ABSTRACT

BAREFOOT, BRICE EDWARD. Increasing the Immunogenicity of Vaccinia Virus Vectors by Insertion of Cowpox Virus A-Type Inclusions. (Under the direction of Dr. Elizabeth A. Ramsburg.)

Prophylactic immunization against infectious pathogens has become a mainstay in the global effort of prevention against harmful diseases. Vaccine design can be better facilitated by the understanding of the effects on the immune responses generated by different vaccine vectors. Cowpox virus (CPXV) produces many infectious forms of virus, one of which is the A-type inclusion (ATI) that contains multiple embedded infectious virions. Vaccinia virus (VV) contains a truncated ATI protein (ATIp) that does not produce an ATI with embedded virus. We report here the results of comparing the *in vivo* pathological consequences and the resulting immunogenicity of wild-type vaccinia virus western reserve (VV-WR) and a recombinant vaccinia virus (rVV) expressing the full-length CPXV ATIp (rVV+ATI). We also enriched virus preparations for ATIs (En-ATIs) from rVV+ATI-infected cells. We evaluated these En-ATIs *in vivo* in parallel with VV-WR and rVV+ATI. We compared the effects of a primary immunization with these novel viruses followed by lethal VV-WR challenge. We also compared the immunological boosting effects of these rVVs in mice that were previously exposed to VV-WR. We found that rVV+ATI and En-ATIs induced cellular and humoral responses comparable to those induced by the VV-WR wild-type vector and these responses provided an equal amount of protection upon lethal challenge. We also found that in the presence of pre-existing immunity from prior exposure to VV-WR, rVV+ATIs and En-ATIs provided protection that was comparable to the wild-type vector. During the primary immunization with the different rVVs, En-ATIs resulted in less

weight loss than wild-type VV-WR and rVV+ATI, thus suggesting that the pathological consequences of En-ATIs might be reduced in comparison with rVV+ATIs and VV-WR. Overall these findings suggest that expression of the full-length CPXV ATIp does not significantly alter the immunogenicity or protection when compared to wild-type VV-WR. En-ATIs also resulted in decreased weight loss, suggesting that the pathological consequences are reduced in comparison to the other rVV vectors. Additional studies will need to be conducted to provide further evidence supporting these conclusions and to further characterize the effects of CPXV ATI protein expressed in rVVs.

Increasing the Immunogenicity of Vaccinia Virus Vectors by Insertion of Cowpox Virus A-Type Inclusions

by
Brice Edward Barefoot

A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the degree of
Master of Science

Immunology

Raleigh, North Carolina

2012

APPROVED BY:

_______________________________ Dr. Susan L. Tonkonogy
Chair of Advisory committee

_______________________________ Dr. Elizabeth A. Ramsburg

_______________________________ Dr. David J. Pickup

_______________________________ Dr. Barbara Sherry

BIOGRAPHY

Brice Barefoot was born on November 30, 1979 in Raleigh, North Carolina and is the son of Donald and Carol Barefoot and older brother to Bret Barefoot. After graduating South Johnston High School in Benson, North Carolina, he attended Johnston Community College, a small community college in rural North Carolina, where he received Associate's degrees in Sciences and Liberal Arts from which he then enrolled at North Carolina State University where he earned a Bachelor of Science degree in Biological Sciences. After graduation, Brice then accepted a full-time position in the Division of Laboratory Animal Resources in the College of Veterinary Medicine Division of Laboratory at North Carolina State University under the direction of Dr. Susan L. Tonkonogy. Approximately six months later, Brice then accepted a position at Duke University as a Research Technician under the direction of Dr. Elizabeth Ramsburg in the Duke Human Vaccine Institute where he has worked for the past five years. Upon his third year he then decided to enroll at North Carolina State University in the Immunology Program. Under the guidance of Dr. Elizabeth Ramsburg, he decided to study the effects of the cowpox virus protein called A-Type Inclusions and how insertion of this protein affects the immunological and pathological efficacy of potential vaccinia vector based vaccine platforms. Upon completion of his Master's degree, Brice will continue with his current research at Duke University with hopes of defining key beneficial aspects of vaccinia virus vaccine platforms.

ACKNOWLEDGMENTS

I would like to thank my advisor and mentor, Dr. Elizabeth Ramsburg and my committee members, Dr. Susan L. Tonkonogy, Dr. David J. Pickup, and Dr. Barbara Sherry for their guidance and support in preparation of my thesis and throughout my time at Duke University and North Carolina State University. I would also like to think the faculty, staff, and students in the Immunology Program for their support, involvement, and friendship during the past two years. I would like to specifically thank Dr. Elizabeth Ramsburg for her encouragement, guidance, and friendship and Dr. Sue Tonkonogy for her academic mentoring and friendship.

Thank you to my fellow lab members; Chris Sample, Kathryn Hudak, Kathleen Athearn and the members of the Duke Human Vaccine Institute at Duke University for their help and encouragement.

Thank you to my mother, Carol Barefoot, father, Donald Barefoot, and the rest of my family and friends for their support and love.

Special thank you to Elizabeth Reynolds for her constant support, friendship, and love.

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LIST OF ABBREVIATIONS

APC:	Allophycocyanin
ATI:	A-Type Inclusion
ATIp:	A-Type Inclusion Protein
BSC-1:	African Green Monkey Kidney Cells
CBP:	Chemokine-Binding Protein
CCR:	Chemokine Receptor
CD:	Cluster of Differentiation
CD4:	Cluster of Differentiation 4
CD8:	Cluster of Differentiation 8
CD62L:	Cluster of Differentiation 62 Ligand
CEV:	Cytoplasmic Enveloped Virion
CPXV:	Cowpox Virus
CPXV-BR:	Cowpox Virus Brighton Red
CrmA:	Complement Receptor Modifier A
CrmB:	Complement Receptor Modifier B
CrmC:	Complement Receptor Modifier C
CrmD:	Complement Receptor Modifier D
CTL:	Cytotoxic T Lymphocyte
DNA:	Deoxyribonucleic Acid
ECTV:	Ectromelia Virus

