Repair of the Vaccinia virus genes B2R and B3R inhibits interferon production upon modified Vaccinia virus Ankara infection

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Inaugural-Dissertation zur Erlangung der Doktorwürde (Dr. rer. biol. vet.)

der Tierärztlichen Fakultät der Ludwig-Maximilians-Universität München

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München 2022

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Lehrstuhl für Virologie

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Gedruckt mit Genehmigung der Tierärztlichen Fakultät

der Ludwig-Maximilians-Universität München

Dekan:	Univ.-Prof. Dr. Reinhard K. Straubinger, Ph.D.
Berichterstatter:	Univ.-Prof. Dr. Dr. h.c. Gerd Sutter
Korreferent:	Univ.-Prof. Dr. Bernd Kaspers

Tag der Promotion: 30. Juli 2022

My Love

For my parents.

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LIST OF ABBREVIATIONS

2’-5’OAS	2’-5’ oligoadenylate synthase
68k-ank	68-kDa Ankyrin-like
AIM2	Absent in melanoma 2
ATCC	American Type Culture Collection
Bcl	B-cell lymphoma
BHK	Baby hamster kidney
BMDCs	Murine bone-marrow derived DCs
cDCs	Conventional DCs
CEF	Chicken embryo fibroblasts
cGAMP	cGAS cyclic GMP-AMP synthase
ChPV	Chordopoxvirinae
CMLV	Camelpox Virus
CPB	Cardiopulmonary bypass
CPE	Cytopathic effect
CPXV	Cowpox virus
CVA	Chorioallantois Vaccinia virus Ankara
DCs	Dendritic cells
DDX3	DEAD-box RNA helicase 3
ECTV	Ectromelia Virus

eIF2α	Eukaryotic translational initiation factor 2 α
eIF2α-p	Phosphorylation eIF2α
EPV	Entomopoxvirinae
ERK2	Extracellular signal-regulated kinase 2
FBS	Fetal bovine serum
FCS	Fetal calf serum
Flt3L	Fms-like tyrosine kinase-3 ligand
Flu-A	Influenza A virus
GFP	Green fluorescent protein
GM-CSF	Granulocyte/macrophage colony stimulating factor
Gt	Goldenticket
HDACs	Histone deacetylases
HIV	Human immune-deficiency virus
HSPV	Horsepox virus
IFI16	Interferon gamma inducible protein 16
IFN	Interferon
IFNAR	Interferon-α/β receptor
IFN-I	Type I IFN
IFN-β	IFN-beta
IFN-γ	IFN-gamma

IL-18	Interleukin-18
IL-18R	IL-18 receptor
IL-1β	Interleukin-1β
IL-1βR	IL-1β receptor
IL-2	Interleukin-2
IMVs	Immature virions
IRF-1	IFN regulatory factor 1
IRF-3	IFN regulatory factor 3
IRK	Interleukin 1 receptor associated kinase
ISGs	IFN stimulate genes
ISRE	IFN-sensitive response element
IκBα	Inhibitor of NF-κB
JAK	Janus kinase (JAK)
STAT	signal transducer of activators of transcription
LGP2	Laboratory of genetics and physiology 2
MAL	MyD88 adapter-like
MAVS	Mitochondrial antiviral-signaling
MDA5	Melanoma differentiation associated gene 5
MERS	Middle East respiratory syndrome
MPXV	Monkeypox Virus

MVA	Modified Vaccinia virus Ankara
MVA-F6	MVA strain F6
MVs	Mature virions
MyD88	Myeloid differentiation primary response 88
NAP1	NAK-associated protein 1
NF-κB	Nuclear factor kappa light-chain enhancer of activated cells
OPXV	Orthopoxvirus
PAMPs	Pathogen-associated molecular patterns
pDCs	Plasmacytoid DCs
p-IkBa	Phosphorylated IkBa
PKR	Protein Kinase
PRANC	Pox protein repeats of Ankyrin C-terminal
PRR	Pattern recognition receptor
RIG-I	Retinoic acid-inducible gene I
RLRs	RIG-I like receptors
RNCV	Racconpox virus
SAMD9	Sterile Alpha Motif Domain Containing 9
SKPV	Skunkpox virus
Slfn	Schlafen

STING	Stimulator of interferon genes
TANK	TRAF family member-associated NF-kB activator
TBK1	TANK-binding kinase-1
TIR	Toll-interleukin receptor
TLRs	Toll-like receptors
TNF	Tumor necrosis factor
TRAFs	Tumor necrosis factor receptor associated factors
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain-containing adaptor protein-inducing interferon-β
VACV	Vaccinia virus
VARV	Variola Virus
VLTF 2	Vaccinia late transcription factor 2
VPXV	Volepox virus
v-Slfn	Viral schlafen
WR	Western Reserve
ZAP	Zinc Finger antiviral protein
β-TrCP	E3 ubiquitin ligase β-transducing repeat-containing protein

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I. INTRODUCTION

Vaccinia virus (VACV), the prototype member of the Orthopoxvirus genus of the Poxviridae, replicates in the cytoplasm of infected cells under the formation of large viral factories. This replication mode more readily produces so-called pathogen-associated molecular pattern (PAMP) that are recognized by pattern-recognition receptor (PRR), leading to the activation of innate immune responses including the production of type I interferons (IFN-I). To antagonize IFN-I production, VACV encodes a series of immunomodulators that can block PRR, cGAS/STING, and downstream factors including IFN regulatory factor 3 (IRF-3) and nuclear factor kappa light-chain enhancer of activated B cells (NF-κB).

Modified Vaccinia virus Ankara (MVA), a highly attenuated strain of VACV, is derived from Chorioallantois Vaccinia virus Ankara (CVA) following more than 516 serial culture passages in chicken embryo fibroblasts (CEF). Nowadays, MVA is widely used in experimental vaccine development targeting infectious pathogens as well as cancer. As unique strain of VACV, MVA also serves as an excellent tool virus in fundament research to study poxvirus biology, such as the role of VACV immunomodulatory genes by inactivation or reinsertion of viral target gene sequences, and by studying putative immune regulatory gene functions.

In contrast to wild-type orthopoxviruses, MVA strongly stimulates the production of IFN-I upon infection, an important feature contributing to the high immunogenicity of non-replicating MVA-based vaccines. IFN-I expression is dependent on the cGAS/STING signaling pathway, in which IRF-3 phosphorylation is one of the hallmarks to certify the activation of cGAS/STING. Conventional

VACV or ectromelia viruses (ECTV) can block the production of IFN-I leading to the inhibition of IRF-3 phosphorylation by interrupting cGAMP in cGAS/STING pathways with viral immune evasion proteins. In the genome of VACV strain Western Reserve (WR), these proteins are encoded by the open reading frames B2R and B3R.

In this study, a recombinant MVA was generated to express the VACV WR gene sequences B2R and B3R (MVA-WR B2R B3R) and used to characterize the role of these immunomodulatory gene sequences upon MVA infection of human THP-1 cells. Infections with the new recombinant MVA-WR B2R B3R could efficiently block the production of IFN-I and IRF-3 phosphorylation in THP-1 cells. Thus, the reinsertion of the VACV genes B2R and B3R into the MVA genome was sufficient to revert the characteristic MVA phenotype of IFN-I production in human cells.

II. LITERATURE REVIEW

1. Vaccinia virus (VACV) and Modified Vaccinia virus Ankara (MVA)

Vaccinia virus (VACV), an Orthopoxvirus in the poxvirus family, was widely used as a vaccine against smallpox disease. More recent evidence suggests that the origin of VACV is much closer to Horsepox virus (HSPV), rather than Cowpox virus (CPXV) (Tulman et al., 2006; Schrick et al., 2017). Modified Vaccinia virus Ankara (MVA), was derived from Chorioallantois Vaccinia virus Ankara (CVA) through more than 500 serial passages in chicken embryo fibroblasts (CEF), which severely restricted host range of MVA (Mayr et al., 1975; Bernard et al., 1996). Like other poxviruses, the ability of VACV to replicate in cell cytoplasm is associated with the formation of viral factories (Nina et al., 2001). However, the replication of MVA is restricted at the stages of the virion assembly in most mammalian cells (Sutter and Moss, 1992; Carroll and Moss, 1997; Drexler et al., 1998). With the capacity to incorporate large foreign gene sequences, MVA has been selected as the one of most promising vaccine vectors against infectious diseases due to its clinical safety and its ability to induce a high efficiency immune response (Verheust et al., 2012). In addition, MVA is also a useful tool to study poxvirus biology (Volz and Sutter, 2017).

1.1 Classification of Orthopoxvirus

Poxviruses are large, enveloped, dsDNA viruses that are grouped into two subfamilies: the *Entomopoxvirinae* (EPV) and *Chordopoxvirinae* (ChPV) (Skinner et al., 2012). EPV mainly infect insects, such as beetles, butterflies, moths, and others (Arif et al.,

2021). Compare to EPV, the ChPV has a much broader host range and is composed of 18 genera: *Avipoxvirus, Capripoxvirus, Centapoxvirus, Cervidpoxvirus, Crocodylipoxvirus, Leporipoxvirus, Macropopoxvirus, Molluscipoxvirus, Mustelpoxvirus, Orthopoxvirus, Oryzopoxvirus, Parapoxvirus, Pteropopoxvirus, Salmonpoxvirus, Sciuripoxvirus, Suipoxviruses, Vespertilionpoxvirus, Yatapoxvirus* (https://talk.ictvonline.org/taxonomy/). Among them, the *Orthopoxvirus* (OPXV) genus is the most important and best characterized poxvirus genus, as it contains representatives that pose a widespread and serious threat to human, domestic, and wildlife health (Silva et al., 2020).

OPXV genus comprises many species, including *Variola virus* (VARV), *Vaccinia virus* (VACV), *Cowpox virus* (CPXV), *Monkeypox virus* (MPXV), *Ectromelia virus* (ECTV) and *Camelpox virus* (CMLV), which are immunologically cross-reactive and cross-protective, so that infection with one member of this genus means the acquisition of protection against any other members (Pauli et al., 2010). This phenomenon is well illustrated by an example from over a hundred years ago, when CPXV-derived agents were used against smallpox disease in Europe (Sanchez-Sampedro et al., 2015). Likewise, immunization with VACV or Modified Vaccinia virus Ankara (MVA) can provide effective protection in a murine model of human smallpox (Walsh and Dolin, 2011; Volz et al., 2018). Another characteristic of OPXVs is that significant DNA sequence similarities are shared among different species. As shown by a phylogenetic study, the *Orthopoxvirus* genus is divided into two large clusters, with strains including VARV, VACV, MPXV, and others forming the Old-World cluster, and the *Racoonpox virus* (RNCV), *Skunkpox virus* (SKPV), and *Volepox virus* (VPXV) forming the New-World cluster, wherein each clade shares high sequence identities and close

ancestral origin (Franke et al., 2017) (Figure 1). Interestingly, OPXVs are descended from a single ancestor. For example, the CPXV isolates forming clusters with similarities to either VARV or VACV (Franke et al., 2017).

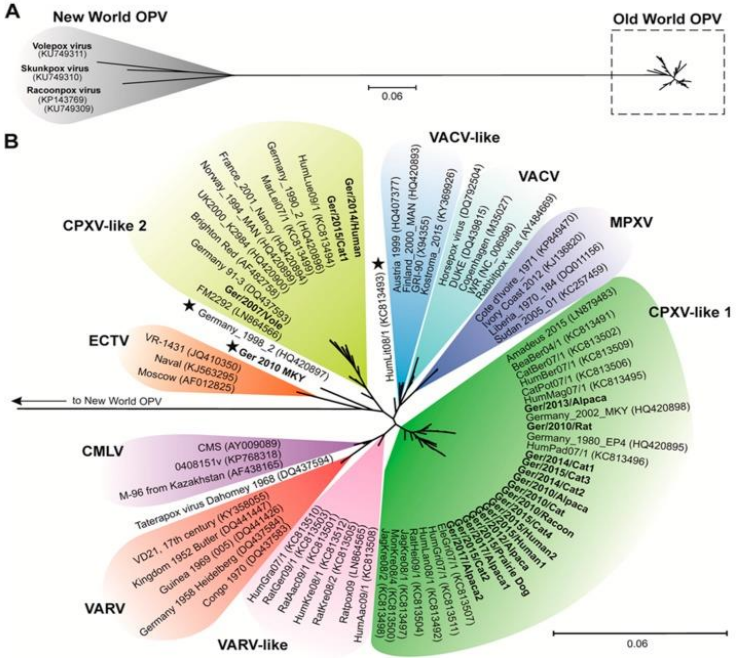

Figure 1 Phylogeny of Orthopoxviruses (OPXVs) (Franke et al., 2017) .

(A) New-World (highlighted grey) and Old-World OPXVs (dotted box) were clearly separated by the unrooted phylogenetic tree. (B) Enlarged unrooted phylogenetic tree of Old-World OPXVs defined the species ectromelia virus (ECTV), monkeypox virus (MPXV), vaccinia virus (VACV), and variola virus (VARV) as monophyletic clades (Franke et al., 2017)

Despite the eradication of smallpox, announced by the WHO in 1980, the emergence or re-emergence of orthopoxviruses in human and animal populations could still impact animal and public health (Silva et al., 2020). MPXV, CPXV, VACV-like viruses circulate in wild and domestic animals in different regions of the world, including Europe, the Middle East, Asia, South America and Africa (Singh et al., 2012). In addition, smallpox was used as a weapon in history, and the threat of poxviruses (especially OPXVs) is still existent (Singh et al., 2012). Therefore, it is necessary to keep precautions in place and remain vigilant regarding potentially emerging pathogenic poxviruses.

1.2 The origin and development of VACV

Smallpox, caused by VARV of the OPXV genus of the *Poxviridae*, is one of the deadliest diseases that has affected mankind over the course of known history. The first strategy to fight smallpox was immunization with the VARV agent itself, known as variolation: immunologically naïve persons inoculated small amounts of pustular fluid/material collected from smallpox patients via cutaneous scratches (Meyer et al., 2020). However, about 2% of individuals who were inoculated in this manner developed smallpox disease after variolation (Eriksen, 2020). A much safer procedure of immunization was found in 1790s by Edward Jenner and contemporaries who successfully used the lesion material from cowpoxvirus infected individuals as the inoculation agent (Smith et al., 2018). Edward Jenner subsequently published this procedure, known as "Inquiry", which marked the beginning of the vaccination era (Baxby, 1999).

