-sensitive mutants with lesions in the vaccinia virus F10 kinase undergo arrest at the earliest stage of virion morphogenesis. *Journal of Virology*, 69(0022-538X SB - M SB - X), pp.6581–6587.
- Trinkle-Mulcahy, Boulon, Lam, Urcia, Boisvert, Vandermoere, Morrice, Swift, et al., 2008. Identifying specific protein interaction partners using quantitative mass spectrometry and bead proteomes. *The Journal of cell biology*, 183(2), pp.223–39. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2568020&tool=pmcentrez&rendertype=abstract> [Accessed December 5, 2014].
- Tscharke, Reading & Smith, 2002. Dermal infection with vaccinia virus reveals roles for virus proteins not seen using other inoculation routes. *Journal of General Virology*, 83(8), pp.1977–1986.
- Turk, Senturk, Dereli & Yaman, 2014. A rare human-to-human transmission of orf. *Int J Dermatol.*, 53(1), pp.1365–4632.
- Turvey & Broide, 2010. Innate immunity. *Journal of Allergy and Clinical Immunology*, 125(2 SUPPL. 2), pp.S24–S32. Available at: <http://dx.doi.org/10.1016/j.jaci.2009.07.016>.
- Ubersax, Woodbury, Quang, Paraz, Blethrow, Shah, Shokat & Morgan, 2003. Targets of the cyclin-dependent kinase Cdk1. *Nature*, 425(6960), pp.859–864.
- Unterholzner, Keating, Baran, Horan, Jensen, Sharma, Sirois, Jin, et al., 2010. IFI16 is an innate immune sensor for intracellular DNA. *Nature immunology*, 11(11), pp.997–1004. Available at: <http://dx.doi.org/10.1038/ni.1932>.
- Unterholzner, Sumner, Baran, Ren, Mansur, Bourke, Randow, Smith, et al., 2011. Vaccinia virus protein C6 is a virulence factor that binds TBK-1 adaptor proteins and inhibits activation of IRF3 and IRF7. *PLoS Pathogens*, 7(9), pp.1–15. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3169548&tool=pmcentrez&rendertype=abstract>

- abstract [Accessed November 7, 2013].
- Upton, Slack, Hunter, Ehlers & Roper, 2003. Poxvirus orthologous clusters: toward defining the minimum essential poxvirus genome. *Journal of virology*, 77(13), pp.7590–7600.
- Uzé, Lutfalla & Gresser, 1990. Genetic transfer of a functional human interferon alpha receptor into mouse cells: cloning and expression of its cDNA. *Cell*, 60(2), pp.225–234.
- Valbuena, López-Sánchez, Vega, Sevilla, Sanz-García, Blanco & Lazo, 2007. Identification of a dominant epitope in human vaccinia-related kinase 1 (VRK1) and detection of different intracellular subpopulations. *Archives of Biochemistry and Biophysics*, 465(1), pp.219–226.
- Valbuena, Sanz-García, López-Sánchez, Vega & Lazo, 2011. Roles of VRK1 as a new player in the control of biological processes required for cell division. *Cellular Signalling*, 23(8), pp.1267–1272. Available at: <http://dx.doi.org/10.1016/j.cellsig.2011.04.002>.
- Valbuena, Vega, Blanco & Lazo, 2006. P53 Downregulates Its Activating Vaccinia-Related Kinase 1, Forming a New Autoregulatory Loop. *Molecular and cellular biology*, 26(13), pp.4782–93. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1489172&tool=pmcentrez&rendertype=abstract>.
- Valderrama, Cordeiro, Schleich, Frischknecht & Way, 2006. Vaccinia Virus-Induced Cell Motility Requires F11L-Mediated Inhibition of RhoA Signaling. *Science*, 311(January), pp.377–381.
- Valentine & Smith, 2010. Inhibition of the RNA polymerase III-mediated dsDNA-sensing pathway of innate immunity by vaccinia virus protein E3. *Journal of General Virology*, 91(9), pp.2221–2229.