EEV: Extracellular Enveloped Virion

EGFR: Epidermal Growth Factor Receptor

ELISA: Enzyme-Linked Immunosorbent Assay

FITC: Fluorescein Isothyocynate

fLUC: firefly luciferase

FSC: Forward Scatter

GTP: xanthine-guanine phosphoribosyltransferase

G-protein: Guanine Nucleotide-Binding Protein

HPAI: Highly Pathogenic Avian Influenza

ICE: IL-1β Cleaving Enzyme

IEV: Intracellular Enveloped Virion

IFN-β: Interferon Beta

IFN-γ: Interferon Gamma

IgG: Immunoglobulin G

IL-1β: Interleukin-1-Beta

IL-1R: Interleukin-1 Receptor

IL-18: Interleukin 18

IL-18BP: Interleukin 18 Binding Protein

IM: Intramuscular

IP: Intraperitoneal

IV: Immature Virion

ITR: Inverted Terminal Repeat

JAK-STAT: Janus Kinase – Signal Transducer Activator of Transcription

MHC: Major Histocompatibility Complex

MOI: Multiplicity of Infection

mRNA: Messenger RNA

MV: Mature Virion

MVA: Modified Vaccinia Ankara

NIAID: National Institute of Allergy and Infectious Disease

NK: Natural Killer

ORF: Open Reading Frame

PAMP: Pathogen Associated Molecular Pattern

PCR: Polymerase Chain Reaction

PE: Phycoerythrin

PFU: Plaque Forming Unit

PRR: Pathogen Recognition Receptor

rMVA: Recombinant Modified Vaccinia Ankara

RNA: Ribonucleic Acid

ROI: Region Of Analysis

rVV: Recombinant Vaccinia Virus

SARS: Severe Acute Respiratory Syndrome

SCID: Severe Combined Immunodeficiency

SCR:	Short Consensus Repeats
SEM:	Standard Error of the Mean
SQ:	Subcutaneous
SSC:	Side Scatter
TCR:	T Cell Receptor
TH1:	T Helper 1
TLR:	Toll-Like Receptor
TNF:	Tumor Necrosis Factor
TNF-α:	Tumor Necrosis Factor-Alpha
TNFR:	Tumor Necrosis Factor Receptor
VCP:	Vaccinia Complement Binding Protein
VV:	Vaccinia Virus
VV-WR:	Vaccinia Virus Western Reserve strain
WHO:	World Health Organization
WNV:	West Nile Virus
WV:	Wrapped Virus
143b:	143 Human Bone osteosarcoma cells

1. INTRODUCTION

1.1. The Evolution and Classification of Poxviruses

Vaccinia virus (VV) and cowpox virus (CPXV) are both members of the complex double stranded DNA virus family *Poxviridae* that has been studied over the past two centuries. The family of *Poxviridae* consists of two subfamilies, *Chordopoxvirinae* and *Entomopoxvirinae,* which differ in the ability to infect either vertebrate or insect hosts. *Chordopoxvirinae*, which can infect a wide range of vertebrates, contains eight genera (*Orthopoxvirus, Parapoxvirus, Avipoxvirus, Capripoxvirus, Leporipoxvirus, Suipoxvirus, Molluscipoxvirus,* and *Yatapoxvirus*). Of all the *Orthopoxviruses*, it is VV and CPXV that have been studied the most because of their use in prophylactic vaccine development for smallpox and other naturally emerging or deliberately altered human pathogens. CPXV is believed to be the closest genetic relative to *Ectromelia*, the causative virus of mouse pox, and is thought to be the ancestral member from which almost all other *Orthopoxviruses* evolved [1, 2]. CPXV has the largest genome of all *Orthopoxvirus*es and contains all the genes that are present in all other *Orthopoxviruses*, whereas other *Orthopoxviruses* have missing, incomplete, or mutated genes in relation to CPXV [1]. VV is very closely related to CPXV, but VV has many truncated or missing genes, many of which have been shown to be associated with host range, virulence, or dissemination [1]. Because of the highly conserved nature of *Orthopoxvirus* proteins between different genera, immunizations with different strains have been shown to result in strong cross protective immunity to other *Orthopoxviruses*. Infection with *Variola* virus, the

causative virus of smallpox, has been shown to result in protective immunity against VV and CPXV [3].

1.2. The Biology of Poxviruses

1.2.1. Poxvirus Structure

Both the morphogenesis and structure of poxvirus virions are unique in comparison to many other viruses. Poxviruses essentially produce a single basic infectious virion, the mature virion (MV), which can acquire additional envelopes and membrane proteins at different stages of virus replication, thus producing different forms of infectious virus. MVs can be thought of as the infectious precursors of enveloped poxvirus virions, which acquire additional membranes by passing through the host cell trans-Golgi apparatus and endosomal cisternae and then by passing through the plasma membrane [4]. MVs are very large in size (approximately 360nm x 250nm) in comparison to many other viruses such as HIV, which is approximately 100nm in diameter [5]. These MVs lack the symmetry features common to other viruses such as helical or icosahedral capsids or nucleocapsids. Poxvirus MVs appear as dense, "brick shaped" membrane-bound particles with a complex internal structure featuring a thick walled, biconcave core [5, 6].

1.2.2. Infectious Forms of Poxviruses

Most poxviruses can generate four forms of infectious virus; 1) the basic non-enveloped cytoplasmic infectious particle called the mature virion (MV) which is the essential infectious unit from which all other uniquely infectious virions are formed, 2) an intracellular enveloped virion (IEV), 3) a plasma membrane bound virion that is found at the cell periphery called a cellular enveloped virion (CEV), and 4) an extracellular enveloped virion (EEV) that is actively propelled from the cell plasma membrane by the formation of virus associated actin derived tails [7]. CPXV also produces these same four forms of infectious virus and in addition produces a large protein matrix called an A-type inclusion (ATI) that can encapsulate multiple infectious MVs that are not converted to IEV, CEV, or EEV [8]. *Entomopoxvirus* ATIs provide a protective capsule that allows the virus to be ingested by insects and pass through the digestive tract until reaching the target tissues, the fat bodies [9]. CPXV ATIs are believed to aid host-to-host transmission and protect the encased MVs from environmental desication and heat [10, 11]. However, CPXV ATIs are still poorly defined in terms of their purpose, function, and effects on the host during infection [12, 13].

1.2.3. The Poxvirus Virion Core

The poxvirus virion core, which may be termed the “nucleocapsid”, is the MV subunit that is comprised of a scaffolding protein that contains the virus genome and transcription apparatus [14]. The core is rectangular shaped and consists of a thick,

bilaminar wall. The outer layer appears ridged and consists of numerous cylindrical subunits with the inner layer appearing more dense and compact with a smooth texture [15]. Upon future maturation, the core is formed into a condensed dumbbell shape and is found to be associated with structures called lateral bodies that provide an ellipsoidal subsurface framework [15, 16]. Once condensed, the core then acquires additional viral membrane proteins to form the infectious MV [4].