After Jenner's successful discovery, this new method of vaccination (derived from vacca, the Latin word for cow) became very prevalent, but limited supplies of vaccine were inadequate for the volume required for immunization (Riedel, 2005). Therefore, the vaccination of VACV derived from CPXV was developed in the 19th century (Riedel, 2005). On the other hand, VACV originating from horsepox (HSPV) was also used to fight smallpox (Meyer et al., 2020). In 1939, Downie reported the distinction of VACV derived from 20th century smallpox vaccine from CPXV (Downie, 1939). Some have reported that modern VACV vaccine strains are more likely HSPV instead of CPXV, which included: (1) John Glover Loy reported a horsepox role in the prevention of smallpox in 1801 (Esparza et al., 2017); (2) Genomic sequencing of a horsepox virus isolated in Mongolia in 1976 indicated that HSPV and VACV share a much closer relationship than other OPXVs (Tulman et al., 2006); (3) Schrick and co-workers indicated an early smallpox vaccine manufactured by Mulford in the United States in 1902 which was based on horsepox (Schrick et al., 2017). Due to the existence of immunological cross-reactivity between CPXV and HSPV, as well as other OPXVs (MPXV, CMPV, ECTV et al), vaccines derived from both viruses provide efficient protection against smallpox (Stern et al., 2016). In summary, although the origin of VACV is complex and it may be impossible to determine a sole ancestor, it can generally be stated that the late 18th VACV vaccines were inclined to derive from CPXV, but early 19th centuries had been gradually replaced with HSPV.

In the middle of 20th century, VACV based vaccine were developed to use in a worldwide campaign against smallpox. These vaccines were mainly including health agency or the country or region named first-generation vaccines, such as NYCBH (USA), Lister (UK,

Europe, Asia, Africa, USA), Tian Tan (China), EM-63 (India), Bern (Germany, Austria), Paris (France, Paris, Syria, Turkey), Copenhagen (Denmark), Dairen (Japan) et al (Sanchez-Sampedro et al., 2015). However, these first-generation smallpox vaccines were still accompanied by a series of adverse reactions such as fever, malaise, mild rash to eczema vaccinatum, post-vaccinial encephalitis, and other reactions (Poland, 2005).

A second generation of vaccines emerged, which had been developed and passaged on live animal cells. The prototypical strains were: Lister derived RIVM (rabbit kidney cells) and Lister/CEP (chicken embryos), NYCBH sourced ACAM2000 (Vero cells) and Western Reserve (WR) (mice and rabbit cells) (Verardi et al., 2012). The complications caused by second-generation vaccines remained. For example, ACAM2000 caused myocarditis in phase II and III clinical trials, although it was licensed in USA (Aysegul and Elizabeth, 2010). In addition, several first- and second-generation VACV strains were used to express different heterologous antigens as vaccine vectors against a wide range of diseases, such as HIV/AIDS, Influenza A and Hepatitis B (Verardi et al., 2012).

The third-generation of smallpox vaccines was engineered in later years by series of passages in cells derived from animal tissues, notably including Lister clone 16m8 (LC16m8), Dairen I strain (DIs), and Modified Vaccinia virus Ankara (MVA) (Jacobs et al., 2009). Compared to previous generations, the third-generation vaccines were much safer due to the loss of host range genes. For example, attenuated MVA can only replicate in primary and established chicken embryo fibroblasts, a quail cell line derived from QT6, and the Syrian hamster cell line BHK-21 (Carroll and Moss, 1997; Drexler et al., 1998). This generation can also be regarded as much

preferable vaccine vector candidates for delivering heterologous antigens to address various viral, bacterial and parasitic infectious diseases, when compared to first- and second-generation vaccines. The fourth-generation smallpox vaccines are generally divided into multiple types, DNA and protein subunit vaccines based on single or recombinant VACV matured virion (MV) specific genes (A27, H3L, L1R) or Enveloped virion (EV) specific genes (A33R, B5R) antigenic sequences, and T-cell epitopes vaccines, used in a DNA-prime, peptide-boost schedule, all of which have been verified to confer protection animal models, despite a lack of testing in clinical trials against smallpox (Verardi et al., 2012).

Another important role, for which VACV is being developed, is as a cancer immunotherapeutic vector and oncolytic virus to fight tumors. Insertion of tumor associated antigens such as MUC1, PSA, and CEA, immunomodulatory genes such as Interleukin(IL)-2, interferon(IFN)-β, and Granulocyte/macrophage colony stimulating factor (GM-CSF), and suicide genes such as cytosine deaminase, and purine nucleoside phosphorylase into VACV as a cancer immunotherapeutic vector are common approaches to effectively induce anti-tumor immunity (Verardi et al., 2012). Modified VACV can also be used as an oncolytic virus to lyse tumor cells. As Breitbach and Hao reported, a recombinant virus consisting of a thymidine kinase (TK) defective VACV (VACV TK-) engineered to express GM-CSF, named JX-594, can degrade the neoplastic cells and is presently undergoing phase II clinical trials against solid tumors (Hwang et al., 2011). Another example is GL-ONC1 (Genelux), a Renilla luciferase-green fluorescent protein (GFP) recombinant VACV Lister which lacks three genes F14.5L, TK, and A56R (VACVΔF14.5L, TK, and A56R) and forms a light-emitting oncolytic virus (Zhang et al., 2007). It has shown the ability to

reduce tumor-specific replication and solid tumor size in a mouse model (Zhang et al., 2007).

1.3 VACV member CVA as the progenitor of MVA

VACV strain Ankara, passaging in donkey-calf-donkey by Turkish vaccine institute, was used to fight against smallpox in Turkey (Mayr et al., 1975). Mayr and Herrlich initiate the passages of VACV Ankara on chorioallantoic membranes of embryonated chicken eggs in 1950s at Munich (Institute of Medical Microbiology, Infectious and Epidemic Diseases in Munich), which led to the new name Chorioallantois Vaccinia virus Ankara (CVA) (Mayr et al., 1975). After 382 further serial passages in CEF, CVA exhibited altered biological properties and displayed an attenuated phenotype similar to MVA, including a decrease of growth capacity in vitro and decreased virulence in vivo (Meyer et al., 1991). After 516 serial passages on primary CEF, this new CVA was named Modified Vaccinia virus Ankara (MVA), which is highly attenuated and no longer possesses the ability to replicate in most mammalian cell lines (Volz and Sutter, 2017).

Compared to wild-type CVA, MVA lacks 26.6 kb of DNA in its genome, including six major deletions sites, a number of small deletions and mutations, and some genes located outside of the six major deletions (Figure 2) (Meyer et al., 1991; Antoine et al., 1998). Among the six major deletions, deletions I-IV were lost in the initial 382 serial passages, while deletion V and deletion VI occurred in the next 190 passages (Meyer et al., 1991; Antoine et al., 1998). Due to these deletions, some defective or truncated genes result in the restriction of MVA's host range, such as C12L (encoding serine protease inhibitor 1, SPI-1), C16L, and K1L (Sutter et al., 1994;

Wyatt et al., 1998; Liu et al., 2019; Peng and Moss, 2020). Several deleted or truncated genes contribute to the lack of virulence and immune evasion of MVA, such as C2L, and N1L (Pires de Miranda et al., 2003; DiPerna et al., 2004). Along with many mutations resulting in gene loss from CVA to MVA, a new open reading frame, called MVA188R, was created by the fusion of a short remnant of the largest known OPXV ORF (CPXV-GRI B22R) (Meisinger-Henschel et al., 2007).

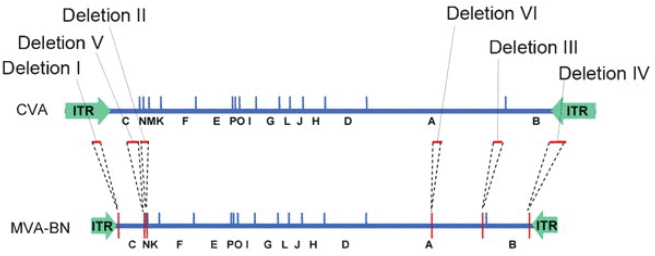

Figure 2 Schematic representation of genomic regions around the six major deletions of MVA (Meisinger-Henschel et al., 2007, with permission)

1.4 The life cycle of VACV and MVA

The infectious form of VACV consists of two types of virions: mature virions (MVs), which are encased by a single lipid membrane and enveloped virions (EVs), which have additional lipid envelopes from the cell's intermediate compartment membranes (Figure 3). MVs enter cells by fusion with the plasma membrane or endocytosis, while the entry of EVs is accomplished by disruption of the outer membrane followed by fusion of the viral inner membrane in the same manner as described for MVs (Moss, 2012). Upon successful entry of MVs or EVs, the virus core containing early transcription factors and DNA-dependent RNA-polymerase is released into the

cytoplasm and early gene expression starts immediately within 20 minutes (Schmidt et al., 2012). In the process of early gene expression, the genome is released into the cytoplasm, known as uncoating of the virion (Kilcher and Mercer, 2015). Subsequently, DNA replication occurs, followed by the transcription of intermediate and late genes within viral factories which are enveloped by the rough endoplasmic reticulum (Nina et al., 2001). Intermediate and late gene products are crucial for DNA packaging and virion morphogenesis, which also includes the process of assembling the structural proteins and early transcription factors into newly formed MVs (Liu et al., 2014a). Most VACV exit as MVs via cell lysis, but a portion of MVs is further enwrapped by two membranes derived from the trans-Golgi or endosomal membranes (Schmelz et al., 1993). These form EVs and leave the cell by microtubule transport or exocytosis (Schmelz et al., 1993).

MVA enters cells in the same manner as VACV, with IMV and EV followed by a cascade-like life cycle, resulting in the synthesis of viral genomic DNA and the expression of early, partial intermediate and partial late genes. However, the virus replication is blocked at the stage of virion assembly, which leads to immature virus particles failing to be released from infected cells (Volz and Sutter, 2017). The ability to efficiently produce all classes of viral proteins (early, intermediate, late) in mammalian-origin cells, combined with MVA's capacity to integrate foreign genes, makes it a unique VACV vector vaccine candidate. Recombinant MVA delivering antigens from various infectious agents, such as human immuno-deficiency virus (HIV), influenza A virus (Flu-A) and Middle East respiratory syndrome (MERS) coronavirus, can induce protective immunity in pre-clinical studies or clinical trials (Kreijtz et al., 2009; Gomez et al., 2011; Koch et al., 2020). The properties of MVA also makes it a

good tool to study host-virus interaction and poxvirus biology (Volz and Sutter, 2017).

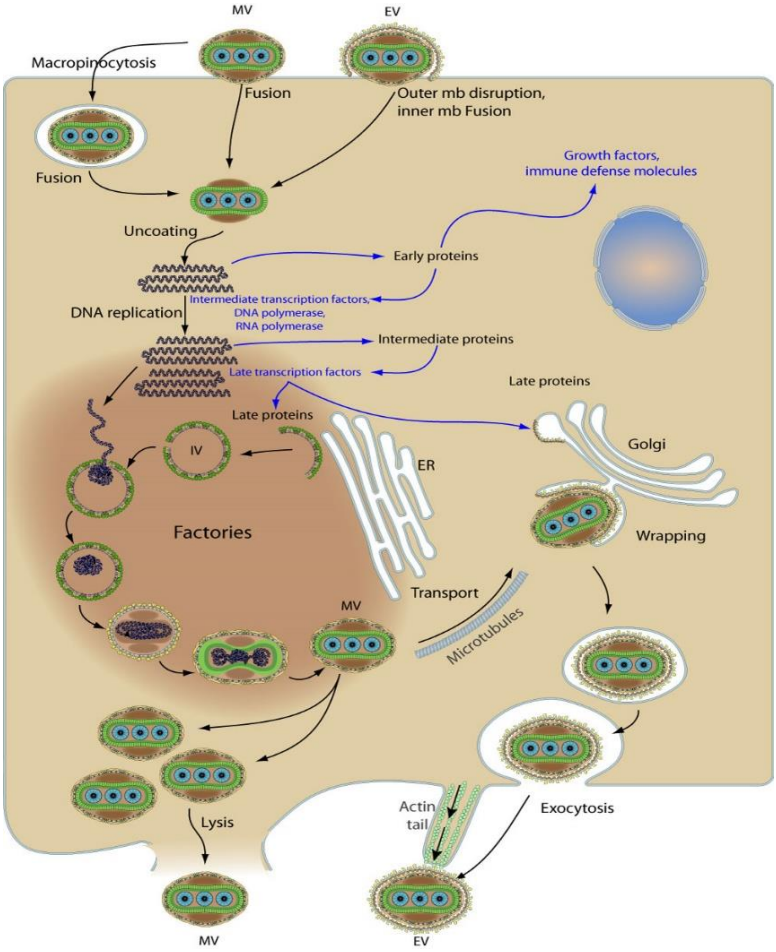

Figure 3 Replication cycle of poxviruses (Source: ViralZone; www.expasy.org/viralzone, SIB Swiss Institute of Bioinformatics; with permission)

2. VACV inhibits induction of type I IFNs

Type I IFNs (IFN-I) are hallmark cytokines in immune responses to viral infection. Upon infection by viruses, the pathogen-associated molecular patterns (PAMPs) will be recognized by the pattern recognition receptor (PRRs) of host cells, which can induce the activation of innate immunity at the early stages of infection (Suresh and Mosser, 2013). Once PRRs sense PAMPs, the downstream signaling pathways of IFN regulatory factor 3 (IRF-3) and nuclear factor kappa light-chain enhancer of activated B cells (NF-κB) will be activated and result in the induction of IFN-I (McNab et al., 2015). In turn, VACV utilizes a series of immune evasion proteins to antagonize the IFN-I production by targeting PRRs, second messenger-cyclic GMP-AMP (cGAMP), or IRF-3 and NF-κB downstream signaling pathways (El-Jesr et al., 2020). The ways by which VACV inhibits IFN-I induction are a major part of the overall immune evasion abilities of VACV.