- Vanderplasschen, Mathew, Hollinshead, Sim & Smith, 1998. Extracellular enveloped vaccinia virus is resistant to complement because of incorporation of host complement control proteins into its envelope. *Proceedings of the National Academy of Sciences of the United States of America*, 95(June), pp.7544–7549.
- Vega, Sevilla & Lazo, 2004. p53 Stabilization and accumulation induced by human vaccinia-related kinase 1. *Molecular and cellular biology*, 24(23), pp.10366–10380.
- Verardi, Titong & Hagen, 2012. A vaccinia virus renaissance: new vaccine and immunotherapeutic uses after smallpox eradication. *Human vaccines & immunotherapeutics*, 8(7), pp.961–970.
- Vermeulen, De Wilde, Van Damme, Vanden Berghe & Haegeman, 2003. Transcriptional activation of the NF-kappaB p65 subunit by mitogen- and stress-activated protein kinase-1 (MSK1). *EMBO Journal*, 22(6), pp.1313–1324.
- Veyer, Maluquer de Motes, Sumner, Ludwig, Johnson & Smith, 2014. Analysis of the anti-apoptotic activity of four vaccinia virus proteins demonstrates that B13 is the most potent inhibitor in isolation and during viral infection. *The Journal of general virology*, 95(Pt 12), pp.2757–68. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/25090990> [Accessed November 28, 2014].
- Wali & Strayer, 1999. Infection with vaccinia virus alters regulation of cell cycle progression. *DNA and cell biology*, 18(11), pp.837–843.
- Wang, Deng, Hong, Akkaraju, Inoue & Chen, 2001. TAK1 is a ubiquitin-dependent kinase of MKK and IKK. *Nature*, 412(6844), pp.346–351.
- Wang, Ludwig, Schuberth, Goldeck, Schlee, Li, Juranek, Sheng, et al., 2010. Structural and functional insights into 5'-ppp RNA pattern recognition by the innate immune receptor RIG-I. *Nature structural & molecular biology*, 17(7), pp.781–7. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3744876&tool=pmcentrez&rendertype=abstract>.
- Wang & Shuman, 1995. Vaccinia virus morphogenesis is blocked by temperature-sensitive mutations in the F10 gene, which encodes protein kinase 2. *Journal of Virology*, 69(0022-538X SB - M SB - X), pp.6376–6388.
- Wang, Zhang, Li, Liu, Mei & Wang, 2010. Structural insights into the assembly and activation of IL-1 β with its receptors. *Nature immunology*, 11(10), pp.905–911.
- Waters, Pober & Bradley, 2013. Tumour necrosis factor in infectious disease. *The Journal of pathology*, 230(2), pp.132–47. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/23460469>.
- Werman, Werman-Venkert, White, Lee, Werman, Krelin, Voronov, Dinarello, et al., 2004. The precursor form of IL-1 α is an intracrine proinflammatory activator of transcription. *Proceedings of the National Academy of Sciences of the United States of America*, 101(8), pp.2434–2439.
- Wesche, Korherr, Kracht, Falk, Resch & Martin, 1997. The Interleukin-1 Receptor Accessory Protein (IL-1RAcP) Is Essential for IL-1-induced Activation of Interleukin-1 Stress-activated Protein Kinases (SAP. *The Journal of biological chemistry*, 272(12), pp.7727–7731.
- Wickramasekera & Traktman, 2010. Structure/Function analysis of the vaccinia virus F18 phosphoprotein, an abundant core component required for virion maturation and infectivity. *Journal of virology*, 84(13),