1.2.4. Poxvirus Genome

VV and CPXV (approximately 194 and 222kb respectively), along with all other poxviruses, have linear double stranded DNA genomes that are replicated as concatemers outside the nucleus in the host cell cytoplasm [17]. CPXV has the largest genome of all *Orthopoxviruses* with both VV and CPXV encoding more than 200 gene products. Cowpox contains all genes found in VV but, although closely related, VV has either deleted, truncated, or mutated genes related to host range factors, immunomodulatory proteins, and the formation of complete ATIs with embedded MVs [8, 9, 18]. All *Orthopoxviruses* have inverted terminal repeats (ITR) which are identical sequences, oriented in opposite directions that occur at both ends of the viral genome [19]. The ITRs contain an A-T rich region that is incompletely base-paired and forms a hairpin loop that connects the two strands of DNA to resolve the genome to its native linear form that is found in the viral core [20]. The genome alone is not infectious because the viral RNA polymerase and viral transcription factors that are associated

with the virion core are not present in the cell cytoplasm [6]. The complete genome sequences have been reported for both VV-WR (GenBank accession#: NC_006998.1) and CPXV (GenBank accession: NC_003663.2) utilizing bacterial artificial chromosome (BAC) cloned DNA sequencing.

1.3. The Life Cycle of Poxviruses

The exact mechanism of attachment and entry of *Orthopoxviruses* into the host cell is not well understood. The ability of *Orthopoxviruses* to enter almost any cell suggests that the viral receptor is a ubiquitous cell surface molecule; however the host protein receptor has not been identified [21]. Depletion of cholesterol in the cell membrane can inhibit or reduce virus penetration [22]. Epidermal growth factor receptor (EGFR) and chemokine receptors (CCR) were once believed to provide a portal of entry into host cells but further research argues against these proposals [23-26]. Thus, the mechanism of poxvirus attachment and entry remains to be clarified.

Although ambiguity remains regarding the specific host cell protein(s) involved in poxvirus attachment and entry, several viral proteins have been implicated in the fusion, entry, and the cell-to-cell spread of *Orthopoxviruses*. MV membrane proteins A21L, A28L, H2 and L5R have been implicated in host cell fusion and entry [27, 28]. EEV membrane glycoproteins A33R, A34R, A36R, and B5R are also associated with infectivity, actin-tail formation, and cell-to-cell spread and dissemination of EVVs [29,

30]. Although B5R is not essential for infectivity [31], we have shown in previous studies, as have other researchers, that polyclonal mouse IgG antibodies that are specific to the ectodomain of B5R can block fusion and subsequent entry of EEVs expressing B5R on their outer envelope and thus prevent cell-to-cell spread and dissemination [31, 32].

1.4. Uncoating of the Virion

In the case of the basic infectious virion, the MV attaches to the plasma membrane of host cells and then the nonglycosylated proteins of the MV lipid membrane fuse with the cell membrane resulting in the formation of the fusion and entry complex, from which the viral core is then released into the cell cytoplasm [28]. Uniquely, in the case of EEVs, the additional membranes acquired previously from the host cell during replication are removed just before virion attachment to the host cell but this process is still poorly defined. Thus, it is the MV surface proteins that facilitate the attachment and insertion of the viral fusion and entry complex.

1.5. Viral Gene Transcription

1.5.1. Early Gene Transcription

After virion fusion and release of the core into the cytoplasm, the viral DNA is extruded through pores in the virion core [28]. The core contains a complete transcription system including an RNA polymerase, enzymes, and other transcription factors that

give the virus the ability to synthesize early viral mRNA, which are then capped, methylated and polyadenylated [33, 34], all within minutes after infection is initiated [35, 36]. Transcription of early viral genes is determined by the consensus promoter core sequence AAAAAATGAAAAAA/TA with an A/G initiator sequence and terminated by the RNA sequence UUUUUNU [37]. Cellular ribosomes and other translational machinery are recruited to the region of the extruded mRNA in the cells cytoplasm and the early transcripts are then translated to early proteins by the host cell machinery [33]. Approximately half of the virus genome is transcribed prior to viral DNA replication [38]. These proteins account for transcription factors of early and intermediate genes, immunomodulatory proteins, and factors involved in viral DNA synthesis [38].

1.5.2. Intermediate Gene Transcription

Viral intermediate gene expression begins directly after DNA replication and are all expressed prior to late gene transcription [35]. The intermediate gene transcription promoter sequence contains a 14bp A/T rich sequence with a TAAA initiator element [35]. At least five of the intermediate genes have been shown to serve as transcriptional regulatory factors for both intermediate and late gene transcription [39]. Many of the intermediate genes are still uncharacterized but are believed to serve as transcription factors for other downstream intermediate and late genes.

1.5.3. Late Gene Transcription

Late gene expression follows intermediate gene expression and continues to the end of the virus life cycle. The late gene promoter consists of a 20bp core that contains consecutive T or A residues and is separated by about 6bp from a conserved TAAAT initiator element [40]. The late genes include virion components, such as transcription elements, that are required for expression of early genes in the next virus life cycle.

1.6. Viral Genome Replication

Poxvirus DNA replication occurs in areas of the cytoplasm that are called either viral factories or viroplasm [41]. Genome replication has been found to begin as early as one to two hours after infection and results in approximately 10,000 copies of the genome depending on the multiplicity of infection [42]. Soon after degradation of the viral core and extrusion of the viral DNA, replication begins at a nick near both ends of the genome, providing the 3' end for initiation [43]. The viral genome is then replicated as concatemers, similar to that of a rolling circle that resembles genome replication in many bacteria. The concatermer of consecutive multiple viral genomes is then resolved into single full-length genomes during late gene transcription [43].