2.1 IFN-I induction upon viral infection

IFN-I, consisting of IFNα, IFNβ, IFNε, IFNκ, and IFNω in most mammalian animals, is the first line of host defense against viral infection (Ali et al., 2019). IFNα and IFNβ are well defined IFN-I, in which IFNβ is produced by almost all types of cells, except for the predominant producers of IFNα by haematopoietic cells and particularly plasmacytoid dendritic cells (Ali et al., 2019). Once IFNα/β are successfully induced, the downstream janus kinase (JAK)–signal transducer of activators of transcription (STAT) (JAK-STAT) signaling pathways are activated by binding IFN-I receptors (IFNAR1/2) and mediate the expression of IFN stimulated genes (ISGs) including Protein Kinase (PKR), 2’-5’ oligoadenylate synthase

(2'-5'OAS), and ISG15 to join antiviral immunity (Majoros et al., 2017). In addition, IFNα/β also play a crucial role in the integration of innate immunity and adaptive immunity, which not only stimulates the maturation and migration of the lymphocytes, including macrophages, dendritic cells (DCs), and natural killer cells (NKs), but also improves the activity of antigen-specific T and B cells (Moretta et al., 2008; Ivashkiv and Donlin, 2014). Therefore, Knowing how IFN-I is induced and produced during infection is a critical step towards a better understanding of the interaction between viruses and their hosts.

The IFN-I signaling is initiated when PAMPs are sensed by host cell PRRs. Viruses produce several PAMPs, including surface glycoproteins, DNA, and RNA molecules, which are important parts of viral particles or viral replication (Mogensen, 2009). PRRs are employed by the host to recognize and sense virus PAMPs, including Toll-like receptors (TLRs), Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs), and cytoplasmic DNA receptors (Bowie and Unterholzner, 2008). TLRs are type I transmembrane proteins with an extracellular ligand-binding domain containing leucine-rich repeats that are responsible for recognition of PAMPs, a single-pass transmembrane domain, and an intracellular Toll-interleukin 1 (IL-1) receptor (TIR) for signalling transduction, which localize to the cell surface and/or reside within intracellular endosomes, multivesicular bodies, lysosomes, and endolysosomes (Goulopoulou et al., 2016). In humans, TLRs consist of 10 members: among these TLRs, one type is present on the cell surface, including TLR1, TLR2, TLR4, TLR5, TLR6, TLR10, and another type expressed in intracellular endosomal- or lysosomal-compartments and the endoplasmic reticulum (ER), such as TLR3, TLR4, TLR7, TLR8, and TLR9 (Yamamoto and Takeda, 2010). Upon TLRs sensed by virus

PAMPs, especially viral DNA and RNA, TIR-domain-containing adaptor proteins that include myeloid differentiation primary response 88 (MyD88), MyD88 adapter-like (MAL), TIR domain-containing adaptor protein-inducing interferon-β (TRIF), and TRIF-related adaptor molecule (TRAM) will be activated and transduce the signal to downstream IRF-3 or NF-κB, which leads to the production of IFN-I (Kawasaki and Kawai, 2014). RLRs belong to the RNA helicases family and comprise RIG-I, melanoma differentiation associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2),which can detect virus RNA in cytoplasm (Kawasaki and Kawai, 2014). When RLRs are activated, IFN-I will be induced by mitochondrial antiviral-signaling (MAVS)-tumor necrosis factor receptor associated factors (TRAFs)-IRF-3/NF-κB axis (Kawasaki and Kawai, 2014). In addition to TLRs, virus DNA also can be sensed by cytoplasmic DNA receptors and stimulate the production of IFN-I, such as cyclic GMP-AMP synthase (cGAS), interferon gamma inducible protein 16 (IFI16), interferon-inducible protein AIM2 and DDX41, and RNA Pol III (Lee et al., 2019). For example, cGAS, belonging to the nucleotidyltransferase family, is a cytosolic DNA sensor that can detect viral cytosolic dsDNA and synthesizes the second messenger cGAMP (Li et al., 2013). Upon binding of cGAMP, the endoplasmic reticulum transmembrane protein named with stimulator of interferon genes (STING) can be activated, causing it to translocate itself and produce an oligomerization of STING that can recruit TANK-binding kinase-1 (TBK1) which clusters upon itself for transautophosphorylation (Cai et al., 2014). Meanwhile, this structure of STING can recruit IRF-3 and lead to IRF-3 phosphorylation by TBK1 (Zhang et al., 2019). Phosphorylated IRF-3 can form dimer and move into the nucleus, leading to the activation of IFN-I (Zhang et al., 2019). TBK1 can also

phosphorylate IκBα, an inhibitor of NF-κB, and activate the translocation of NF-κB into the nucleus (Durand et al., 2018).

2.2 VACV inhibition of IFN-I induction by intracellular immunomodulators

During the infection, VACV utilizes a number of proteins, named immunomodulators, to resist host defense (Albarnaz et al., 2018; Albarnaz et al., 2022) . To block IFN-I induction, different viral immunomodulators are employed by VACV (Figure 4): (1) Antagonism of PRRs through E3, C4, and C16; (2) targeting the second messenger cGAMP using B2; (3) inhibition of IRF-3 downstream signaling pathway by A46, K7, C6, and N2; (4) blocking of NF-κB downstream signaling pathway with A46, A52, K7, B14, N1, C4, M2, A49, K1, and F14. Due to a domain similar to B-cell lymphoma (Bcl)-2 family proteins that relate to apoptosis, A46, K7, C6, N2, A52, B14, N1, A49 and F1 are defined as VACV Bcl-2 like proteins (Albarnaz et al., 2018). Among these Bcl-2 like proteins, A46 and K7 can interfere with both IRF-3 and NF-κB signaling pathways by targeting different adaptors (Bowie et al., 2000 ; Benfield et al., 2013). A52 and B14 are more involved in the inhibition of NF-κB than apoptosis (Graham et al., 2008).

2.2.1 VACV antagonism of PRRs

VACV E3L, a host range gene, is required for replication in most mammalian cell lines (Elizabeth et al., 1996). Its protein product E3, consists of an N-terminal Z-DNA-binding domain and a C-terminal RNA-binding domain, both of which are responsible for virulence (Chang and Jacobs, 1993; Kim et al., 2003). The C-terminal domain can prevent RIG-I and MAD-5 by binding 5’-ppp

poly(A-U) RNA transcribed from dsDNA dependent on RNA POL III and resulting in the inhibition of IFN-I induction (Valentine and Smith, 2010). Interestingly, the Z-DNA-binding domain has no ability to directly bind dsDNA, but can inhibit necroptosis via DNA-dependent activator of IFN-regulatory factors (DAI) in IFN-treated cells (Valentine and Smith, 2010; Koehler et al., 2017). In addition, the C-terminal domain can antagonize PKR and 2'-5'OAS by binding dsRNA and interact with Interferon-stimulated gene 15 (ISG15) (Guerra et al., 2008; Albarnaz et al., 2018).

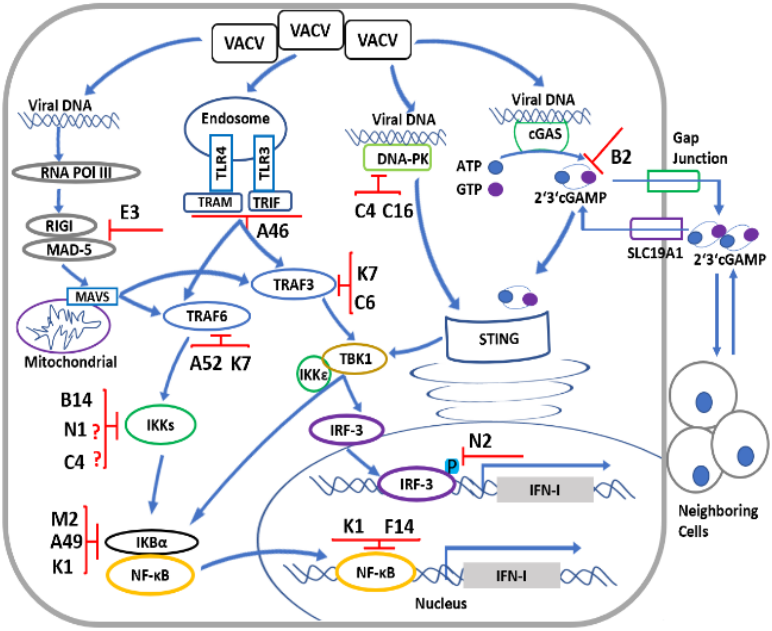

Figure 4 IFN-I induction signaling pathways and antagonism by intracellular immunomodulators of VACV

VACV C4 and C16, sharing 43% amino acid identity, can block DNA-PK by binding to the Ku subunit and resulting in the inhibition of the IRF3 signaling pathway, cytokine production, and immune cell recruitment (Scutts et al., 2018). Notably, C4 and C16 share conserved aromatic ring residues in their C-terminal domain for Ku binding (Scutts et al., 2018). In vivo, the existence of both proteins affects virulence when subjects are immunized by intradermal and intranasal infection, respectively (Scutts et al., 2018). In addition, C4 can also block the NF-κB signaling pathway at, or downstream of, IκB kinase (IKK) by an unknown mechanism (Ember et al., 2012).

2.2.2 VACV targeting of the second messenger-cGAMP

VACV protein B2, a recently reported poxvirus immune nuclease (Poxin), can block the second messenger 2′, 3′-cGAMP, which plays a pivotal role in the activation of the STING-TBK1-IRF3 axis and antiviral response by being transported into neighboring cells via gap junctions and by transporter SLC19A1 (Eaglesham et al., 2019). Poxin binds and cleaves 3′-5′ bond of 2′, 3′-cGAMP and converts it into linear Gp[2′-5′] Ap[3′]. The crucial residues are H17, Y138 and K142 and their functions are similar to the general acid-base reaction when the 3′-5′ bond of 2′, 3′-cGAMP is cleaved (Eaglesham et al., 2019). 293T cells stably expressing B2 blocked cGAS/STING signaling through 2′, 3′-cGAMP and downregulated IFN-β receptor expression (Eaglesham et al., 2019). However, B2 deleted VACV (VACVΔB2R) can still block the production of IFN-β in A549 cells, compared with wild-type VACV, suggesting that VACV has another different immunomodulator to inhibit IFN-I. Moreover, B2 is dispensable for the virus in vitro replication, in vivo replication of VACV ΔB2R in mice is attenuated

40-fold compared to wild-type VACV, highlighting the importance of B2R for virulence in vivo (Eaglesham et al., 2019).

In some orthopoxviruses, such as CPXV, CMLV, and ECTV, the conserved B2R gene is fused with the B3R gene to form a new ORF that expresses an intact protein called viral schlafen (v-Slfn), (Eaglesham et al., 2019). CMLV v-Slfn, encoded by gene 176R, cannot alter virus replication in murine NIH-3T3 cells, but still contributes to orthopoxvirus virulence (Gubser et al., 2007). ECTV v-Slfn can block the IFN-I induction by interrupting cGAS/STING axis, which is also accompanied by the blocking of IRF-3 phosphorylation (Hernáez et al., 2020). In addition, ECTV v-Slfn is a crucial virulence factor. When v-Slfn was deleted from ECTV, the release of IFN-I was able to efficiently protect animals during infection (Hernáez et al., 2020).

While conserved in most mammalian poxvirus, both B2 and B3 are not produced as functional proteins by MVA, which is due to deletions and nonsense mutations in the B2R and B3R genes (Eaglesham et al., 2019). In addition, also VARV, the causative agent of human smallpox, lacks a functional B2 protein.

2.2.3 VACV inhibition of the IRF-3 downstream signaling pathway

The VACV protein A46 can bind to several downstream adaptor molecules of TLRs and Interleukin (IL)-1β receptors (IL-1βs) including the TIR, MyD88, MAL, TRIF, and TRAM by a novel β-sheet fold that contains a myristic acid binding pocket located in the N-terminal domain which result in the failure of IFN-I induction through IRF3 and NF-κB signaling pathways (Stack et al., 2005; Lysakova-Devine et al., 2010). In addition, VACV WR lacking A46 was attenuated in

an intranasal model, which indicated that A46 is required for virulence (Stack et al., 2005).

Both VACV C6 and K7 proteins block the IFN-I induction dependent on IRF-3 and NF-κB signaling pathways by interacting with different scaffold adaptor proteins of TBK1/ IKKε (Unterholzner et al., 2011; Benfield et al., 2013). For C6, the binding scaffold adaptor proteins are NAK-associated protein 1 (NAP1), TRAF family member-associated NF-kB activator (TANK) and similar to NAP1 TBK1 adaptor (SINTBAD) (Unterholzner et al., 2011). In addition, C6 also plays a role to block the production of ISG by antagonism of histone deacetylases (HDACs) associated with IFN-sensitive response elements (ISREs) (Lu et al., 2019). K7 can bind the DEAD-box RNA helicase 3 (DDX3) adaptor of TBK1/IKKε and directly interacting with the IFN-β promoter (Schroder et al., 2008). Therefore, K7 can antagonize the production of IFNβ dependent- and independent-IRF-3 signals. In addition, K7 can counteract TRF6 to inhibit the activation of IKKs that phosphorylate IκB to activate NF-κB (Schroder et al., 2008). Due to this block in immune response, VACV lacking C6 or K7 exhibited decreased virulence in vivo, despite no effects on infected cells in vitro (Unterholzner et al., 2011; Benfield et al., 2013).

The VACV protein N2, another virulence factor, also has the ability to block the IRF-3 signaling pathway further downstream following IRF3 phosphorylation and translocation into the nucleus, but the exact mechanism is still unknown (Ferguson et al., 2013).

2.2.4 VACV interference with NF-κB downstream signaling pathway

The VACV protein A52 can interfere with NF-κB signaling pathway by associating with Interleukin 1 receptor associated kinase (IRK) 2 (IRAK2) and TRAF6 (Bowie et al., 2000). A52 inhibits NF-κB signal activation dependent on TLRs by interacting with IRAK-2 more than with TRAF6 (Keating et al., 2007). The 36 residues in the A52 N-terminal domain are not necessary to inhibit NF-κB activation (Graham et al., 2008).

The B14 protein, another NF-κB inhibitor, has the ability to bind IKKs IKK-beta and prevent IKK-beta phosphorylation (Chen et al., 2008). VACV lacking B14 has normal growth kinetics in cell culture, but can induce an increased infiltration of cells into the infected lesion and a smaller lesion size compared with wild-type VACV by the intradermal infection model (Chen et al., 2006). This suggests B14 is non-essential for VACV replication in vitro, but contributes to virulence.

VACV N1 is also an antagonist of the NF-κB signaling pathway, but its target is still unclear, due to the dispute whether N1 binds IKK (Chen et al., 2006). N1 also can block apoptosis by binding BH3-only proteins including Bid, Bad and BAX, which depend on N1 forming a homodimer structure with a constitutively open surface groove similar to the grooves of other anti-apoptotic Bcl-2 proteins that can bind the BH3 motifs (Cooray et al., 2007). The VACVΔN1 can replicate in cultured cells, but N1 is important for VACV virulence in vivo comparing with wild-type and revertant controls, based on intranasal and intradermal murine models (Bartlett et al., 2002).