References

- pp.6846–60. Available at:
<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2903294&tool=pmcentrez&rendertype=abstract>.
- Wiebe, Nichols, Molitor, Lindgren & Traktman, 2010. Mice deficient in the serine/threonine protein kinase VRK1 are infertile due to a progressive loss of spermatogonia. *Biology of reproduction*, 82(1), pp.182–93. Available at:
<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2802121&tool=pmcentrez&rendertype=abstract>.
- Wiebe & Traktman, 2007. Poxviral B1 kinase overcomes barrier to autointegration factor, a host defense against virus replication. *Cell host & microbe*, 1(3), pp.187–97. Available at:
<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1978190&tool=pmcentrez&rendertype=abstract> [Accessed December 1, 2014].
- Willis, Langland & Shisler, 2011. Viral double-stranded RNAs from vaccinia virus early or intermediate gene transcripts possess PKR activating function, resulting in NF-kappaB activation, when the K1 protein is absent or mutated. *The Journal of biological chemistry*, 286(10), pp.7765–7778. Available at:
<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3048664&tool=pmcentrez&rendertype=abstract> [Accessed December 11, 2014].
- Witteck, Hanggi & Hiller, 1984. Mapping of a gene coding for a major late structural polypeptide on the vaccinia virus genome. *Journal of Virology*, 49(0022-538X SB - M SB - X), pp.371–378.
- Wood, 1958. The Role of Endogenous Pyrogen in the Genesis of Fever. *The Lancet*, 2(7037), pp.53–57.
- Wormbase.org, 2016. Wormbase.
http://www.wormbase.org/species/c_elegans/gene/WBGene00017895?from=http://www.wormbase.org/db/misc/biblio?name=WBGene00017895&category=&class=Gene&abstract=WBPaper00027981#WBPaper00027981. Available at:
http://www.wormbase.org/species/c_elegans/gene/WBGene00017895?from=http://www.wormbase.org/db/misc/biblio?name=WBGene00017895&category=&class=Gene&abstract=WBPaper00027981#WBPaper00027981.
- Wu, Li, Shao, Avey, Fu, Gillen, Hand, Ma, et al., 2015. Inhibition of cGAS DNA Sensing by a Herpesvirus Virion Protein. *Cell Host and Microbe*, 18(3), pp.333–344.
- Wu, Peisley, Richards, Yao, Zeng, Lin, Chu, Walz, et al., 2013. Structural basis for dsRNA recognition, filament formation, and antiviral signal activation by MDA5. *Cell*, 152(1-2), pp.276–289. Available at:
<http://dx.doi.org/10.1016/j.cell.2012.11.048>.
- Wu, Peisley, Tetrault, Li, Egelman, Magor, Walz, Penczek, et al., 2014. Molecular imprinting as a signal-activation mechanism of the viral RNA sensor RIG-I. *Molecular Cell*, 55(4), pp.511–523. Available at:
<http://dx.doi.org/10.1016/j.molcel.2014.06.010>.
- Wu, Sun, Chen, Du, Shi, Chen & Chen, 2013. Cyclic GMP-AMP Is an endogenous second messenger in innate immune signaling by cytosolic DNA. *Science (New York, N.Y.)*, 339(6121), pp.826–831. Available at:
<http://www.ncbi.nlm.nih.gov/pubmed/23258412>
<http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3855410&tool=pmcentrez&rendertype=abstract>.
- Xia, Sun, Chen, Pineda, Jiang, Adhikari, Zeng & Chen, 2009. Direct activation of protein kinases by unanchored polyubiquitin chains. *Nature*, 461(7260), pp.114–119. Available at:
<http://dx.doi.org/10.1038/nature08247>.
- Xu, Johnson, Liggitt & Bevan, 2004. Cellular and humoral immunity against vaccinia virus infection of mice. *Journal of immunology (Baltimore, Md. : 1950)*, 172(10), pp.6265–6271.
- Xu, Wang, Han, Li, Zhai & Shu, 2005. VISA is an adapter protein required for virus-triggered IFN-beta signaling. *Molecular Cell*, 19(6), pp.727–740.
- Yamamoto, Sato, Hemmi, Uematsu, Hoshino, Kaisho, Takeuchi, Takeda, et al., 2003. TRAM is specifically involved in the Toll-like receptor 4-mediated MyD88-independent signaling pathway. *Nature immunology*, 4(11), pp.1144–1150.
- Yamamoto, Sato, Mori, Hoshino, Takeuchi, Takeda & Akira, 2002. Cutting edge: a novel Toll/IL-1 receptor domain-containing adapter that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *Journal of immunology (Baltimore, Md. : 1950)*, 169(12), pp.6668–6672.
- Yamaoka, Courtois, Bessia, Whiteside, Weil, Agou, Kirk, Kay, et al., 1998. Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation. *Cell*, 93(7), pp.1231–1240.
- Yan, Hao, DeLucia, Swanson, Florens, Washburn, Ahn & Skowronski, 2015. CyclinA2-Cyclin-dependent Kinase Regulates SAMHD1 Protein Phosphohydrolase Domain. *The Journal of biological chemistry*, 290(21), pp.13279–92. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/25847232>.