1.7. Virion Maturation and Release

The early stages of virion assembly take place in areas of the cytoplasm called virus factories, which has also been termed viroplasm [6]. The first shapes to appear are

“half-moon” like crescents, which are the formation of immature virions (IVs), the precursors of MVs. The IVs are composed of a honeycomb protein lattice and consist mainly of viral core components [6, 14, 44]. The IVs are then packaged with the condensed genome and the transcription apparatus late in the viral life cycle. The IVs are then processed into a more compact cylindrical brick shape that results in the first form of infectious viral progeny, the MVs [45]. In VV, infectious MVs can then either remain as MVs and be released upon cell lysis, or they can be transported by host cell microtubules to be wrapped by additional viral proteins and host cell associated membranes derived from virus-modified trans-Golgi or endosomal cisternae [45, 46]. MVs that acquire additional membranes derived from the host are then referred to as either wrapped virus (WVs) or intracellular enveloped virus (IEV). The viral proteins that are associated or acquired by the IEVs are A27, A33, A34, A36, A56, B5, E2, F12, F13, and K2 [47]. IEVs are then transported to the cell periphery by host cell microtubules to be retained on the cellular surface as cellular enveloped virus (CEV) or to be released as extracellular enveloped virus (EEV). EEVs are then either released from the cell by proteolytic cleavage or actin-tail propulsion, thus inducing cell-to-cell spread and dissemination of the virus that results in the next round of infection [48-50]. The actin-tail propulsion system is produced by IEV proteins (A33, A34 and A36) that use host cell cortical actin to form motile microvilli on the cell surface that propel or “kick” the EEVs from the cell to aid virus dissemination and cell-to-cell spread [50].

CPXV produces MVs, IEVs, CEVs, and EEVs, but also has the ability to produce a unique protein that can encapsulate multiple MVs into a spherical protein matrix that essentially adds a fifth, distinct form of virus that could possibly serve as an additional mode of virus transmission. These protein capsules that contain multiple MVs have been termed A-type inclusions (ATIs) [51]. The function of these ATIs that contain multiple infectious MVs have not been clearly defined but they have been presumed to possibly aid viral dissemination within the host and increase protection and durability of the virus outside of the host. *Entomopoxviruses* produce ATIs that help the virus pass through the upper digestive tract of insects so that the virus can be released by the alkaline pH in the gut in order to target nearby fat bodies [13]. Mouse pox, raccoon pox, and fowl pox also produce ATIs but the function of ATIs in those viruses has yet to be elucidated.

1.8. The Immune Response to Poxvirus Infection

The innate inflammatory response, in combination with the adaptive immune response, is essential for the reduction of viral replication and subsequent clearance of primary poxvirus infections. However, exactly how VV activates the innate immune pathways is just beginning to be characterized. The conserved innate immune system is the first line of defense against invading pathogens, which recognizes pathogen-associated molecular patterns (PAMPs) through a set of receptors called pattern recognition receptors (PRRs)[52]. It is the innate response that reduces viral replication and

dissemination until the antigen-stimulated adaptive B cell and T cell responses are generated [10, 53]. The innate immune response can also influence the adaptive response to *Orthopoxvirus* infections [53], as it has been shown that type I IFNs induce dendritic cell (DC) maturation by up-regulating the expression of costimulatory molecules, which in turn leads to efficient homing to secondary lymphoid organs and priming of vaccinia-specific CD4 and CD8 T cells [54]. Vaccinia virus PAMPs have been shown to be recognized by toll-like receptor 2 (TLR2) and myeloid differentiation response gene 88 (MyD88) dependent pathways which lead to the production of pro-inflammatory and cytokines such as interleukin-6 (IL-6), interleukin-1 (IL-1), and interferon-β (IFN-β) which have been shown to be critical in the innate anti-viral response to VV in mice [53].

The acquired adaptive response generates poxvirus specific antiviral antibodies produced by B cells and cytotoxic functions by T cells. While the adaptive response helps aid viral clearance and recovery from primary infections, the clearance of secondary infections is almost solely mediated by the previously acquired or "learned" B cell and T cell response. Vaccinia virus specific CD4 T cell dependent antibody production is more important for clearing VV infections than the VV specific CD8 T cell response [55-57]. However, in a primary infection in the absence of CD4 T cells and antibodies, CD8 T cells can play an important role in reducing infection and disease in mice in comparison to naïve mice [55]. Vaccinia virus specific antibodies can reduce

virus dissemination by a number of different mechanisms: by preventing binding surface viral proteins and preventing attachment, inhibiting VV from uncoating, aggregating virions together, enhancing uptake by Fc mediated endocytosis, or by enhancing lysis of infected cells in combination with complement proteins [58]. Even in the presence of VV specific CD8 T cells, B cell deficient mice have severe consequences such as increased viral load, dissemination, and mortality [59]. In humans, it has been shown that vaccinia specific antibody levels persist for decades; vaccinia specific memory B cells are maintained for more than 50 years and are able to mount a vigorous antibody response upon re-vaccination with Dryvax® [60]. Recent reports have shown that antibodies against both the surface proteins of MVs and EEVs are essential for optimal protective immunity against *Orthopoxvirus* infections [61]. For example, it has been shown that the EEV membrane protein B5R induces neutralizing antibodies that reduce cell-to-cell spread and disease in mice by inhibiting attachment and fusion of EEVs [62].

Orthopoxviruses induce potent and long lasting CD4 and CD8 T cell responses that play a very important role in protection [56]. Infection in CD4 deficient mice result in delayed viral clearance and increased mortality [63]. The role of CD4 T cells with poxvirus infections is very clear-cut as they are essential for virus specific antibodies [64]. The recognition and elimination of virus-infected cells by virus specific CD8 cytotoxic T lymphocytes (CTL) is an adaptive effector mechanism used to clear virus infections [65].

Many studies have emphasized the importance of T cells in the clearance of infected cells and recovery from poxvirus infections. With primary infections the VV specific CD8 T cell response plays an important role in clearance of infection and recovery [63]. The humoral response has been shown to be more important in secondary poxvirus infections as mice are able to clear the virus efficiently in the absence of the CD8 T cells [63, 64]. In the absence of the VV specific humoral response, CD8 T cells can provide protection against a second infection but both VV induced disease and recovery time are increased compared to hosts with intact humoral immunity [64]. While the CD8 T cell response appears to be more important in the clearance and recovery from primary infections, it is the humoral response that is the mainstay of protection against subsequent poxvirus infections with CD8 T cells having a secondary role to the humoral immunity in contributing to clearance and recovery from infection.

In summary, primary poxvirus infections are maintained at a manageable level by the innate response prior to the induction of the adaptive response that will subsequently clear the remaining infection, which is mostly mediated by the CD8 T cell response. The clearance of subsequent poxvirus infections is managed by the humoral response with the aid of virus specific CD8 T cells.

1.9. Host Immune System Evasion by Poxviruses

The highly evolved mammalian immune system has many mechanisms in place that can reduce or stop the spread of various pathogens including viruses. Many viruses have found ways to modulate such immune mechanisms to benefit their survival. Poxviruses encode multiple proteins that interfere with the induction of the innate antiviral response [18]. These viral proteins can be grouped into three categories: 1) virokines, which resemble host cytokines, 2) viroceptors, which are soluble viral proteins that bind and sequester immune ligands, and 3) viral intracellular proteins that interfere with host signaling pathways and inhibit downstream induction of host anti-viral responses [18]. Below is a summary of many of the viral immunomodulatory proteins.