VACV M2 can inhibit the translocation of NF-κB and reduce extracellular signal-regulated kinase 2 (ERK2) phosphorylation induced by phorbol myristate acetate (Gedey et al., 2006). Recombinant MVA-M2L can decrease the phosphorylation of ERK2 and restore the NF-κB-inhibitory phenotype like VACV infection in HEK293 cells (Gedey et al., 2006).

VACV A49, an intracellular protein expressed in early stages of VACV, showed the ability to inhibit the degradation of phosphorylated IkBa (p-IkBa) by binding E3 ubiquitin ligase β-transducing repeat-containing protein (β-TrCP) (Mansur et al., 2013). The research on mutant A49 elucidates the mechanism by which A49 inhibits the elimination of p-IkBa by ubiquitylation: phosphorylation of serine 7 in the A49 N terminal domain by IKKs, which results in β-TrCP only recognizing and binding the phosphorylated A49, instead of p-IkBa, and the failure to translocate NF-κB to the nucleus (Neidel et al., 2019). In vivo characterization of VACVΔA49 indicates that A49 is a VACV virulence factor, but this role is independent from its function as NF-κB by inhibitor (Neidel et al., 2020).

VACV F14 can block post-translational modifications of the NF-κB components P65 in the nucleus and leads to the reduction of cytokine gene transcription, including the expression of TNF-α, CXC10, and IFN-I (Gubser et al., 2004; Albarnaz et al., 2021). Generally, P65 goes through post-translational modifications to trigger activation of cytokines. For example, acetylated modification in specific locus by acetyltransferases cardiopulmonary bypass (CPB) and p300 (CPB paralogue, also known as CREBBP and EP300) after NF-κB is translocated into the nucleus (Albarnaz et al., 2021). However, F14 mimics the p65 translocation domain to bind the DNA domain and acetyltransferase, which leads to the interruption of

P65 acetylation by CPB (Albarnaz et al., 2021). In addition, F14 can contribute to VACV virulence (Albarnaz et al., 2021).

VACV K1, encoded by the prototype VACV host range gene K1L, also inhibits NF-κB activation by inhibition of IκBα degradation and p65 acetylation (Gillard et al., 1985; Gillard et al., 1986; Sutter et al., 1994; Shisler and Jin, 2004; Bravo Cruz and Shisler, 2016). In a complementary function with VACV protein C7, K1 can safeguard VACV replication and late gene expression in most mammalian cell lines by preventing IFN-I induction, blocking ISG product IFN regulatory factor 1 (IRF-1) production, and interacting with Sterile Alpha Motif Domain Containing 9 (SAMD9), even though there is no amino acid sequence similarity between K1 with C7 (Meng et al., 2009; Backes et al., 2010; Zwilling et al., 2010; Meng et al., 2012; Liu and McFadden, 2015). K1 can also interact with the host protein tryptophan-aspartic acid repeat 6 (WDR6), but the replication of a mutant VACVΔC7ΔK1 cannot be rescued in WDR6$^{-/-}$ Hela cells (Sivan et al., 2015; Sivan et al., 2018).

3. VACV regulatory or immunomodulatory proteins conserved in MVA

MVA is unable to replicate in most mammalian cells and its viral life cycle is blocked at the assembly stage (Volz and Sutter, 2017). In the process of MVA attenuation serial passage, many VACV regulatory genes were lost, inactivated or modified by deletions and mutations that occurred in the MVA genome (Meyer et al., 1991; Antoine et al., 1998). However, remaining MVA genes still have the potential to antagonize the host defense encoding, e.g. the IFN-I resistance proteins E3, K3, C6, K7 and A46, anti-apoptosis protein F1, and cytokine or chemokine inhibitory proteins A41, C12 and

B16 (Volz and Sutter, 2017) (Figure 5). Among these immunomodulatory proteins, E3 is a multi-functional protein that not only contributes to IFN-I resistance, but also supports the inhibition of apoptosis, in addition to its host range function (Garcia et al., 2002; Hornemann et al., 2003; Ludwig et al., 2005; Ludwig et al., 2006; Albarnaz et al., 2018).

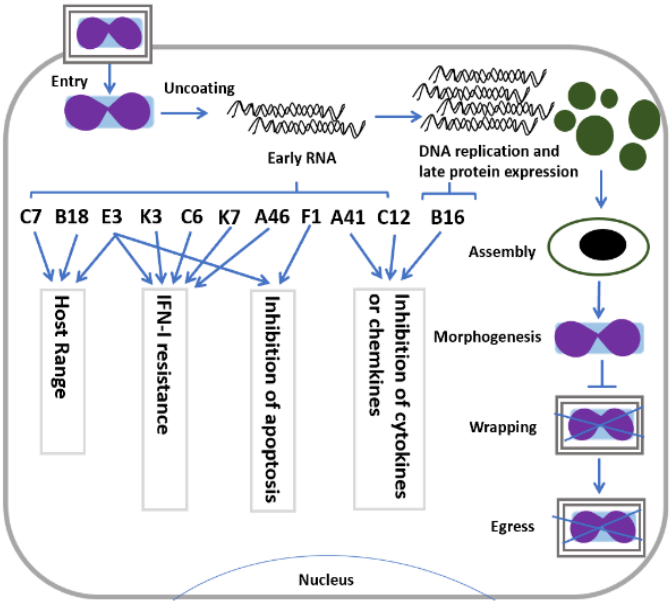

Figure 5 MVA proteins with potential to antagonize host defense mechanisms in mammalian cells

MVA produces three host range related viral proteins, E3, C7, and B18 (68k-ank). In human cells, MVA E3 controls viral intermediate RNA, and affected life cycle (Ludwig et al., 2006). When E3 is deleted from MVA, viral intermediate RNA can form RNase A/T1-

resistant dsRNA and activate 2'-5'OAS/RNase L and PKR pathways, which resulted in the failure of late transcription due to the inability to produce essential viral intermediate proteins, as demonstrated for vaccinia late transcription factor 2 (VLTF 2). Similarly, C7L-deleted MVA cannot express late proteins in human and murine cells and trigger the phosphorylation of eukaryotic translational initiation factor 2 α (eIF2α), which is alleviated by reinserting K1L gene into MVAΔC7 genome (Backes et al., 2010). B18, known as 68-kDa Ankyrin-like (68k-ank) protein of MVA, consists of ANKs and F-box-like PRANC (pox protein repeats of Ankyrin C-terminal) domains that form a Cullin-1-based SCF ubiquitin ligase by binding of cellular Skp1 (Sperling et al., 2008). When the B18(68k-ank) is deleted from MVA, the transcription and translation of intermediate and late viral genes is reduced in human and murine cells (Sperling et al., 2009).

MVA proteins can interfere with IFN-I by two ways: (1) inhibition of IFN production by E3, K7, A46, and C6 and (2) blocking of ISGs induced by IFN-I using E3 and K3. E3 is conserved in MVA and its C-terminal domain still can bind dsRNA to inhibit IFN-I dependent RIG-I, MAD-5, and PKR signal (Valentine and Smith, 2010). K7 and A46 can affect IRF-3 and NF-κB pathways by binding different targets, but C6 only blocks the IRF-3 signal (Stack et al., 2005; Unterholzner et al., 2011; Benfield et al., 2013). K3 can act as a pseudosubstrate with amino acid similarity to eIF2α to competitively bind PKR that has been activated by accumulated dsRNA, and leads to the inhibition of eIF2α phosphorylation (eIF2α-p) which blocks cellular and viral protein synthesis (Carroll et al., 1993). The N-terminus of K3 is mainly required for the inhibition of eIF2α-p (Gale et al., 1996).

Apoptosis is a process of programmed cell death in response to a viral infection, which can be composed of two ways, the intrinsic or the extrinsic pathways induced by intracellular damages or extracellular death ligands such as Fas ligand (FasL) or tumor necrosis factor (TNF)-α (Nichols et al., 2017). In the intrinsic pathway, DNA damage, energy stress, or hypoxia serve as the intracellular stimuli and trigger the interaction of BH3-only proteins, such as Bid, Bim, and Bad, with their effectors Bax and Bak, which oligomerize and assemble pores at the mitochondrial membrane (Ren et al., 2010). When macrophages are infected by MVA, Bax/Bak dependent mitochondrial apoptosis, along with TNF-α receptor induced apoptosis occurs, as well as the activation of necroptosis-pathway (Klaas et al., 2021). STING also plays an important role in stimulation of TNF- α that triggers the apoptosis (Klaas et al., 2021). In order to antagonize apoptosis, E3 and F1 are employed. E3 inhibits apoptosis by binding dsRNA, for example, the cells expressing E3 are able to avoid dsRNA induced apoptosis (Garcia et al., 2002). F1 appears to inhibit apoptosis at multiple stages: (1) interaction with Bak, but not Bax and prevention of its oligomerisation and the release of cyt-c (Wasilenko et al., 2005); (2) blocking of the signal transduction to Bax by binding the BH3-only protein Bim (Taylor et al., 2006); (3) binding of pro-caspase-9 and caspase-9 and interruption of the downstream pathway after the both apoptotic caspases (Zhai et al., 2010). F1L-deleted MVA (MVA-ΔF1L) can induce high level apoptosis in HeLa cells and in mouse embryonic fibroblasts (MEFs) (Fischer et al., 2006). Additionally, inactivation of F1 in MVA is suggested to promote vaccine immunogenicity as an MVA-HIV-C vector vaccine improves the magnitude of HIV-1-specific $CD8^+$ T cell response (Perdiguero et al., 2012). However, this study lacks

appropriate control viruses to confirm the proposed effector of F1L deletion (Perdiguero et al., 2012).

Cytokines function as the bridge between innate immunity and the following adaptive immune response, and chemokines are produced in order to recruit leukocytes to the sites of infection or inflammation (Finlay and McFadden, 2006). MVA still expresses C12 (conserved with VACV WR C12L, similar with variola Bangladesh D7L), A41, and B16 proteins (equivalent with VACV WR B15 and VACV Cop B16) in early and late stages to antagonize some cytokines and chemokines (Volz and Sutter, 2017): (1) interaction with IL-18 using C12 to prevent IL-18 reaching cellular receptor lL-18R (Born et al., 2000); (2) binding of IL-1β by B16 to interrupt the binding of IL-1β with its' receptor IL-1βR (Alcami and Smith, 1992); (3) targeting for CC chemokines CCL21, CCL25, CCL26 and CCL28 by A41 (Bahar et al., 2008).

4. Modification of regulatory/immunomodulatory genes of MVA

MVA possesses a number of advantages that allow it to be a safe and effective vaccine vector candidate against infectious diseases, including high genetic stability, large capacity for foreign DNA, high expression level for foreign antigens, and high immunogenicity to stimulate acute and memory immune response (Volz and Sutter, 2017). However, some immunomodulatory proteins, such as B16, C12, A46, C6, K7, and F1, are retained in MVA and could still have the potential to antagonize the host immune response when recombinant MVA vaccines delivers antigens against infectious diseases. One of the strategies to investigate these gene functions and their possible immunomodulation is to inactivate these genes in

the MVA genome (Volz and Sutter, 2017). In addition, the reinsertion of VACV regulatory genes into the MVA genome can be used to study the underlying mechanisms of VACV host range and immunomodulation (Bradley and Terajima, 2005; Ryerson and Shisler, 2018; Peng and Moss, 2020).

4.1 Growth restrictions of MVA associated with gene inactivation

In addition to CEF, MVA still can replicate in primary CEF, chicken embryo fibroblast cell lines LSCC-H-31 and DF-1, and in baby hamster kidney (BHK)-21 cells, which are classified as permissive cells for MVA (Meyer et al., 1991; Drexler et al., 1998; Wyatt et al., 1998). Inactivation of the E3 protein in MVA (MVA-ΔE3L) results in abortive MVA replication in CEF during late protein expression, even though DNA replication still occurs (Hornemann et al., 2003). MVA-ΔE3L was unable to counteract IFN-I or apoptosis, which again indicated that E3 plays a critical role in antagonism of host responses (Hornemann et al., 2003). D4R is also indispensable to the replication of MVA in DF-1 cells (Ricci et al., 2011). The MVA-ΔD4R cannot replicate in DF-1 cells, but this deficient replication is rescued in D4R complementing DF-1 cells (cDF-1) (Ricci et al., 2011). MVA F13L also influences the growth capacity of MVA (Sanchez-Puig and Blasco, 2005). Deletion of the F13L gene in MVA reduces the plaque size in BHK-21 cells compared to wild-type MVA (Sanchez-Puig and Blasco, 2005). The original growth phenotype is restored by reinsertion of the F13L gene in the original locus of the MVA genome (Sanchez-Puig and Blasco, 2005).

4.2 Improved MVA immunogenicity following regulatory gene inactivation

B16R deleted MVA (MVA-ΔIL-1βR) can induce significantly higher levels of VACV- specific $CD8^+$ memory T-cells than wild-type MVA when used as a vaccine in a mouse model (Staib et al., 2005). MVA-ΔIL-1βR confers higher levels of protection against lethal challenge with VACV WR 4-6 months after vaccination, when compared with non-mutant and revertant control MVA (Staib et al., 2005). Similarly, MVA lacking the A41 protein enhances the splenic $CD8^+$ T-cell responses in immunized mice and provides better protection post challenge with a 300 LD_{50} dose of VACV WR than control viruses (Clark et al., 2006). When A41L and B16R are deleted from MVA-B, a HIV vaccine candidate in which MVA delivers HIV-1 Env, Gag, Pol and Nef antigens from clade B, the magnitude and polyfunctionality of HIV-1-specific $CD4^+$ and $CD8^+$ T-cell immune responses are enhanced (Garcia-Arriaza et al., 2010). Similarly, MVA-B lacking C6L and K7R was reported to improve the magnitude and quality of HIV-1-specific $CD4^+$ and $CD8^+$ T cell in acute and memory immune responses, albeit this study lacks revertant control viruses for C6L and K7R MVA mutant viruses (Garcia-Arriaza et al., 2013).