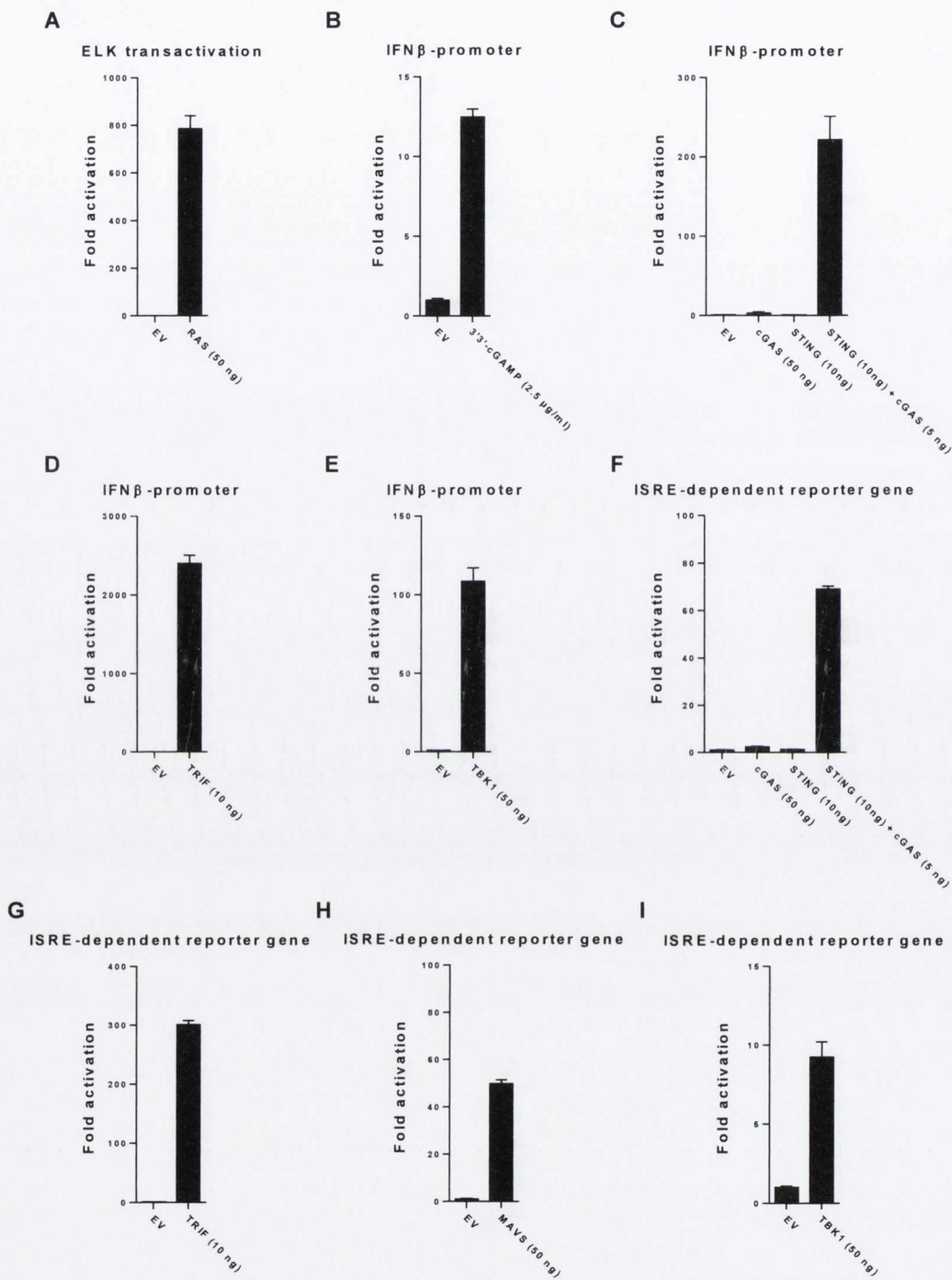
- Yan, Krishnan, Greenlund, Gupta, Lim, Schreiber, Schindler & Krolewski, 1996. Phosphorylated interferon- α receptor 1 subunit (IFN α R1) acts as a docking site for the latent form of the 113 kDa STAT2 protein. *The EMBO journal*, 15(5), pp.1064–1074.
- Yang, Cao, Martens, Porcella, Xie, Ma, Shen & Moss, 2015. Deciphering Poxvirus Gene Expression by RNA Sequencing and Ribosome Profiling. *Journal of Virology*, 89(13), pp.6874–6886. Available at: <http://jvi.asm.org/lookup/doi/10.1128/JVI.00528-15>.
- Yang, Fan, Wadzinski, Sakurai & Richmond, 2001. Protein phosphatase 2A interacts with and directly dephosphorylates RelA. *Journal of Allergy and Clinical Immunology*, 276(51), pp.47828–33.
- Yang, Tajkhorshid & Chen, 2010. Functional interplay between acetylation and methylation of the RelA subunit of NF- κ B. *Molecular and cellular biology*, 30(9), pp.2170–2180.
- Ye, Arron, Lamothe, Cirilli, Kobayashi, Shevde, Segal, Dzivenu, et al., 2002. Distinct molecular mechanism for initiating TRAF6 signalling. *Nature*, 418(6896), pp.443–447.
- Yeung, Hoberg, Ramsey, Keller, Jones, Frye & Mayo, 2004. Modulation of NF- κ B-dependent transcription and cell survival by the SIRT1 deacetylase. *The EMBO Journal*, 23(12), pp.2369–2380.
- Yin, Lamothe, Darnay & Wu, 2009. Structural basis for the lack of E2 interaction in the RING domain of TRAF2. *Biochemistry*, 48(44), pp.10558–10567.
- Yoneyama, Kikuchi, Natsukawa, Shinobu, Imaizumi, Miyagishi, Taira, Akira, et al., 2004. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nature immunology*, 5(7), pp.730–737.
- Yoneyama, Suhara, Fukuhara, Fukuda, Nishida & Fujita, 1998. Direct triggering of the type I interferon system by virus infection : activation of a transcription factor complex containing IRF-3 and CBP / p300. *EMBO Journal*, 17(4), pp.1087–1095.
- Yoon, Kurnasov, Natarajan, Hong, Gudkov, Osterman & Wilson, 2012. Structural Basis of TLR5-Flagellin Recognition and Signaling. *Science*, 335(6070), pp.859–864.
- Yu, Chang, Liang, Chiang, Lee, Liao & Lin, 2012. Dengue virus targets the adaptor protein MITA to subvert host innate immunity. *PLoS Pathogens*, 8(6).