1.9.1. Inhibition of Host Complement

The complement pathway leads to the production of C3 convertase, which can promote inactivation of viruses and destruction of virus infected cells [66]. VV encodes for a secreted protein called vaccinia virus complement control protein (VCP). VCP is functionally similar to mammalian host complement regulatory proteins [67, 68]. VCP inhibits the classical and alternative pathways of complement activation through its ability to bind, inactivate, and increase the decay of the complement cascade proteins C4B and C3B [68, 69]. This form of complement inhibition leads to a reduction in pathogen opsonization and formation of the membrane attack complex, which ultimately leads to an increase in virus replication and disease [67-69]. Deletion of the

VCP protein in vaccinia has been shown to correlate to a decrease in virulence in guinea pigs and rabbits [69, 70].

1.9.2. Inhibition of TNF

Tumor necrosis factor (TNF) is a pro-inflammatory cytokine with pleotropic properties. TNF leads to fever, vascular permeability, production of other pro-inflammatory cytokines such as IL-1 and IL-6, and can also induce death of infected cells [18]. Many *Orthopoxviruses* can encode soluble TNF receptor (TNFR) homologs that can bind and sequester host secreted TNF, thus resulting in decreased host anti-viral TNF activity and subsequent downstream responses [18]. Variola virus has only one soluble TNFR and CPXV has three TNFR Type II homologs, which are named cytokine response modifier (Crm) B, C and D [71, 72]. In most strains of VV, the TNFR homolog open reading frame (ORF) is interrupted, thus rendering it unable to produce a functional TNFR homolog [73]. These TNFR homologs are important virulence factors for poxviruses as they can block TNF-α mediated cell cytolysis and suppress induction of the anti-viral state in both infected and uninfected cells. Deletion of the CPXV TNFR homologs lead to a reduction in virus replication, virulence, and disease in mice [74].

1.9.3. Inhibition of IL-1β

Interleukin-1-beta (IL-1β) is a cytokine that is an important mediator of the inflammatory response and other cellular responses such as cell proliferation,

differentiation, and apoptosis of infected cells [75]. *Orthopoxviruses*, including VV and CPXV, encode soluble homologs of the interleukin-1 receptor (IL-1R) that bind IL-1β, thus preventing further downstream signal induction of inflammatory and anti-viral response [74]. Although not present in VV, CPXV produces a protein named CrmA that can inhibit the processing of IL-1β to its mature form thus inhibiting its pleotropic pro-inflammatory properties as a host defense cytokine [71]. The deletion of CrmA in CPXV has been shown to increase virus replication, virulence, and disease in mice [76].

1.9.4. Inhibition of IL-18

The clearance and recovery of poxvirus infections is best facilitated by the induction of the host T helper 1 (Th1) response that leads to the activation of NK cells and the production of interferon-gamma (IFN-γ), which have important roles in reducing viral spread and replication [77, 78]. Interleukin-18 (IL-18) is an important cytokine that induces the production of IFN-γ in NK cells, macrophages, and T cells that results in polarization toward a Th1 response, which is better tailored toward virus clearance and protection [78, 79] in comparison to the host T helper 2 (Th2) response, which leads to a suppression of the inflammatory and CD8 T cell responses [80]. The potent anti-viral properties of IL-18 can induce protection in mice infected with VV [81]. IL-18 binding protein (IL-18BP) is a soluble inhibitor of IL-18 that is produced by both humans and mice and functions as an inhibitor of the early Th1 cytokine response in order to reduce severe inflammation [82]. *Orthopoxviruses* encode IL-18BP homologs that can bind and

inhibit IL- 18 [83, 84]. The deletion of VV CL12 protein, an IL-18BP homolog, leads to reduced virulence and disease mice [85].

1.9.5. Inhibition of Interferons

Both type I and II interferons (IFN) bind their cognate receptors and activate the Janus kinase signal transductor activation of transcription (JAK-STAT) pathway to induce an anti-viral response in both infected and uninfected cells [86]. Many *Orthopoxviruses* secrete proteins that share sequence homology with the extracellular domain of type I and type II IFN receptors, thus enabling these viral homologs to bind to and sequester host IFNs [87, 88]. Deletion of either VV type I IFN or type II IFN receptor homologs greatly reduces virus replication and virulence *in vivo* [89, 90].

1.9.6. Inhibition of Chemokines

Chemokines are host cell secreted proteins that bind to guanine nucleotide-binding protein (G-protein) receptors on leukocytes and mediate the migration of immune cells, such as monocytes and lymphocytes, into sites of infection or inflammation [91]. Poxviruses encode several chemokine-binding proteins (CBP) that mimic soluble host cell chemokines and bind their cognate G-protein receptors to interfere with the activation and chemotaxis of leukocytes to the site of infection. The deletion of these CBPs in certain strains of VV has been shown to increase virulence and inflammation in mice [92]. VV strains Western Reserve (WR) and Copenhagen do not contain CBPs and

insertion of these proteins decreases lymphocyte trafficking, virus replication, and virulence in an intranasal infection model in mice [92]. These results suggest that production of viral CBPs is not advantageous for VV, but it has been suggested that host disease may be due to the influx of cytotoxic cells and production of inflammatory cytokines [92]. How poxvirus CBPs promote infection and survival in the host is unknown.

1.10. Poxvirus Host Range

Many poxviruses are restricted to a narrow range of hosts that are capable of supporting viral replication and survival in selected host cells. Poxvirus pathogenicity seems to be mostly based on species-specific genes rather than the genes that are common to most poxviruses [93]. Some poxviruses, like *Variola* (smallpox), *Ectromelia* (mouse pox) or camel pox remain mostly restricted to one host species and rarely cause zoonotic infections [93]. Poxvirus tropism is not dependent on specific cell surface receptors. Instead tropism appears to be regulated by intracellular factors needed for virus replication and by the ability of the virus to modulate intracellular signaling pathways that regulate antiviral processes downstream after virus entry [93]. Even though poxviruses can initiate infection in a wide range of species, production of MVs is limited to only a few species that can fully accommodate viral replication. VV encodes many genes that regulate the host antiviral response, thus allowing viral replication to proceed. The E3L gene product, required for replication of VV in HeLa cells, a human