C12L deleted-MVA (MVAΔC12L) can induce two to three-fold increase of $CD8^+$ and $CD4^+$ T-cell responses to different VACV epitopes, compared to MVA wild- type, especially magnifying the percentage of anti-VACV cytotoxic $CD8^+$ T cells during the acute phase of immune responses (Falivene et al., 2012). MVAΔC12L can also trigger efficient memory immunity in an intranasal challenge model by VACV WR (Falivene et al., 2012). MVAΔC12L recombinant virus with HIV antigens Env, Gag, Pol and Nef induces significant

increases in immune response when vaccinated with a DNA prime/MVAΔC12L boost schedule in a mouse model (Falivene et al., 2012). Based on the mutant virus MVAΔC12L, A44L and A46L are simultaneously deleted to generate MVAΔC12L/ΔA44L-A46R, which is reported to enhance T-cell responses against VACV epitopes in the acute/memory phases and to elicit higher levels of cytokines, such as IL-12, IFN-γ, IL-1β, and IFN-β, than wild-type MVA (Holgado et al., 2016).

MVA gene N2L with an in-frame deletion that causes the loss of five amino acids (aa 31–35) compared to VACV is suggested to retains its function to inhibit the immune response, when tested as recombinant MVA-HIV antigen vaccine candidate. The MVAΔN2L mutant virus enhanced innate, adaptive and memory immune response against HIV specific antigens (Garcia-Arriaza et al., 2014). Despite the unknown function of A40R, MVAΔA40R as a HIV vaccine vector also shows an increase in the mRNA levels of IFN-β, IFN-induced genes, chemokines, and a higher magnitude of acute and memory HIV-1-specific $CD4^+$ and $CD8^+$ T-cell immune responses in comparison to parental MVA-B (Perez et al., 2020). Yet, it should be noted that all these studies using mutated MVA-HIV vector viruses lack revertant control viruses to further confirm the proposed immune enhancing effects of gene deletions (Falivene et al., 2012; Garcia-Arriaza et al., 2014).

Some MVA regulatory proteins are necessary to maintain unimpaired immunogenicity and protective capacity when used as a vaccine vector. MVAΔF1 and MVAΔE3 result in the reduction of protective capacity against challenge with mousepox at three weeks after vaccination, compared to wild type MVA (Volz et al., 2018). Interestingly, MVAΔF1, MVAΔE3 and wild type MVA can equally

protect mice against a lethal ECTV challenge infection at two days after vaccination, suggesting the importance of inducing virus-specific $CD8^+$ T cells for rapid protection (Volz et al., 2018). In apparent discrepancy to results from studies with MVA-HIV deletion mutant viruses, the simultaneous deletions of -up to 15 genes in MVA, including A41L, A42R, A43R, A44L, A45R, A46R, B7R, B8R, B9R, B10R, B11R, B12R, B13R, B14R, and B15R, failed to significantly enhance immunogenicity of a recombinant MVA delivering mycobacterium tuberculosis antigens suggesting the need to further evaluate the immunomodulatory impact of selected MVA gene function (Cottingham et al., 2008; Varga et al., 2015).

4.3 Expansion of MVA host range by reinserting VACV regulatory or immunomodulatory genes

In the MVA genome, the K1L gene is truncated, restricting the ability of MVA to productively grow in RK-13 cells. However, replication can be restored when VACV K1L gene is reinserted into the MVA genome (Meyer et al., 1991; Wyatt et al., 1998; Staib et al., 2000; Staib et al., 2003). The K1 protein, has been extensively researched including the use of several MVA-K1L recombinant viruses that can form plaques and productively replicate in RK13 cells (Sutter et al., 1994). The replication of MVA can be rescued in a RK-13 cell line that stably expresses K1, and the ANKS play a key role to MVA's replication in RK-13 cells, especially ANK1, ANK2, ANK3 and ANK5, but with no relation to the interaction with a GTPase-activating protein, ACAP2 (Sutter et al., 1994; Wyatt et al., 1998; Meng and Xiang, 2006). In addition, CP77, a poxvirus host range gene in Chinese hamster ovary (CHO) cells, is either deleted or fragmented in all sequenced VACV strains, and can complement the K1 function in RK13 cells (Ramsey-Ewing and Moss, 1996).

Serine protease inhibitor 1 (SPI-1), encoded by VACV Copenhagen C12L, is another host range factor that can be restored in MVA to rescue its replication selectively in the human cell line MRC-5 but not in other cells of human origin (Liu et al., 2019). This finding suggested the presence of one or more additional host range genes to support the replication in human cells.

Recently, the C16L/B22R duplicated gene has been identified as a second host range gene to support the replication of MVA in human cell lines (Peng and Moss, 2020). In the MVA genome, C16L is truncated at the left end and B22R at the right end harbours a 45-bp deletion. When either of gene was reintroduced into MVA combined with C12L, replication capability was fully restored in the MRC-5 and A549 cell lines, and similar growth capacity was achieved in permissive CEF (Peng and Moss, 2020). This finding unveils the mystery of which VACV genes, in addition to C12L, support MVA replication in human cells. Moreover, either C16 and C12 or both proteins can restore the ability of MVA to form MV (Peng and Moss, 2020). A follow-up study by Peng et al. identified the zinc-finger antiviral protein (ZAP) as a restricted factor for MVA replication in A549 cells, and the C16 protein can interact with ZAP in cytoplasm when MVA-C16 infects A549 cells, which was also confirmed by $ZAP^{-/-}$ A549 cells infected with MVA (Peng et al., 2020).

D10, similar to D9 as a decapping enzyme of VACV, is also conserved in MVA and can accelerate the degradation of host mRNAs to reduce competition for translation factors and diminish the activation of innate immune responses (Susan Parrish and Moss, 2006). D10 deleted VACV can result in delayed onset of early and late gene expression and persistence of viral and cellular

mRNAs (Liu et al., 2014b). Recently, spontaneous mutations in D10 were reported to enhance the replication of MVA in monkey cells BS-C-1 during serial passages (Erez et al., 2021). The substitutions of Cys25Tyr, Ala226Thr, and His233Tyr occurring at the N- or C-terminus of D10 distal to the active site, leads to the increases of viral mRNA and proteins in BS-C-1 cells when compared to parental MVA (Erez et al., 2021). However, the mutations of D10 also caused diminished decapping activity in BS-C-1 cells, resulting in a similar phenotype to D10 active-site mutations in MVA that are shown to completely prevent decapping activity and viral protein synthesis (Erez et al., 2021). Thus, D10 might have other functions in addition to the activity as decapping enzyme.

4.4 Immunomodulatory consequences of VACV gene reinsertions in the MVA genome

A recombinant MVA containing an 5.2kb VACV DNA fragment (MVA/5.2kb; Meyer et al., 1991) that includes the VACV genes N2L, M1L, M2L, K1L, K2L, K3L, and K4L, can inhibit the activation of MVA-induced apoptosis and NF-κB in human THP-1 and monocyte derived-dendritic cells (HMDDCs) (Ryerson and Shisler, 2018). However, the production of cytokines (IL-6) and chemokines (IL-8) remains similar during MVA/5.2kb infection, although TNF-α dependent on NF-κB decreases (Ryerson and Shisler, 2018). In addition, the phenotype of HMDDCs infected MVA/5.2kb is much more similar to infected wild type MVA than to wild-type WR (Ryerson and Shisler, 2018). In vivo, MVA/5.2kb induces fewer VACV-specific T cells compared to wild type MVA, suggesting decreased immunogenicity of MVA/5.2kb (Ryerson and Shisler, 2018).

VACV O1, encoded by the O1L gene, is fragmented in MVA, and a crucial factor to induce sustained activation of extracellular signal regulated kinase 1/2 (ERK1/2) in infected human 293T cells. When O1L is reinserted in the locus of MVA O1L, ERK1/2 activation is restored in 293T cells, but no increase of replication or spread of MVA is observed (Schweneker et al., 2012). When O1L was deleted from VACV CVA, the virus demonstrated reduced cytopathic effects (CPE) and reduced virulence in intranasally infected mice (Schweneker et al., 2012). Thus, the O1 protein appears necessary to retain ERK1/2 activation in MVA infected human cells and to enhance virulence of CVA in vivo (Schweneker et al., 2012). Moreover, phosphorylated ERK2 can activate NF-κB, which can be inhibited by M2 that is fragmented in MVA (Gedey et al., 2006). When M2 was reinserted in MVA, the “wild-type” NF-κB-inhibitory phenotype can be restored in human 293T cells (Schweneker et al., 2012).

N1 is a NF-κB inhibitor, but a frameshift mutation in MVA results in being shortened by four amino acids and a completely different 23 amino acid segment in the C-terminus (Dai et al., 2014). Recombinant MVA expressing N1L (MVA-N1L) can lead to reduced levels of IFN-I mRNAs in infected conventional DCs and a much lower levels in activation of p-TBK1 and p-IRF3 at 4 and 8 h post-infection, when compared to wild-type MVA (Dai et al., 2014). Quantitative analysis of IFN-I shows MVA-N1L induces lower amounts of IFN-α and IFN-β compared with wild-type MVA (Dai et al., 2014). These results indicate MVA-N1 can inhibit the IFN-I induction in conventional DCs (cDCs).

III OBJECTIVES

VACV B2 plays a crucial role in blocking the production of IFN-I dependent on cGAS-STING signaling pathway. In ECTV, B2 (P26 domain) and B3 (Slfn domain) are fused to form the v-Slfn protein, which is another inhibitor of cGAS-STING and leads to abortion of IFN-I production during infection. In MVA, B2 and B3 are inactivated in MVA, due to the existence of deletions and mutations in the B2R and B3R genes. In addition, MVA can stimulate the production of IFN-I and activate the phosphorylation of IRF-3 in human THP-1 cells. Based on these characteristics, we consider the question whether an MVA with reinserted B2R B3R genes can block the production of IFN-I dependent on phosphorylated IRF-3 in human THP-1 cells.

To solve this question, we established the following objectives:

(i) Generation and quality control of a recombinant MVA by insertion of the B2R and B3R genes of VACV strain Western Reserve (WR) (MVA-WR B2R B3R)

(ii) Characterization of IFN-β mRNA in human THP-1 cells during infection with recombinant MVA-WR B2R B3R virus

(iii) Characterization of IFN-β protein in human THP-1 cells infected with recombinant MVA-WR B2R B3R virus

(iv) Characterization of IRF-3 phosphorylation in human THP-1 cells during infection with recombinant MVA-WR B2R B3R virus

IV MATERIAL and METHODS

1. Oligonucleotide primers

Table 1 lists the oligonucleotide primers to amplify the different genes for MVA (Vaccinia virus Acambis 3000 Modified Virus Ankara, Genbank: AY603355), WR (Vaccinia Virus WR, GenBank: AY243312), and new generation of recombinant MVA-WR B2R B3R virus. The primers of IFN-β and GAPDH genes were designed according to Homo sapiens IFNB1 (Genbank: NM_002176.4) and Homo sapiens GAPDH (GenBank: NM_001256799.2), respectively. All oligonucleotide primers were synthesized by Eurofins Genomics (Ebersberg, Germany).

Table 1 Oligonucleotide primers

Primer	sequence
B2R B3R-S-F	5’ – GAGGAACCCAATTACGACAAG– 3’
B2R B3R-S-R	5’ – CACGTGTCGATTCATCAAGG– 3’
E3L-F	5’ – GATCTATATTGACGAGCGTTCTG– 3’
E3L-R	5’ – GTTGTCATAAACCAACGAGGAG– 3’
IFN-β-F (human)	5’ – TGCTCTCCTGTTGTGCTTCTCC – 3’
IFN-β-R (human)	5’ – CAGTGACTGTACTCCTTGGCCTTC – 3’
GAPDH-F (human)	5’ – AGCCACATCGCTCAGAACAC – 3’
GAPDH-R (human)	5’ – GAGGCATTGCTGATGATCTTG – 3’

* s means specific primers

2. Antibodies

Table 2 Antibodies

Antibodies used for Titration		
Specificity	Dilution	Company
Rabbit anti-VACV	1:2000	Acris GmbH, Arnbruck, Germany
Goat anti-rabbit	1:5000	Jackson Immuno Research, West Grove, USA
Antibodies used for Western blot		
Specificity	Dilution	Company
Rat anti-C7	1:200	Acris GmbH, Arnbruck, Germany
Goat anti-rat	1:4000	BioLegend, San Diego, USA
P-IRF-3 (S396) rabbit mAB	1:1000	Cell Signaling, Danvers, USA
HRP-linked Goat anti-rabbit IgG	1:2000	Cell Signaling, Danvers, USA
Rabbit anti-GAPDH mAB	1:1000	Cell Signaling, Danvers, USA
IFN-β Antibodies used for ELISA		
Specificity	Dilution	Company
Capture antibody	1:120	R&D systems, Minnneapolis MN; USA
Detection antibody	1:60	R&D systems, Minnneapolis MN; USA

3. Plasmid constructions

MVAΔB2R B3R transfer plasmid: Flanked by MVA B1R and MVA B4R, the EGFP regulated by the late promotor P11 was inserted as a marker gene into a PCR-blunt plasmid (Zero Blunt™ PCR Cloning Kit, Invitrogen™). The resulting MVA B1R-P11 EGFP-MVA B4R backbone plasmid was purified using the Miniprep Kit (Qiagen) and confirmed by sequencing (Eurofins sequence service).

MVA-WR B2R B3R transfer plasmid: Synthesis of MVA B1R-WR B2R B3R-MVA B4R by Eurofins Genomics (Ebersberg, Germany) was inserted into PCR-Blunt plasmids (Zero Blunt™ PCR Cloning Kit, Invitrogen ™). The MVA-WR B2R B3R transfer plasmid was purified using the Miniprep Kit (Qiagen) and confirmed by sequencing (Eurofins sequence service).

4. Cells

4.1 Cell culture

The DF1 and THP-1 cell lines were obtained from American Type Culture Collection (ATCC) and cultured in recommended culture medium (see Table 3) at 37°C, 5% CO^2 (HeraCell 150 CO^2 incubator): DF-1 cells grew in Dulbecco's Modified Eagle's Medium (SIGMA-ALDRICH) containing 5% heat-inactivated fetal calf serum (SIGMA-ALDRICH) and 1% MEM Non-Essential-Amino Acid Solution (SIGMA-ALDRICH); Suspension THP-1 cells were cultured in RPMI-1640 (Anprotec) containing 10% FBS (Anprotec). Each cell line was passaged two times per week with Trypsin-EDTA (SIGMA-ALDRICH) when the cell density reached 90%.