- Yuen, Flesch, Hollett, Dobson, Russell, Fahrer & Tschärke, 2010. Analysis of A47, an immunoprevalent protein of vaccinia virus, leads to a reevaluation of the total antiviral CD8⁺ T cell response. *Journal of virology*, 84(19), pp.10220–9. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2937773&tool=pmcentrez&rendertype=abstract> [Accessed November 7, 2013].
- Zandi, Rothwarf, Delhase, Hayakawa & Karin, 1997. The I κ B kinase complex (IKK) contains two kinase subunits, IKK α and IKK β , necessary for I κ B phosphorylation and NF- κ B activation. *Cell*, 91(2), pp.243–252.
- Zarembek & Godowski, 2002. Tissue expression of human Toll-like receptors and differential regulation of Toll-like receptor mRNAs in leukocytes in response to microbes, their products, and cytokines. *Journal of immunology (Baltimore, Md. : 1950)*, 168(2), pp.554–561.
- Zeng, Sun, Jiang, Chen, Hou, Adhikari, Xu & Chen, 2010. Reconstitution of the RIG-I pathway reveals a signaling role of unanchored polyubiquitin chains in innate immunity. *Cell*, 141(2), pp.315–330. Available at: <http://dx.doi.org/10.1016/j.cell.2010.03.029>.
- Zhang, Clark, Lawrence, Pegg & Cohen, 2014. An unexpected twist to the activation of IKK β : TAK1 primes IKK β for activation by autophosphorylation. *The Biochemical journal*, 461(3), pp.531–7. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4206954&tool=pmcentrez&rendertype=abstract>.
- Zhang, Keshan, Liu, Kong, Shang & Liu, 2014. Human Infection with Orf Virus from Goats in China, 2012. *Vector-Borne and Zoonotic Diseases*, 14(5), pp.365–367. Available at: <http://online.liebertpub.com/doi/abs/10.1089/vbz.2013.1445>.
- Zhang & Moss, 1991. Vaccinia virus morphogenesis is interrupted when expression of the gene encoding an 11-kilodalton phosphorylated protein is prevented by the Escherichia coli lac repressor. *Journal of Virology*, 65(11), pp.6101–6110.
- Zhang, Niu, Li, Chu, Shen, Zhang & Li, 2015. Mechanistic insight into the functional transition of the enzyme guanylate kinase induced by a single mutation. *Scientific reports*, 5, p.8405. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/25672880>.
- Zhang, Park, Li, Byun, Jeon, Lee, Hong, Kim, et al., 2013. PHF20 regulates NF- κ B signalling by disrupting recruitment of PP2A to p65. *Nature communications*, 4(May), p.2062. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3942884&tool=pmcentrez&rendertype=abstract>.
- Zhang, Xu, Shi, Wu, Zhang, Sun, Chen & Chen, 2013. Cyclic GMP-AMP containing mixed Phosphodiester