cervical cancer cell line, is a double-stranded RNA binding protein that is believed to act by binding and sequestering double-stranded RNA. K3L, required for replication in baby hamster kidney (BHK) cells, encodes a protein that acts as a competitive inhibitor of the double-stranded RNA-dependent protein kinase (PKR) [94-98]. The K1L gene in VV encodes a protein composed or a series of ankyrin-like repeats that are common to host transcription factors and cell cycle regulators [99]. The mechanism by which the K1L protein acts is still mostly uncharacterized but it is been shown to inhibit nuclear factor kappa B (NF-κB) activation by inhibitor kappa B (IκB) degradation, which would in turn lead to the failure to activate several host anti-viral defenses [100]. In modified vaccinia Ankara (MVA), the K1L gene has been truncated through serial passaging in chicken embryonic fibroblast (CEF) cells, resulting in limited replication in many mammalian cells [101]. VV also contains a C7L gene that encodes a protein similar to the K1L gene and has been shown to limit replication in a manner similar to the K1L protein [102].

Many host range mechanisms and restrictions of poxviruses are still poorly understood. A better understanding of poxvirus host range genes will enable researchers to engineer tissue specific or attenuated viruses, such as MVA and the New York City Board of Health vaccinia strain (NYVAC), that may be better suited as viral vaccine vectors in terms of safety.

1.11. Poxvirus A-Type Inclusions

As mentioned earlier, CPXV is able to produce what is essentially a fifth form of virus, called the A-type inclusion (ATI) in addition to the four other forms of virus: MVs, IEVs, CEVs, and EEVs. ATIs are well-defined, proteinaceous bodies that are produced under the control of a late promoter in the viral replication cycle and can contain multiple infectious MVs embedded in the ATI matrix protein [103]. Using electron microscopy, CPXV ATIs with embedded infectious MVs can be clearly visualized within infected cells [8]. We used confocal microscopy in **Figure 1** to identify BSC-1 cells infected with recombinant vaccinia virus (rVV) that result in the production of ATIs with the embedded virus (indicated by arrows). Very little is known about the function of ATIs or how they may benefit poxviruses. The different forms of infectious virus produced by poxviruses suggest the possibility that each form of virus has a distinct role in promoting and sustaining viral infection, replication, and transmission of virus. For example, neutralizing antibodies against EEVs fail to neutralize MVs because the EEV surface proteins are absent from MVs, and neutralizing antibodies against MVs cannot neutralize EEVs because the additional EEV envelope shields the MV surface proteins from specific antibodies [45]. ATIs are thought to promote virus stability for transmission between hosts, although this has not been determined experimentally. We hypothesize that the ATI protein matrix may act as a durable container of MVs that protects the embedded MVs from immune recognition by MV specific antibodies.

We also believe that the ATI may facilitate a periodic release of virus, promoting a more long-lived infection rather than a localized acute infection.

1.11.1. Characterization of A-Type Inclusions

ATIs are large well-defined structures that appear in the cytoplasm during the late stages of viral replication in some *Orthopoxviruses* such as cowpox, raccoon pox, mouse pox, and entomopox. The ATI is used as a common morphological marker for differentiation between CPXV and other *Orthopoxviruses* that lack ATIs, such as VV and *Variola* [103-105]. The ATI protein, encoded by the A25 gene, is one of the most abundant viral products, accounting for roughly four percent of all viral proteins and is produced rapidly during late gene transcription [106]. CPXV ATIs are composed of a single 160kDa protein [105] that is antigenically similar to the truncated 94K ATI protein (ATIp) produced in VV, which does not result in production of an ATI with embedded infectious MVs [103].

1.11.2. Mechanism of Virus Embedding into the A-Type Inclusion

Approximately ninety percent of the viral progeny in VV consist of MVs that have a single membrane and are released upon cell lysis [107]. Less than ten percent of the MVs are converted to IEVs, which are MVs that have acquired additional membranes from the trans-Golgi or endosomal cisternae [104, 108]. IEVs are then trafficked to the cell surface where their outer membranes fuse with the cell plasma membrane and are

released as either EEVs or CEVs [29]. The remaining MVs that are not converted to IEVs, CEVs, or EEVs remain in the cell cytoplasm to be released upon lysis or to be embedded by the ATIp [8]. This leads to an obvious question; how are MVs that are not converted to EEV or CEV embedded into the ATIp matrix?

Some of the first identified MV surface proteins were the highly conserved 60kDa 4a, 4b, and P4c structural proteins [8, 109, 110]. It has been shown that the P4c protein is among the last proteins to be incorporated into the outer surface membranes of MVs [111]. The 4a and 4b proteins that are generated by proteolytic cleavage of P4a and P4b serve as two of the major structural proteins of the viral core in all poxvirus virions [112]. The P4c protein has been implicated as a factor for directing the association of MVs with the ATI protein [8, 105]. The P4c protein associates with the already present MV surface protein A27 (A27p) and with the N-termini of the ATIp [113]. The P4c protein is an MV exclusive protein, suggesting that MVs that acquire the p4c protein many not be directed into the pathway of becoming IEVs, CEVs, or EEVs [114]. The mechanism of P4c interaction with the ATIp N-termini has not yet been characterized.

How MVs are embedded into the ATIp matrix is not clearly understood. The CPXV ATIp forms an electron-dense matrix in which MVs are embedded [10]. The embedding of virus into the ATIp matrix appears to be an active and directed process rather than one of chance. The ATIp has been shown to be myristylated on multiple lysine and arginine

residues throughout the protein even though sequence analysis shows that the ATIp lacks glycine rich myristylation motifs [10]. It seems that myristylation may function to either stabilize the protein matrix or drive protein-protein interactions between the ATI proteins during the embedding process [10]. It is also possible that the myristic acid residues could result in altered hydrophobicity of the protein that may facilitate aggregation of the ATIp associated MVs. Truncation of the C-termini of the ATIp in VV could result in loss of myristylation residues that may be important for driving the ATIp aggregation process. How the truncated portion of the C-termini results in the aggregation of the ATIp and formation of ATIp matrix with embedded MVs has not been determined.