Table 3 Medium/ additives / company

Medium/ additives	Company
Fetal Calf Serum (FCS)	SIGMA-ALDRICH, St. Louis, USA
MEM Non-Essential-Amino Acid Solution	SIGMA-ALDRICH, St. Louis, USA
Fetal Bovine Serum (FBS)	Anprotec, Bruckberg, Germany
Dulbecco's Modified Eagle's Medium (DMEM)	SIGMA-ALDRICH, St. Louis, USA
RPMI 1640	Anprotec, Bruckberg, Germany

Trypsin-EDTA	SIGMA-ALDRICH, St. Louis, USA

4.2 Cell counting

DF1 cells were diluted (1:2 or 1:4) with Trypan blue solution for dead cell staining before cell viability counting using a Neubauer Chamber. Viable THP-1 cells were also counted in the same way after centrifuging at 1,500 rpm for 5 min (Centrifuge 5810R, Eppendorf).

5. Virus

5.1 Generation of recombinant virus

In this study, the generation of MVA-WR B2R B3R based on MVA strain F6 (MVA-F6) was performed in a two steps procedure: First MVAΔB2R B3R was generated and then used to construct MVA-WR B2R B3R in a second step. The details of the production of recombinant MVA-WR B2R B3R virus are shown in Figure 6.

To generate MVAΔB2R B3R virus, the MVAΔB2R B3R transfer plasmid was transfected into DF1 cells that were infected with MVA-F6 at an MOI of 0.1 for 1 h using Lipofectamine 2000 (Fisher Scientific). Transfected and infected DF1 cells were incubated at 37° C for 48 h until green fluorescent foci appeared, whereupon plates were screened for these foci using a fluorescence microscope (Olympus CKX41) to successfully isolate MVAΔB2R B3R viruses. The green foci were aspirated and diluted for a series of 1:10 to use for new rounds of infection in DF-1 cells. The screening and isolating steps were repeated several times until no unexpected plaques displaying the wild type MVA-F6 phenotype could be observed, and the MVAΔB2R B3R virus stock could be amplified and harvested.

Once MVAΔB2R B3R virus was successfully constructed, the generation of MVA-WR B2R B3R was initiated by transfection of MVA-WR B2R B3R transfer plasmid in DF-1 cells followed by infection with MVAΔB2R B3R at an MOI of 0.1 after 1 h. Similarly, the screening and isolating steps were repeated several times until no green fluorescent foci were visible anymore, then recombinant MVA-WR B2R B3R virus was amplified and harvested. To confirm MVAΔB2R B3R and MVA-WR B2R B3R viruses, the distinguished PCR products were amplified by specific B2R B3R primers (Table 1), then purified by PCR purification Kit (Qiagen) and sequenced (Eurofins sequence service).

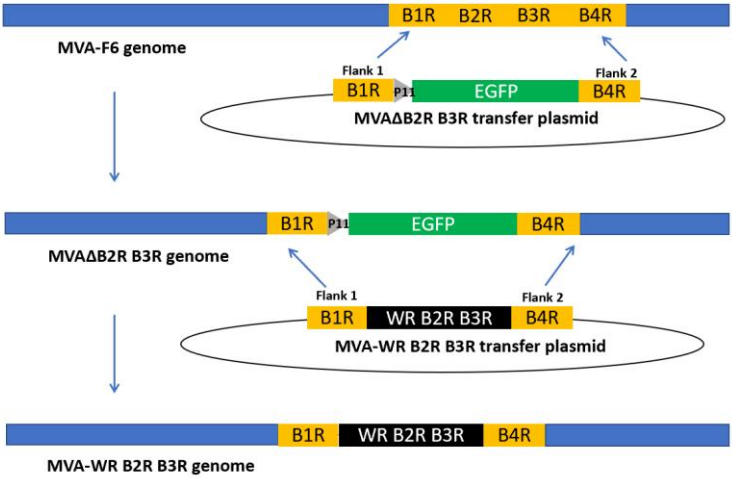

Figure 6 Schematic overview of MVA-WR B2R B3R generation

Blue long boxes, orange small boxes, green boxes with gray triangle, and black small boxes respectively represent genome of MVA-F6 or VACV-WR,

the open reading frames of MVA, P11 EGFP fragment, and open reading frames of WR.

5.2 Virus amplification and purification

MVAΔB2R B3R and MVA-WR B2R B3R were propagated in DF-1 cell monolayers. DF-1 cells were infected with MVAΔB2R B3R or MVA-WR B2R B3R at an MOI of 0.1 and incubated for two to three days at 37°C until widespread cytopathic effects could be observed. Then, 20 Flasks of DF-1 cells infected with MVAΔB2R B3R or MVA-WR B2R B3R were frozen and thawed three times at -20°C and harvested in 200 ml bottles for centrifugation at 4°C 15,000 rpm to 3 h (Avanti J-26XP, Beckman Coulter). The supernatant was discarded and the pellet was resuspended in 10 ml of 10 mM Tris-HCl, pH 9.0 buffer. Then, the resuspended virus suspension was sonicated three times for 60 sec and centrifuged at 1,500 rpm for 5 min at 4°C. This step was repeated three times and the supernatant was collected after each centrifugation. In this process, a 36% sucrose cushion was made by adding moderate sucrose in 10 mM Tris-HCl 9.0 The supernatant was purified using the 36% sucrose cushion via centrifugation at 4°C 15,000 rpm for 2 h (OptimaTMLE-80K Ultracentrifuge, Beckman Coulter). Finally, the pellet was resuspended in 1 ml 10 mM Tris-HCl, pH 9.0 buffer and frozen at -80°C.

5.3 Titration

The titration of MVAΔB2R B3R and MVA-WR B2R B3R was performed on 90% confluent DF-1 cells in 6-well plates. DF-1 cell monolayers were infected with 1ml/well of MVAΔB2R B3R or MVA-WR B2R B3R using dilutions from 10^{-4} to 10^{-10} and incubated for 2 h

at 37°C. Then, the DF-1 cells were washed with PBS after the virus inocula had been removed and 2 ml 2% FCS DMEM were added to each well for incubation at 37 °C for 48 h. Fixation and permeabilization of DF-1 cells were performed with acetone-methanol (1:1) at room temperature for 5 min. Next, the cells were blocked for 1 h at room temperature (RT) with PBS containing 3% FCS. The next was removing the blocking buffer and incubating with a primary antibody (Table 2) for 1 h at RT. Then, DF-1 cells were washed three times using PBS and incubated with a secondary antibody (Table 2) for 1 h at RT. Washing steps were repeated three times using PBS and virus plaque were stained with 0.5 ml peroxidase substrate (KPL True blue) per well. The plaques were counted after approximately 10 min and titers were calculated as plaque forming units (PFU)/ml according to the formula $\text{Conunt Plaque numbers}(20-100)/\text{Virus Dilution magnitude}$. For example, if 50 virus plaques were counted at a viral dilution of 10^{-7}, then the virus titer is $5x\ 10^{8}$ PFU/ml.

6. Polymerase chain reaction (PCR) and reverse transcription (RT)-PCR

DF-1 cells were infected with MVA-F6, MVAΔB2R B3R, MVA-WR B2R B3R, or VACV-WR viruses respectively, at an MOI of 0.1 for 24 h. Then, the infected DF-1 cells were harvested and used to isolate genomic DNA by QIAmp DNA Mini Kit (Qiagen). DNA concentration was determined by NanoDrop® (PEQLAB Biotechnology GmbH). Polymerase chain reaction (PCR) was performed using a 25 µl reaction mixture containing 1-3 µl of 100 ng DNA per sample, 2.5 µl of 10x PCR buffer (Biozym), 0.5 µl of 10 µM forward Primer (Table 1), 0.5 µl of 10 µM reverse Primer (Table 1), 0.5 µl dNTP's, 0.5 µl of Taq® enzymes (Biozym), and 17.5-19.5 µl of distilled water. The PCR

was run using a PEQSTAR 2 thermocycler (PEQLAB Biotechnology GmbH): Initial denaturation 3 min at 95°C, followed by 26-30 of cycles including denaturation at 95°C for 30 sec, annealing for primer-hybridization at 57°C for 30 sec, and elongation at 72°C for 45 sec, and a final elongation at 72°C for 5 min.

THP-1 cells were respectively infected with MVA-F6, MVA-WR B2R B3R, and VACV-WR viruses, at an MOI of 4 for 6 h. Afterwards, the infected THP-1 cells infected with different viruses were harvested to isolate total RNA using RNeasy Plus Mini Kit (Qiagen). RNA concentration was determined by the NanoDrop® (PEQLAB Biotechnology GmbH). The isolated total RNA was converted to cDNA using Omniscript reverse transcriptase (Qiagen). The cDNA was used to amplify IFN-β, E3L, and GAPDH using above mentioned PCR protocol (primers see Table 2).

7. Gel electrophoresis

PCR products were sized-separated by 1.5% agarose (Biozym) gel and stained with GelRed (Biotium). DNA was mixed with 6x loading dye (biotechrabbit, Biozym) before loaded onto the gel. 100 bp DNA standard (biotechrabbit, Biozym) was applied as molecular weight marker. Gels were run in 1x TAE buffer (Bio-Rad) at 50 V for one hour and nucleic acid was detected using Chemi Doc™ Imaging System (Bio-Rad).

8. Western blot (WB)

To obtain cell lysates for WB, THP-1 cells were infected with MVA-F6, MVA-WR B2R B3R, and VACV-WR viruses at an MOI of 4 for 4 h. Then, the THP-1 cells infected with different viruses were harvested by centrifugation at 1,500 rpm (Centrifuge 5417R, Eppendorf) for 5

min and lysed on ice using 4x laemmli sample buffer (Bio-Rad) with 10% beta-Mercaptoethanol (Applichem GmbH), and immediately boiled at 95℃ for 10 min. Samples were loaded into 12% SDS-PAGE gels and underwent protein electrophoresis in 1X Tris/Glycin/SDS running buffer (Bio-Rad) at 90-120 V for approximately 120 min. Afterwards, proteins were transferred to a 0.2 µm nitrocellulose blotting membrane (Amersham™ Protran™, GE Healthcare Life Science) with 1x transfer buffer using Trans Blot Turbo system (Bio-Rad). The membrane was blocked with 1X Tris buffered saline (TBS) with 0.1% Tween®20 (Promega) (TBS/0.1% Tween) and 5% BSA (SIGMA-ALDRICH) at RT for 1 h. After the blocking step, the membrane was incubated at 4°C overnight with the appropriate dilution of primary antibody (Table 2). The next day, the membrane was washed three times with TBS/0.1%Tween and incubated with appropriate dilution of secondary antibody (Table 2). Again, the membrane was washed three times with TBS/Tween®20 before ECL Plus kit (Pierce™ ECL Plus Western Blotting Substrate, Fisher Scientific) was added according to manufacturer's instructions, and protein bands were analyzed using ChemiDoc™ MP Imaging System (Bio-Rad).

9. Enzyme-linked Immunosorbent assay (ELISA)

THP-1 cells were infected with MVA-F6, MVA-WR B2R B3R, and VACV-WR viruses at an MOI of 4 for 24 h. Afterwards, supernatants were harvested by centrifugation (Centrifuge 5810R, Eppendorf) at 1,500 rpm for 5 min. According to the manufacturer's instructions (R&D Systems, DuoSet ELISA), the ELISA assay was performed using the harvested supernatants to quantitively measure the human IFN-β. The plates were analyzed by Tecan plate reader (Tecan™).

Statistical analyses were performed by GraphPad Prism 5 (GraphPad Software).

10.Statistical analysis

Standard student's test (two-tailed) was used for analyzing results in Figure 9. In all cases, significance was achieved if $p<0.05$.

V RESULTS

1. Confirm recombinant MVA-WR B2R B3R virus

To generate recombinant MVA-WR B2R B3R viruse, MVAΔB2R B3R served as the parental virus and the MVA B2R B3R DNA sequence was replaced by WR B2R B3R DNA sequence using homologous recombination (Figure 7A). The correct insertion of WR B2R B3R into recombinant MVA-WR B2R B3R's genome was confirmed by PCR using specific B2R B3R primer (Figure 7B). The correct insertion of WR B2R B3R in MVA genome were verified with the same size to WR B2R B3R in VACV WR genome by sequencing (Eurofins Sequence service). The MVA-F6 and VACV-WR served as the control viruses (Figure 7).

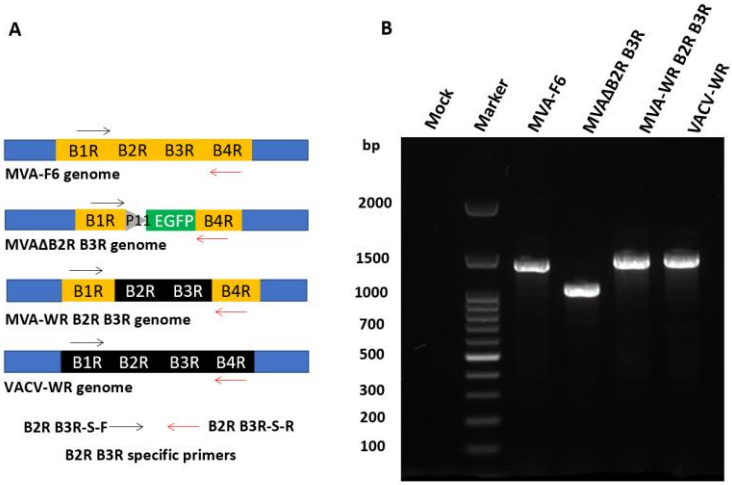

Figure 7 PCR to confirm recombinant MVA-WR B2R B3R virus

(A) Schematic overview of PCR to confirm recombinant MVA-WR B2R B3R using specific primers B2R B3R. (B) PCR results to confirm B2R B3R genes in different viruses. Uninfected DF-1 cells served as cells control (Mock). Expected sizes of the PCR products were: MVA-F6=1387bp, MVAΔB2R B3R=1054bp, MVA-WR B2R B3R=1477bp, and VACV-WR=1477bp

2. MVA-WR B2R B3R blocks IFN-β mRNA in human THP-1 cells

Human monocytic THP-1 cells are a typical cell line employed to study MVA infection in human monocyte/macrophages-like cells. MVA readily infects THP-1 cells and induces measurable amounts of cytokines or chemokines at 24h post infection despite being unable to productively replicate in THP-1 cells (Lehmann et al., 2009). In contrast, infection with VACV-WR fails to induce the production of cytokines or chemokines (Lehmann et al., 2009). Similarly, IFN-β can be efficiently stimulated by MVA in murine cDCs, but is blocked by VACV-WR (Dai et al., 2014). After 6h infection by MVA, IFN-I mRNA was significantly increased in murine cDCs, compared to VACV WR (Dai et al., 2014). In this study, we also confirmed that IFN-β mRNA can be easily detected when THP-1 cells are infected with MVA-F6 at an MOI of 4 for 6h post infection (Figure 8). On the contrary, infections with VACV-WR resulted in clearly reduced levels of IFN-β mRNA in human THP-1 cells. Similar to VACV-WR infection, the new recombinant MVA-WR B2R B3R virus also inhibited the production of IFN-β mRNA (Figure 8). The E3L mRNA served as infection control to indicate that THP-1 cells had been infected by the different viruses as indicated. The GAPDH mRNA served as the cells control to show that each sample included THP-1 cells.