References

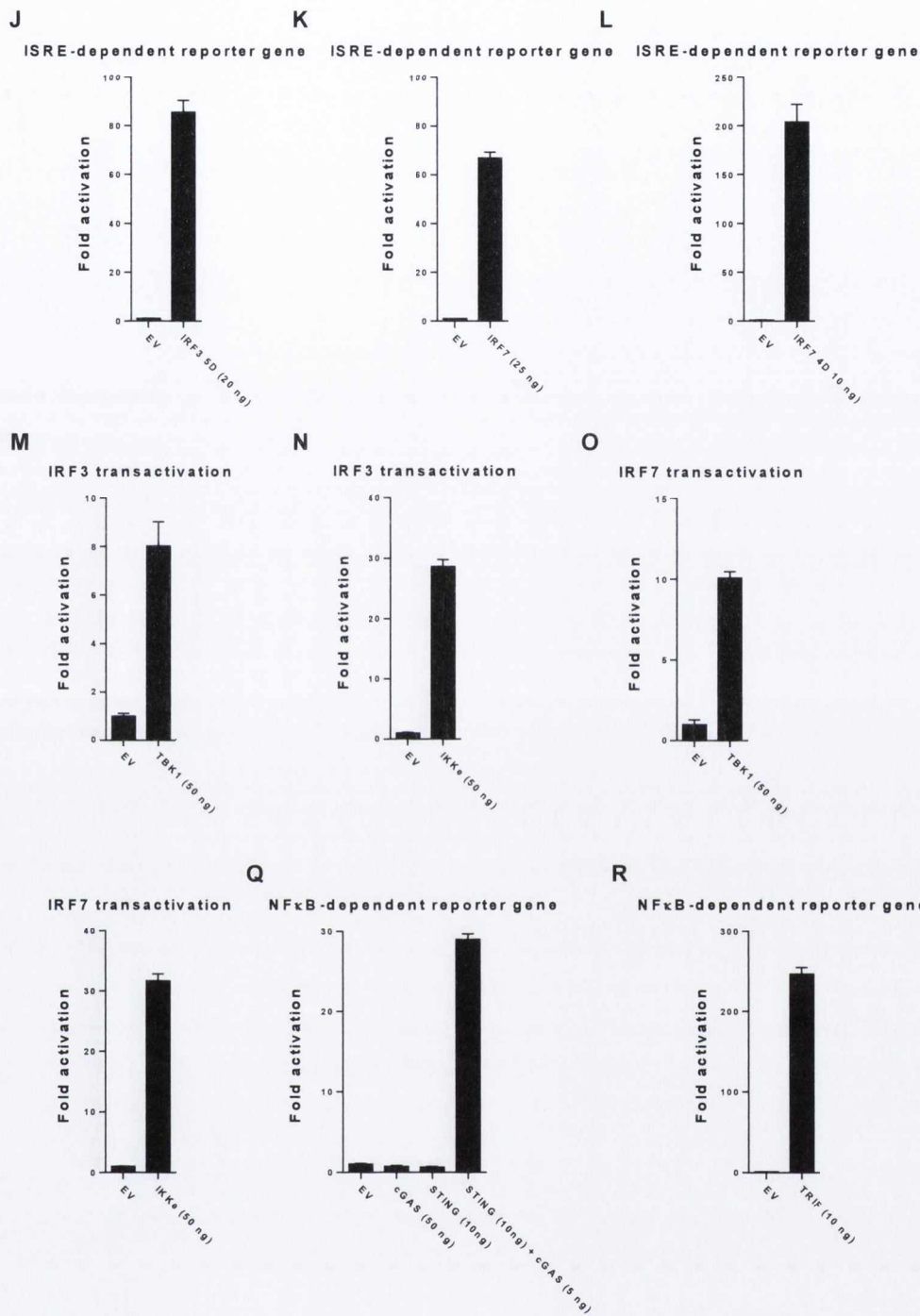
- linkages is an endogenous high-affinity ligand for STING. *Molecular Cell*, 51(2), pp.226–235. Available at: <http://dx.doi.org/10.1016/j.molcel.2013.05.022>.
- Zhang, Wu, Du, Xu, Sun, Chen, Brautigam, Zhang, et al., 2014. The cytosolic DNA sensor cGAS forms an oligomeric complex with DNA and undergoes switch-like conformational changes in the activation loop. *Cell Reports*, 6(3), pp.421–430. Available at: <http://dx.doi.org/10.1016/j.celrep.2014.01.003>.
- Zhang, Yuan, Bao, Lu, Kim & Liu, 2011. The helicase DDX41 senses intracellular DNA mediated by the adaptor STING in dendritic cells. *Nature Immunology*, 12(10), pp.196–196.
- Zhao, De Trez, Flynn, Ware, Croft & Salek-Ardakani, 2009. The adaptor molecule MyD88 directly promotes CD8 T cell responses to vaccinia virus. *Journal of immunology (Baltimore, Md. : 1950)*, 182, pp.6278–6286.
- Zhong, Voll & Ghosh, 1998. Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. *Molecular cell*, 1(5), pp.661–671.
- Zhong, Yang, Li, Wang, Li, Diao, Lei, He, et al., 2008. The Adaptor Protein MITA Links Virus-Sensing Receptors to IRF3 Transcription Factor Activation. *Immunity*, 29(4), pp.538–550. Available at: <http://dx.doi.org/10.1016/j.immuni.2008.09.003>.
- Zhu, Martinez, Huang & Yang, 2007. Innate immunity against vaccinia virus is mediated by TLR2 and requires TLR-independent production of IFN- β . *Blood*, 109(2), pp.619–625.
- Zhu, Yu, Shi, Nelson & Luo, 2009. CSR1 induces cell death through inactivation of CPSF3. *Oncogene*, 28(1), pp.41–51. Available at: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2918272&tool=pmcentrez&rendertype=abstract> [Accessed December 17, 2014].
- Züst, Cervantes-Barragan, Habjan, Maier, Neuman, Ziebuhr, Szretter, Baker, et al., 2011. Ribose 2'-O-methylation provides a molecular signature for the distinction of self and non-self mRNA dependent on the RNA sensor Mda5. *Nat Immunol*, 12(2), pp.137–143. Available at: <http://www.ncbi.nlm.nih.gov/pubmed/21217758>.