1.11.3. Vaccinia and A-Type Inclusions

At least two genes have been implicated in the formation of ATIs with embedded MVs: 1), the A25 gene encoding the ATIp and 2), the A26 gene encoding the P4c packaging protein [8, 113]. CPXV encodes both the full-length P4c and ATI proteins, which enables it to form ATIs with embedded MVs. VV retains the functional 60kDa P4c protein [8] that is needed for the directing of MVs into the ATI matrix but contains a truncated ATIp. The VV ATIp homolog encoded by the A25L gene contains a deletion of two adenylate residues resulting in a C-terminally truncated 94kDa ATIp. This truncation renders VV unable to produce the full-length ATIp that is required to embed MVs even thought the P4c protein still associates with the N-termini of the truncated

ATIp [115]. Restoration of the ATIp in VV-WR by replacing the truncated ATIp with the full-length CPXV ATIp has been shown to produce VV ATIs with embedded MVs [8, 113].

1.11.4. Release of Infectious Virus from A-Type Inclusions

In order for ATI embedded MVs to initiate infection of host cells, the virus must first be released from the ATIp matrix. Although this process is highly uncharacterized, there are several proposed models of virus release. One suggests that ATIs are able to initiate an infection by attaching to the host cell surface. After attachment, it is possible that the ATIp barrier is degraded by host cell surface proteases to release MVs in close proximity to the host cell. The serine protease trypsin, commonly found in the digestive tract, has been shown to cleave the ATIp into several fragments [103]. The presence of serine proteases on the surface of many leukocytes [116, 117] could possibly degrade the ATIp to release the embedded MVs. Following this model, the ATIp could be partially degraded to release a few MVs to infect host cells or all MVs could be released at once. If only a few of the embedded MVs were released, the partially degraded ATI could possibly move on to infect other cells and act as a delivery system to infect many cells over a period of time. Phagocytic cells such as macrophages or dendritic cells could internalize ATIs with embedded MVs. Once internalized, the infectious MVs could be released upon degradation of the ATI matrix, thus allowing embedded virus to infect the host cell. There is the possibility that ATIs are disrupted by different pH gradients

found throughout different compartments of the body that may result in the release of virus, such as the acidic pH of the digestive tract.

Currently, the mode of virus release from the ATIp matrix is poorly characterized. There are many possible mechanisms to explain how, when, and where ATIs with infectious MVs are released and how they initiate infection. In our studies, we designed experiments to begin to determine how infectious MVs are released from ATIs.

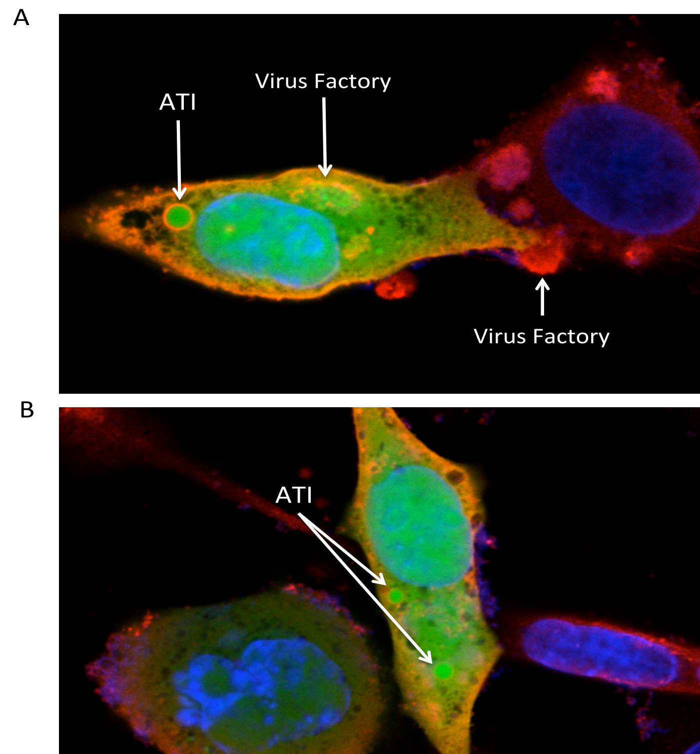


Figure 1. Production of VV ATIs in BSC-1 cells. BSC-1 cells were grown to 50% confluency on microscope slide coverslips in 30mm tissue culture dishes and then infected with rVV+ATIs at a multiplicity of infection (MOI) of 10 pfu for 1 hour. The infection mixture was then removed and replaced with a transfection mixture containing a plasmid with the vaccinia A5 core protein fused to mGFP (A5-mGFP) under the control of its native promoter. Thirty-six hrs post-infection (PI), 35hrs post transfection, cells were fixed with a 3% paraformaldehyde-PBS solution, incubated with the appropriate antibody (as described in the **Materials and Methods**), and analyzed by immunoflourescent confocal microscopy for VV ATIs with embedded MVs. Embedded MVs will have the A5-mGFP core protein incorporated and will fluoresce green at 395nm. To visualize the ATI protein, cells were permeated with 0.5% Triton-X 100 detergent-PBS and incubated with a polyclonal mouse IgG antibody specific for the ATIp followed by an anti-mouse IgG antibody conjugated to phycoerythrin (PE) that will bind to the polyclonal mouse IgG and fluoresce red upon excitation. Hoechst stain was used to stain the nuclei of the infected cells that will fluoresce blue upon excitation. VV ATIs should appear circular with a green interior and circumscribed by a red "halo-like" exterior. Both (A) and (B) are representative images of infected/transfected cells that have ATIs in the cytoplasm. Arrows indicate cytosolic ATIs, virus factories (sites of replication).

1.12. Vaccine Development and Vaccinia

The 9/11 attacks along with numerous emerging and re-emerging infectious pathogens brought about the realization of the need for advancement toward better prophylactic prevention and protection from naturally occurring pathogens or deliberately altered pathogens. The National Institute of Allergy and Infectious Disease (NIAID) strategic plan for biodefense research is based on three approaches toward accomplishing this goal: 1), progress toward a better understanding of these dangerous pathogens and how these pathogens result in disease; 2), devise studies to determine the role of the human immune system in response to these pathogens; 3) develop safe and effective prophylactic strategies to combat diseases caused by such emerging, reemerging, and weaponized pathogens [118]. The use of live virus vector based vaccines has been proposed for the design of novel vaccines that are able to elicit long lived and protective humoral and cell mediated immune responses. Many properties of poxvirus-derived vectors make them ideal candidates for the development of vaccines. Some of the top priorities of future multivalent prophylactic vaccine development include the ability of vaccines to accommodate multiple foreign antigens from multiple pathogens that generate long-lasting immunity after immunization and cause minimal adverse side effects in both healthy and immunocompromised individuals [119]. Other crucial aspects of practical vaccine development include cost of production, temperature stability, and easy administration. Poxvirus vaccine vectors meet many of these requirements and are at the top of many lists of potential vaccine vectors [120].