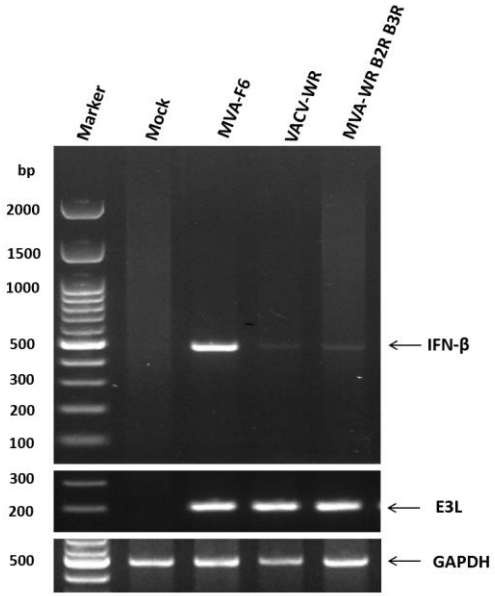

Figure 8 MVA-WR B2R B3R blocks the IFN-β mRNA in human THP-1 cells

THP-1 cells were infected with the different viruses at an MOI of 4 for 6h, including MVA-F6, VACV-WR, and MVA-WR B2R B3R. Total RNA isolated from infected THP-1 cells was used to detect IFN-β mRNA by RT-PCR. Uninfected THP-1 cells served as negative control (Mock). E3L and GAPDH served as infection and cell control, respectively.

3. MVA-WR B2R B3R blocks the production of IFN-β in human THP-1 cells

The chemokines or cytokines induced by MVA in human monocytic THP-1 cells can be measured by ELISA (Lehmann et al., 2009). In murine cDCs derived from BMDCs, IFN-β was accumulated by MVA until more than 20h post infection, but was blocked by VACV-

WR (Dai et al., 2014). In this study, the supernatants of THP-1 cells infected with different viruses at an MOI of 4 for 24h post infection, including MVA-F6, VACV-WR, and MVA-WR B2R B3R, were used to perform ELISA. IFN-β can be induced to significantly increased levels (more than 600 pg/ml) upon MVA-F6 infection when compared to VACV-WR or MVA-WR B2R B3R infections ($p<0.0001$, Figure 9). MVA-WR B2R B3R was able to block IFN-β comparable to VACV-WR, which is consistent with the results of blocking of IFN-β mRNA seen previous Figure 8.

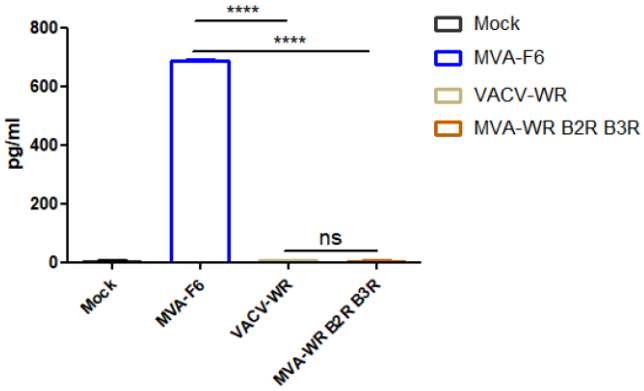

Figure 9 Drastically reduced amounts of IFN-β in supernatants from MVA-WR B2R B3R infected human THP-1 cells

THP-1 cells were infected with the different viruses at a MOI of 4 for 24h, including MVA-F6, VACV-WR, and MVA-WR B2R B3R. Supernatants of infected THP-1 cells were used to measure IFN-β by ELISA. Uninfected THP-1 cells served as negative control (Mock). Data are means ± SD (n = 4). **** stands for $P<0.0001$, and ns means non-significant difference.

4. MVA-WR B2R B3R blocks the phosphorylation of IRF-3 in human THP-1 cells

cGAS is an important DNA sensor that can recognize DNA from PAMPs and itself and produce the second messenger cGAMP (Li et al., 2013). Once cGAMP is activated, the IFN-I can be induced by IRF-3 and/or NF-Kb signal dependent on STING (Cai et al., 2014; Motwani et al., 2019). cGAS/STING-mediated cytosolic DNA-sensing pathway is the most dominant signal axis to induce the production of IFN-I during MVA infection in murine cDCs (Dai et al., 2014). In cGAS/STING pathway, IRF-3 phosphorylation is the crucial step before triggering the production of IFN-I (Cai et al., 2014). To compare IRF-3 phosphorylation in protein level during the MVA infection in THP-1 cells, the lysates from THP-1 cells that are affected by different viruses at a MOI of 4 for 4h post infection, including MVA-F6, VACV-WR, and MVA-WR B2R B3R, were performed via Western Blot using monoclonal antibody against phospho-IRF3. Our data show that MVA-F6 can induce the phosphorylation of IRF-3, but VACV-WR cannot, which is in line with previous reports (Figure 10) (Dai et al., 2014). Similar to VACV-WR, MVA-WR B2R B3R can also block the phosphorylation of IRF-3 (Figure 10). This result is also in agreement with Hernáez who showed that v-Slfn can inhibit the phosphorylation of IRF-3 (Hernáez et al., 2020). IRF-3 signals were detectable in each lysate and remained at same level, serving as a control for the signaling pathway. C7 is expressed in the early phage of the VACV life cycle and served as the control of infection. GAPDH served as cell control.

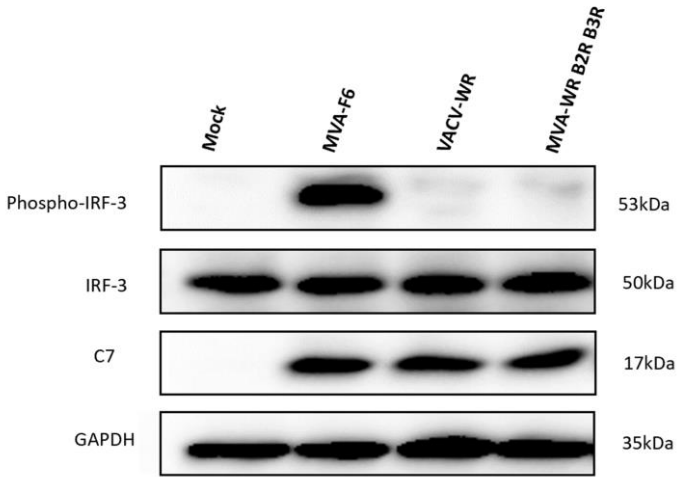

Figure 10 MVA-WR B2R B3R blocks the phosphorylation of IRF-3 in human THP-1 cells

THP-1 cells were infected with the different viruses at an MOI of 4 for 4h, including MVA-F6, VACV-WR, and MVA-WR B2R B3R. Western Blot analysis was performed using different monoclonal antibody against phospho-IRF3, IRF-3, C7, and GAPDH. Uninfected THP-1 cells served as negative control (Mock). IRF-3, C7, and GAPDH served as control of signal pathway, infection, and cell, respectively.

VI DISCUSSION

Upon viral infection, the host immune responses are activated to defend the invading pathogen. The first line of immune response is characterized by (i) phagocytosis of pathogen associated structures by professional phagocytes such as macrophages, monocytes or neutrophils, (ii) antigen processing and presentation by professional antigen presenting cells such as DCs, (iii) induction of cytokines including IFN-I and chemokines resulting in the migration of leukocytes to the site of infection, and (iv) the priming of antigen-specific $CD4^+$ and $CD8^+$ T cells (Price et al., 2013). Several poxviruses, including wild-type Vaccinia virus (VACV), have viral genes ("host range" genes) which are expressed at an early stage of infection allowing to control and inhibit the host cell immune response (Smith, 1993). Modified Vaccinia virus Ankara (MVA), an attenuated Vaccinia virus strain, lost some of immunomodulatory genes (immune evasion and virulence factors) during serial passages on CEF cells (Carroll and Moss, 1997; Antoine et al., 1998). Due to these features, MVA is commonly used as a vector platform expressing viral and bacterial antigens. Furthermore, MVA is utilized as a tool to investigate the biological nature of related poxviruses that cause severe diseases (e.g. Variola virus). The viral genes of interest are therefore inserted in or deleted from the MVA genome.

In this study, a recombinant MVA expressing the two VACV Western Reserve (WR) proteins B2 and B3 (MVA-WR B2 B3) could be generated successfully by homologous recombination. Furthermore, it could be demonstrated that recombinant MVA-WR B2 B3 blocks the activation of type-I IFN response in human THP-1 cells. This could be shown by reduced levels of IFN-β mRNA as well as low levels of secreted IFN-β in the supernatant of MVA-WR B2 B3

infected THP-1 cells. Furthermore, we could demonstrate that MVA-WR B2 B3 blocks the phosphorylation of IRF-3. Taken together, these data show the importance of VACV WR B2 and B3 to inhibit the host cell immune response following infection.

The production of high levels of IFN-I following infection with a certain pathogen is one important mechanism of infected cells. Viruses developed several mechanisms to bypass this activation by expressing viral genes to interfere at certain stages of this pathway. Recently, VACV B2 had been identified as a nuclease to cleave cGAMP and thus, blocking the production of IFN-I dependent on the cGAS/STING signaling pathway (Eaglesham et al., 2019). In the genome of some orthopoxviruses such as CPXV and ECTV, the two genes B2R and B3R fuse to generate a new ORF expressing v-Slfn which can also block the IFN-I production by interferring with the cGAS/STING signaling pathway (Eaglesham et al., 2019). In ECTV, v-Slfn is composed of two domains: P26, homologous to B2 and located at the N-terminus, and Slfn, homologous to B3 and located at the C-terminus (Eaglesham et al., 2019). P26 plays a crucial role to cleave 2'3'cGAMP resulting in the inhibition of IFN-I (Eaglesham et al., 2020). It is believed that p26 is evolved from insect viral proteases and integrated into metazoans, resulting in the addition of p26 to poxvirus from metazoan during infection (Eaglesham et al., 2020). Like ECTV, CPXV and MPXV also have the ability to express the intact v-Slfn and cleave 2'3'cGAMP (Eaglesham et al., 2019). v-Slfn, a protein widely distributed from mammals to viruses, participates in the control of cell proliferation and immune responses (Casa-Espero´n, 2011). Schwarz et al., firstly reported and indicated that Slfn can regulate the cell growth and T cell proliferation, because the inhibition of cell growth and thymic development were observed when Slfn was over- or abnormal-

expressed (Schwarz et al., 1998). Mavrommatis et al. stated that the Slfn family was regulated by IFNR triggering JAK-STAT signal pathway through binding IFN-I (Mavrommatis et al., 2013). However, it is still unknow whether Slfn also plays a role in inhibiting IFN-I.

In MVA, B2 and B3 are inactivated due to deletions and mutations. Unlike VACV and other poxviruses, MVA induces a strong IFN-I response in murine macrophages/DCs and human monocytic THP-1 cells via phosphorylated IRF-3, because it lacks specific immunomodulators to resist IFN-I. For examples, murine BMDCs produce elevated levels of IFN-I during MVA infection (Dai et al., 2014). In THP-1 cells, IFN-I can be stimulated by MVA at early stages during infection (Lehmann et al., 2009). Based on these characteristics, we considered weather a recombinant MVA with reinserted B2R and B3R genes can block the production of IFN-I dependent on phosphorylated of IRF-3 in human THP-1 cells. In new generation of recombinant MVA-WR B2R B3R virus, can normally express B2 and B3 proteins that inhibit the production of IFN-I in human THP-1 cells. Based on the property that P26 (homologous to B2) blocks IFN-I production by cleaving of 2'3'cGAMP, it is hypothesized that B2 plays the critical role in inhibiting IFN-I production in the recombinant MVA-WR B2R B3R virus.

In recent years, cGAS/STING pathway is confirmed to play a crucial role in the antagonism of MVA infection and induction of IFN-I in murine cDCs (Dai et al., 2014). cDCs from Flt3L (fms-like tyrosine kinase-3 ligand)- cultured BMDCs can produce IFN-α and IFN-β in response to MVA infection, but plasmacytoid DCs (pDCs) from Flt3L-cultured BMDCs cannot (Dai et al., 2014). This result indicates the properties of cDCs and pDCs to induce IFN-I are different in

response to MVA infection. cDCs cultured by Granulocyte/macrophage colony stimulating factor (GM-CSF) that can induce $CD11c^{+}B220^{-}PDCA1^{-}$ cDCs from BMDCs can secrete lots of IFN-α and IFN-β 8h post infection by MVA at a MOI of 10 (Dai et al., 2014). Even MOI at a lower MOI of 0.25, IFN-α and IFN-β can also be detected in GM-CSF cultured cDCs (Dai et al., 2014). However, no IFN-α or IFN-β can be observed in GM-CSF cultured cDCs infected with VACV at same levels of MOI (Dai et al., 2014). It is concluded that MVA can induce the production of IFN-I in cDCs instead of pDCs, but VACV cannot. In addition, IFN-α and IFN-β mRNAs are respectively increased 6-fold and 105-fold in MVA infected cDCs (Dai et al., 2014). However, VACV only induces 2-fold and 6-fold increases in IFN-α and IFN-β mRNAs when the same cultured cDCs are infected with VACV (Dai et al., 2014). In this study, we detect the IFN-I induction in monocytic THP-1 cells that are infected with different viruses at a MOI of 4, including MVA-F6, VACV-WR, and MVA-WR B2R B3R. Our data indicate MVA-F6 can significantly induce the transcription of IFN-β mRNA in human monocytic THP-1 cells, but VACV-WR cannot. Like VACV-WR, recombinant MVA-WR B2R B3R is able to block the transcription of IFN-β mRNA in human THP-1 cells. These data could be verified by ELISA. Upon infection of THP-1 cells with MVA-F6, high levels of secreted IFN-β could be detected in the supernatant, whereas no detectable levels of IFN-β could be seen in the supernatant of VACV-WR or MVA-WR B2R B3R infected cells.