Appendix

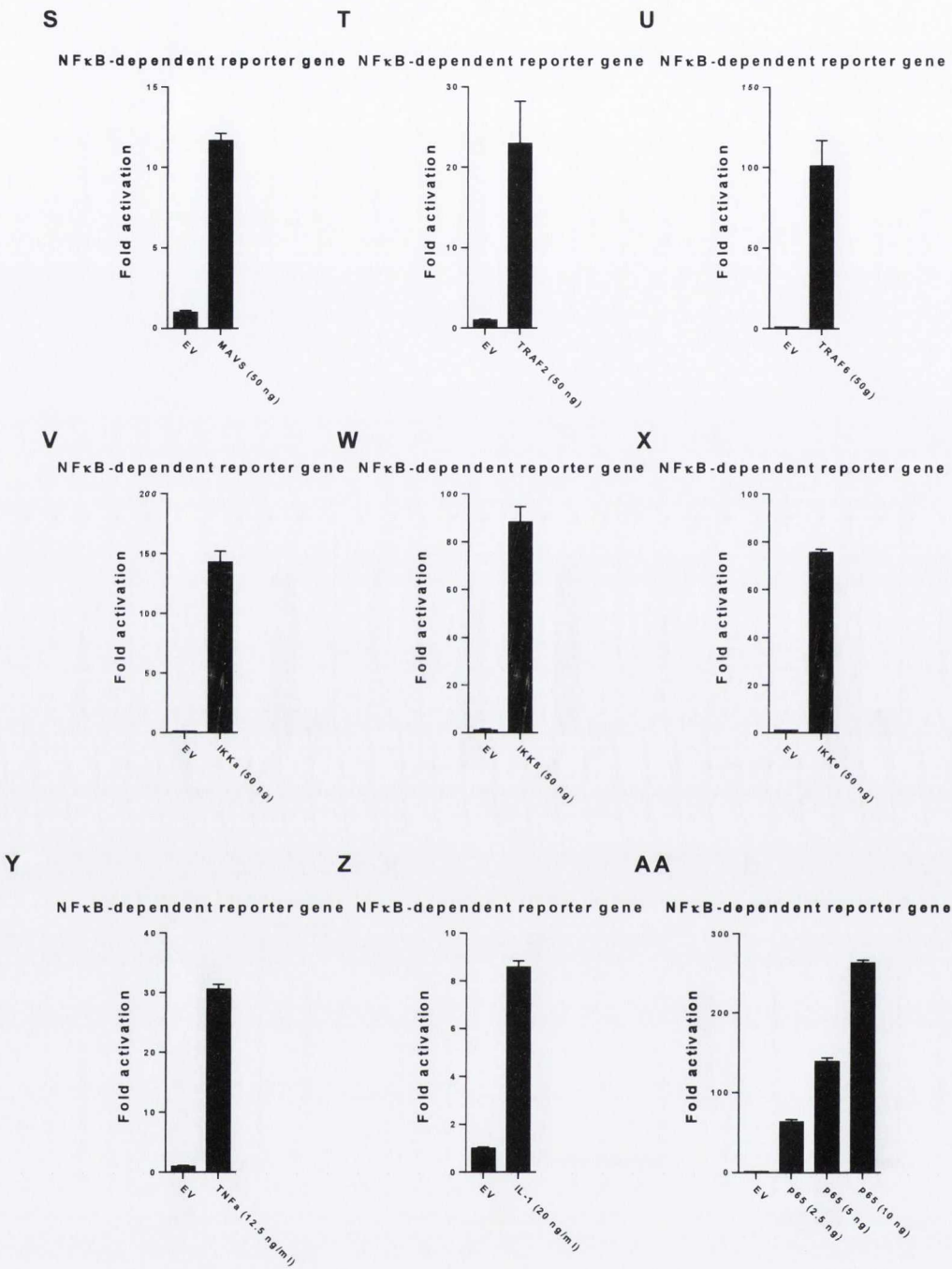
Activation levels of reporter gene assays



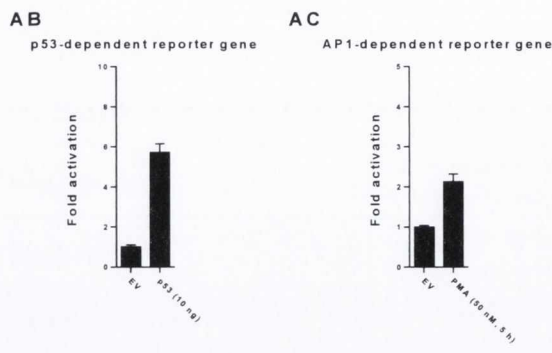
Appendix 1 Reporter gene assay activation levels (see page LIX for legend)



Appendix 1 Reporter gene assay activation levels (see page LIX for legend)



Appendix 1 Reporter gene assay activation levels (see next page for legend)



Appendix 1 Reporter gene assay activation levels

Individual experiments representative of average activation levels for reporter gene assays performed in (A, C-AC) HEK 293T cells or (B) HEK 293T cells stably expressing STING are shown. Cells were co-transfected with renilla luciferase control, empty vector control (EV) and a firefly luciferase reporter plasmid under the control of (B-E) an IFN β -promoter, (F-L) ISRE-elements, (Q-AA) NF κ B-elements, (AB) p53-elements, or (AC) an AP1-promoter. (A, M-P) Cells were transfected with renilla luciferase control, EV, a pFA-firefly luciferase reporter plasmid under the control of a Gal4-promoter, and (A) an elk-Gal4 fusion protein, (M,N) IRF3-gal4 fusion protein, or (O,P) an IRF7-gal4 fusion protein. The firefly luciferase reporter plasmids were activated by overexpression of indicated components of signalling pathways or stimulation with 3'3'-cGAMP, TNF α or IL-1 for 24 h. Cells were harvested 24 hpt or 24 h post-stimulation, firefly luciferase activity was measured and normalised to renilla luciferase activity. Assay values were scaled relative to EV, which was set to 1. Means of single experiments in triplicate determination are shown with their SDs.