STING can affect the production of IFN-I. The cDCs, derived from STING Goldenticket (Gt) mutant mice with a single nucleotide variant of STING, abrogates the production of IFN-I in response to MVA infection (Dai et al., 2014). Notably, the IRF-3 phosphorylation is also blocked along with the abrogation of IFN-I in

STING mutant cDCs (Dai et al., 2014). In vivo, this synergy of STING and IRF-3 phosphorylation can be observed in STING mutant and IRF-3 knockout mice that are infected with MVA (Dai et al., 2014). Collectively, these results indicate that IFN-I induction by MVA in murine cDCs is dependent with STING and IRF-3 phosphorylation. Given that the STING/IRF-3 pathway can activate by cGAMP produced by cGAS during DNA virus infection, cDCs from cGAS knockout ($cGAS^{-/-}$) mice are generated to detect how IFN-I affects by MVA infection (Dai et al., 2014). In $cGAS^{-/-}$ cDCs, IFN-I induction is abolished, and TBK1 and IRF3 phosphorylation is absent, which supports that cGAS is the crucial sensor in response to MVA infection (Dai et al., 2014). Therefore, the complete signaling pathway to sense MVA and induce IFN-I in murine cDCs is cGAS-STING-TBK1/IRF-3. Moreover, endosomes and lysosomes also acting as MVA sensors to induce IFN-I are confirmed (Dai et al., 2014). However, MVA replication is not required to induce IFN-I, because inactivated MVA by UV-treatment is still able to activate cGAS/STING pathway in murine cDCs (Dai et al., 2014).

In murine cDCs derived from BMDCs, IRF-3 is phosphorylated during MVA infection and reaches to the peak 4h post infection (Dai et al., 2014). In this study, we used MVA to infect THP-1 cell at an MOI of 4 and determined how IRF-3 phosphorylation was affected 4h post infection. To detect phosphorylation of IRF-3, we performed a western blot analysis. It is verified that MVA induced the phosphorylation of IRF-3 at an MOI of 4 for 4h in THP-1 cells. However, the phosphorylation of IRF-3 was blocked in THP-1 cells during the early stages of VACV-WR infection, consistent with VACV-WR being unable to activate IFN-I dependent on IRF-3 phospharylation in murine cDCs (Dai et al., 2014). Compared to VACV-WR, the recombinant MVA-WR B2R B3R virus is also able to

inhibit the phosphorylation of IRF-3. This result is in line with the reports of Hernáez et al. who indicated that ECTV v-Slfn (homologous to B2 fused with B3) plays the dominant role in blocking the IFN-I dependent on cGAS/STING signaling pathway (Hernáez et al., 2020).

Further perspectives

Our data indicate that expression of VACV WR B2 and B3 is a crucial factor to inhibit IFN-I immune response in THP-1 cells. However, we do not know whether the IFN-I blocking function of recombinant MVA-WR B2 B3 is dependent on B2, B3 or B2-B3 protein. Therefore, further investigations need to be done. In a next step, recombinant MVA expressing either B2 (MVA-WR B2) or B3 (MVA-WR B3) will be generated and analysed in regard to their ability to block IFN-I production. One advantage of MVA is its good immunogenicity which allows its use as a viral vector vaccine to defend against infectious diseases. Therefore, we will investigate how MVA-WR B2 B3, MVA-WR B2 and MVA-WR B3 affects immunogenicity in a mouse model.

VII SUMMARY

Repair of the Vaccinia virus genes B2R and B3R inhibits interferon production upon Modified Vaccinia virus Ankara infection

VACV WR B2 can block the production of IFN-I by acting as a nuclease to cleave cGAMP that can stimulate cGAS/STING signal pathway. In some orthopoxviruses, such as ECTV, CPXV, and CMLV, the B2R ORF and B3R ORF are fused to a new intact ORF that can express v-Slfn,. In ECTV, v-Slfn is verified with the ability to block the production of IFN-I. During inhibition of IFN-I by v-Slfn, the phosphorylation of IRF-3 dependent on the cGAS/STING signaling pathway is blocked.

The goal of this study was to determine how recombinant MVA-WR B2R B3R virus affects the production of IFN-I in human THP-1 cells. In this study, recombinant MVA-WR B2R B3R virus was generated by homologous recombination. During infection with MVA, expression of IFN-β was significantly increased in human THP-1 cells, whereas it was blocked in VACV-WR infected THP-1 cells. Like VACV-WR, recombinant MVA-WR B2R B3R virus is capable of blocking IFN-β production in human THP-1 cells. With the process of IFN-β inhibition, recombinant MVA-WR B2R B3R virus blocks IRF-3 phosphorylation, which plays an important role in the cGAS/STING signaling pathway.

To the best of our knowledge, this is the first time that recombinant MVA-WR B2R B3R has been reported to block IFN-I production in human THP-1 cells. This expands the knowledge on the use of VACV regulatory factors in recombinant MVA virus contributing to a better understanding of poxvirus biology.

VIII ZUSAMMENFASSUNG

Die Wiederherstellung der Vaccinia Virusgene B2R und B3R hemmt die Interferonproduktion bei einer Infektion mit dem Modifizierten Vacciniavirus Ankara

VACV WR B2 kann die Produktion von IFN-l blockieren, indem es als Nuklease fungiert und cGAMP spaltet, welches wiederum den cGAS/STING Signalweg stimulieren kann. Bei einigen Orthopockenviren, wie z.B bei ECTV, CPXV und CMLV, sind die beiden ORFsB2R und B3R zu einem neuen intakten ORF fusioniert, der die Expression von v-Slfn ermöglicht. Bei ECTV ist v-Slfn in der Lage, die Produktion von IFN-l zu blockieren. Bei der Inhibierung von IFN-l durch v-Slfn wird die vom cGAS/STING vermittelte IRF-3 Phosphorylierung verhindert.

Das Ziel dieser Studie war es, herauszufinden, wie das rekombinante MVA-WR B2R B3R Virus die Produktion von IFN-l in humanen THP-1 beeinflusst. In dieser Studie wurde ein rekombinantes MVA-WR B2R B3R durch homologe Rekombination erzeugt. Während der Infektion mit einem nicht rekombinanten MVA war die Expression von IFN-β in humanen THP-1 Zellen, signifikant erhöht, wohingegen VACV-WR die IFN-β Produktion blockierte. Das rekombinante MVA-WR B2R B3R Virus ist wie auch VACV-WR in der Lage, die IFN-β Produktion in humanen THP-1 Zellen zu blockieren. Während der Hemmung von IFN-β blockiert das rekombinante MVA-WR B2R B3R Virus die IRF-3 Phosphorylierung, die eine wichtige Rolle im Verlauf des cGAS/STING Signalwegs spielt.

Nach unserem Kenntnisstand ist dies das erste Mal, dass rekombinantes MVA-WR B2R B3R die IFN-l Produktion in humanen THP-1 Zellen blockiert. Diese Erkenntnis erweitert das Wissen über den Einsatz von VACV Immunmodulatoren in rekombinanten MVA Viren und trägt somit zu einem besseren Verständnis der Biologie der Pockenviren bei.

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X APPENDIX

1. Consumables/plasticware, chemicals, and reagents

Descriptions	Supplier
Cell culture flasks (25/75/175 cm^2)	Sarstedt, Nümbrecht, Germany
6-/24-/96-well flat bottom plates	Sarstedt, Nümbrecht, Germany
Serological pipettes (5/10/25 ml)	Sarstedt, Nümbrecht, Germany
Micro tubes (1.5 ml/2.0 ml)	Sarstedt, Nümbrecht, Germany
Falcon (10 ml/50 ml)	Sarstedt, Nümbrecht, Germany
Biosphere® Filter Tips (20 µl/100 µl/200 µl/1000 µl)	Sarstedt, Nümbrecht, Germany
Eppendorf PCR tubes	Eppendorf AG, Hamburg, Germany
ProtranTM AmershamTM Nitrocellulose membrane, 0.2 µm	GE Healthcare, München, Germany
BSA	SIGMA-ALDRICH, St. Louis, USA
6 x loading dye	Biozym, Hessisch Oldendorf, Germany
100bp DNA ladder	Biozym, Hessisch Oldendorf, Germany
Agrose	Biozym, Hessisch Oldendorf, Germany
GelRed	VWR, Radnor, USA
TAE buffer 50X	Fisher Scientific, Waltham, USA
Lipofectamine 2000	Fisher Scientific, Waltham, USA
4X Laemli sample buffer	Bio-Rad, Hercules, USA
beta-Mercaptoethanol	AppliChem, Darmstadt, Germany
TAE buffer 50X Fisher	Scientific, Waltham, USA
10X Tris/Glycine/SDS	Bio-Rad, Hercules, USA
Tween®20	Promega, Madison, USA
Hydrochloride acid (HCl)	Carl-Roth GmbH, Karlsruhe, Germany
96% Ethanol (C_2H_5OH)	Carl-Roth GmbH, Karlsruhe, Germany

99% Methanol	Carl-Roth GmbH, Karlsruhe, Germany
Aceton(CH_4O)	Carl-Roth GmbH, Karlsruhe, Germany
Sodium chloride (NaCl)	Carl-Roth GmbH, Karlsruhe, Germany
Tris ultrapure ($C_4H_{11}NO_3$)	AppliChem, Darmstadt, Germany
Glycin ($C_2H_5NO_2$)	AppliChem, Darmstadt, Germany
Acrylamide	AppliChem, Darmstadt, Germany
SDS	AppliChem, Darmstadt, Germany
Ammoniumpersulfate	AppliChem, Darmstadt, Germany
TEMED	SIGMA-ALDRICH, St. Louis, USA
PBS	In-house production, LMU, München, Germany
Distilled water	In-house production, LMU, München, Germany

2. Solutions and buffers

Solutions and buffers	Conditions
Transfer buffer 1X	25 mM Tris base 200 mM glycine 20% methanol distilled water
TBS 10X (pH 7.4)	200 mM Tris base 1.4 M NaCl distilled water
TBS 0.1% Tween20	TBS 1X 0.1% Tween20 distilled water
1.5 M Tris-HCl (pH 8.8)	1.5 M Tris base distilled water
1.0 M Tris-HCl (pH 6.8)	1.0 M Tris base distilled water
10 mM Tirs-Hcl (pH 9.0)	10 mM Tris base distilled water
36% sucrose	36% sucrose 1.0 M Tirs-Hcl (pH 9.0)

12% SDS-PAGE gels	4.0 ml (30% acrylamide) 2.5 ml (1.5 M Tris-HCl pH 8.8) 0.1 ml (20% SDS) 0.1 ml (10% ammoniumpersulfate) 4 µl TEMED 3.3 ml distilled water
5% SDS-PAGE gels	0.83 ml (30% acrylamide) 0.63 ml (1.0 M Tris-HCl pH 6.8) 0.05 ml (20% SDS) 0.05 ml (10% ammoniumpersulfate) 5 µl TEMED 3.4 ml distilled water

3. Commercial kits

Descriptions	Supplier
Plasmid Plus Omniscript RT Kit	Qiagen, Hilden, Germany
QIAquick Gel Extraction Kit	Qiagen, Hilden, Germany
QIAquick PCR purification Kit	Qiagen, Hilden, Germany
Quick Ligation Kit	New England Biolabs, Ipswich, USA
Pierce BCA Protein Assay Kit	Fisher Scientific, Waltham, USA
QIAamp DNA Mini Kit	Qiagen, Hilden, Germany
Maxi Kit RNeasy Plus Mini Kit	Qiagen, Hilden, Germany
Spin Miniprep Kit	Qiagen, Hilden, Germany
Taq DNA Polymerase, 5 U/µl	Biozym, Hessisch Oldendorf, Germany
Humman IFN-beita Duoset ELISA Kit	R&D Systems, Minneapolis, MN
Zero Blunt PCR clonging Kit	Thermo Fisher Scientific, Massachusetts, USA
KPL True BlueTM peroxidase Substrate	Srea care, Milfords, MA USA
Pierce ECL Plus Western Blotting substrate	Fisher Scientific, Waltham, USA

4. Laboratory equipment and software

Equipment	Supplier
UV Transilluminator	UVP, Upland, USA
Trans Blot® Turbo™ Transfer System	Bio-Rad, Hercules, USA
Safety Work Bench BDK-SK 1200	BDK, Sonnenbühl, Germany
NanoDrop® ND-1000 PEQLAB	Biotechnology GmbH, Erlangen, Germany
Olympus CKX41	Olympus Life Sciences, Hamburg, Germany
Heraeus Kendro HeraCell 150 CO^2 Inkubator	Heraeus, Hanau, Germany
Mini Vortex	Mixer Fisher Scientific, Waltham, USA
Tecan plate reader	Tecan™, Männedorf, Switzerland
PEQSTAR	PEQLAB Biotechnology GmbH, Erlangen, Germany
Chemi DOCTM Imaging System	Bio-Rad, Hercules, USA
GraphPad Prism 5	GraphPad Software, San Diego, USA
Centrifuge 5810R, 5417R	Eppendorf AG, Hamburg, Germany
Avanti J-26 XP centrifuge	Beckman Coulter, Krefeld, Germany
Optima LE-80K ultracentrifuge	Beckman Coulter, Krefeld, Germany

XI. ACKNOWLEDGEMENTS

It's my great honor to be here writing the acknowledgements. I would like to thank everyone who supported me during my doctoral thesis. In particular, so many thanks to:

Prof. Gerd Sutter gave me the opportunity to work in here for a doctoral degree pursuit. I really appreciate that he help me so much since the first day when I study here. Due to his scientific design, patient guidance, and consisitent encouragement, I learned how to start scientific projects, consider questions, and overcome difficulties during the research.

Dr. Ellen Link, my supervisor, guided me finish the project and revised my dissertaion. Her precise attitudes made me know how to be a scientific reseracher.

All staffs in Lehrstuhls für Virologie (Michael Lehmann, Marlowe Peter, Christine Brandmüller, Alina Tscherne, Wanda Schroppa and Nina Hechtberger) assisted me so much in the period of doctoral study.

China Scholarship Council (CSC) supports my study and life in Munich, Germany. Without this support, it would have been impossible for me to work and live in here.

My parents and friends encourged me when I had difficulties in study and life. They supported my decision and gave me confidence at every stage of my academic work. I appreciate them everything and wish I could show them just how much I love them. Thanks again for their love that gives me.