List of suppliers

Appendix

Name	Address
5 Prime GmbH	Max-Volmer-Str. 8, 40724 Hilden, Germany
BD Biosciences	Edmund Halley Road, Oxford Science Park, OX4 4DQ, Oxford, United Kingdom
BioLegend	4B Highgate Business Centre, 33 Greenwood Place, London, NW5 1LB, United Kingdom
Bio-Rad	Fannin Ltd, Fannin House, South County Business Park, Leopardstown, Dublin 18, Ireland
Biosciences Ltd.	3 Charlemont terrace, Crofton Road, Dun Laoghaire, Co. Dublin, Ireland
BIOSYNTH AG	Rietlistr. 4, 9422 Staad, Switzerland
Biotium, Inc.	3159 Corporate Place, Hayward, CA 94545, 510-265-1027, USA
Brennan and Company	61 Birch Avenue, Stillorgan Industrial Park Stillorgan, Co Dublin, Ireland
Calbiochem	via Merck Millipore
Cell Signaling Technology Europe, B.V.	Schuttersveld 2, 2316 ZA Leiden, The Netherlands
Cruinn Diagnostics Ltd.	5b/6b Hume Centre, Parkwest Industrial Estate, Nangor Road, Dublin12, Ireland
Eurofins Genomics	Anzinger Str. 7a, 85560 Ebersberg, Germany
Fisher Scientific UK Ltd.	Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG, United Kingdom
GE Healthcare	3050 Lake Drive, Citywest Business Campus, Dublin 24, Ireland
Integrated DNA Technologies, BVBA	Interleuvenlaan 12A, 3001 Leuven, Belgium
Invitrogen	via Biosciences or Fisher Scientific
Invivogen	5, rue Jean Rodier, 31400 Toulouse, France
Labtech International Ltd	2 Birch House, Brambleside, Bellbrook Ind. Est., Uckfield, East Sussex, TN22 1QQ, United Kingdom
LGC Standards	Queens Road, Teddington, Middlesex, TW11 0LY, United Kingdom
LI-COR Biotechnology - UK Ltd	St. John's Innovation Centre, Cowley Road, Cambridge, CB4 0WS, United Kingdom
Merck Millipore	Millipore Ireland B.V., Tullagreen, Carrigtwohill, County Cork, Ireland
MyBio Ltd.	Kilkenny Research and Innovation Centre, St. Kierans College, Kilkenny, Ireland
National Diagnostics	via Biosciences
New England Biolabs	240 County Road, Ipswich, MA 01938-2723 978-927-5054, USA
PBL Assay Science	131 Ethel Road West, Suite 6, Piscataway NJ 08854, USA
PeproTech EC Ltd.	PeproTech House, 29 Margravine Road, London W6 8LL, United Kingdom
Promega Corporation	2800 Woods Hollow Road, Madison, WI 53711 USA
QIAGEN Ltd.	Skelton House, Lloyd Street North, Crawley, ManchesterUK, United Kingdom
Name	Address
R&D Systems	19 Barton Lane, Abingdon Science Park, Abingdon, OX14 3NB, United Kingdom

Appendix

Name	Address
Roche Diagnostics Ltd.	Charles Avenue, Burgess Hill, West Sussex, RH15 9RY, United Kingdom
Santa Cruz Biotechnology	European Support Office, Bergheimer Strasse 89-2, 69115 Heidelberg, Germany
Sigma-Aldrich Company Ltd	Fancy Road, Poole, Dorset, BH12 4QH, United Kingdom
Source BioScience LifeSciences	1 Orchard Place, Nottingham Business Park, Nottingham, NG8 6PX, United Kingdom
Stratagene/Agilent Technologies Ireland Ltd.	Unit 3 Euro House, Euro Business Park, Little Island, Cork, Ireland
Takara Bio Europe/Clontech	2 Avenue du Président Kennedy, 78100 Saint-Germain-en-Laye, France
Thermo Fisher Scientific	81 Wyman Street, Waltham, MA 02451, USA
VWR International Ltd.	Hunter Boulevard, Magna Park, Lutterworth, Leicestershire, LE17 4XN, United Kingdom