However, safety concerns are still a major concern with the use of live-virus-based vaccines, particularly with many replication competent rVVs. Unacceptable side effects were demonstrated during the World Health Organization (WHO) directed smallpox vaccination program with roughly one percent of the vaccinated population suffering moderate to severe side effects including myopericarditis, eczema, encephalitis and even death [3]. Thus, poxvirus based vaccine vectors with improved safety profiles are needed for effective vaccination of young, old, and immunocompromised individuals.

1.13. Purpose of This Study

The overall objective of this study is to characterize and understand how ATIs with embedded MVs alter immune recognition and host pathogenicity. Our goal is to improve the efficacy of poxvirus vaccine platforms by employing ATIs. Specifically, we aim to develop vaccines that can provide: 1), broad spectrum activity against multiple pathogens; 2), cross protective immunity against pathogens that can genetically drift or be deliberately altered, such as influenza or smallpox [118]; 3), a platform for future vaccine applications. Vaccinia virus-based vaccines offer extraordinary capacity to encode multiple foreign proteins, suggesting that VV is one of the best viral vectors for use as broad-spectrum vaccines. Past VV-based vaccines, while highly effective at inducing immunity against smallpox, suffered from a high rate of moderate to severe complications such as generalized vaccinia and inadvertent inoculations at a rate of 250 to 500 cases per million immunized or life-threatening complications such as

myopericarditis, postvaccinial encephalitis, progressive vaccinia, and eczema vaccinatum at 10 to 25 cases per million [3].

The primary purpose of this study is to determine if the incorporation of ATIs containing embedded MVs in VV can alter the pathological effects and the immune response to VV. If ATIs can enhance the efficacy of VV without increased pathological effects it may be possible to construct a new generation of VV-based vaccine vectors or improve existing VV-based vaccine platforms. To better understand the function and purpose of the ATIs in VV, we repaired the truncated ATI gene in VV. We also inserted the bioluminescent gene encoding firefly luciferase (fLUC) that will enable us to track virus replication and dissemination by *in vivo* imaging. Earlier studies have shown that the formation of ATIs with embedded MVs requires the ATI and P4c genes together with the highly conserved structural surface genes encoding A27 and A17 to produce a virus with the capacity to make ATIs with embedded MVs [8, 121]. We have constructed a novel form of VV that has the capacity to produce ATIs with embedded infectious MVs and a virus that mimics wild-type VV-WR, with both viruses expressing fLUC. Specifically, we want to determine: 1), if the rVV that has the capacity to produce ATIs with embedded MVs is more effective than wild-type vaccinia at inducing anti-vaccinia B cell and T cell responses; 2), the effects of ATIs with embedded MVs on dissemination, replication, and pathogenicity. This study will help define the immunological effects and biological properties of CPXV ATIs in the context of VV and

how we might be able to apply them to VV and other poxviral vaccine vectors. These ATIs with embedded infectious virus may enable us to produce poxviral vaccine platforms that possess the safety of replication-defective or host cell restricted viruses, such as MVA and NYVAC, with the immunologic efficacy of replication-competent wild-type virus based vaccines.

2. MATERIALS AND METHODS

2.1 Recombinant Vaccinia Virus Construction

All recombinant VVs were made in David Pickup's lab at Duke University using standard techniques outlined by McKelvey et al [8]. The approach for the isolation of vaccinia virus recombinants involves homologous recombination between a transfected plasmid that contains the genes of interest and the replicating viral DNA in infected cells. Briefly, 143b human bone osteosarcoma cells were grown to 80% confluency in tissue culture plates and infected with wild-type VV-WR and then transfected with pGEM7zf+ plasmid containing a cassette of genes to be inserted into the genome. DNA Inserts containing the full-length P4c and ATI genes under the control of their native promoters were subcloned into the pGEM7zf+ plasmid using standard cloning techniques described by McKelvey et al [8]. All transfected cassettes contain xanthine-guanine phosphoribosyltransferase (GTP) under both early and late VV promoters (see introduction for sequence) for recombinant virus selection and isolation along with fLUC under an early promoter for fluorescent imaging of viruses. All insert cassettes were flanked by homologous VV-WR sequence from the N-termi of rpol32 and the C-termi of A27L. These flanking sequences provide points for homologous recombination between the viral genome and plasmid to occur. Recombinant virus plaques were isolated by GTP selection, cultured, sequenced, and checked for protein expression by western blot and by (S^{35}) methionine labeling.

2.2 ATI Purification

rVV construction and ATI purification followed the procedure described by Patel et al [103] . The constructs are listed in **Table 1**. Briefly, 143b human osteosarcoma cells were cultured to 80% confluency and infected at an MOI (multiplicity of infection) of 10 with rVV+ATI that has the ability to produce ATIs with embedded MVs. The infected cell cultures were then harvested 36hrs later just before detachment by scrapping infected cells from the tissue culture plates and the cells were pelleted by centrifugation (10k rpms/30mins/4^{c}/Sorvall GSA rotor) and resuspended in sterile 1x PBS. The culture suspensions were then disrupted using a Dounce homogenizer to disrupt the cells and release the ATIs from the cells. The homogenized suspension was then washed with 1x PBS, pelleted (600g/15mins/4^{c}), resuspended in 1x PBS, and then sonicated for 5 mins in an ultrasonic water bath (Branson - Sonifier®). The virus suspension was then layered onto a 38% sucrose gradient and spun using high-speed centrifugation (30k rpms/60mins/SW41 rotor). The pellet was then resuspended in 1x PBS and fractionated by density gradient centrifugation (39k rpms/56hrs/4^{c}/SW41 rotor) in 40% Nycodenz density gradient media. ATI fractions were collected from the bottom of the density gradient. Each fraction collected was analyzed by PAGE and by phase-contrast microscopy. The ATIs formed a visible band at a density of 1.24g/ml. The yield of the ATI preparation was then estimated by using spectroscopy and Bradford's reagent. Infectious ATIs were then enumerated by using a plaque assay as described below.

2.3 Determination of Viral Titers

All recombinant and wild-type viruses were titrated on BSC-1 cells (African green monkey kidney cells) or 143B cells. BSC-1 cells were plated on 6-well tissue culture plates and grown to 90% confluence in DMEM supplemented with 10% Heat-Inactivated fetal bovine serum (FBS) with penicillin-streptomycin. All viruses were sonicated using a pulse water bath sonicator (Branson 2510) for 10mins and then log10 serial diluted in sterile glass tubes in unsupplemented DMEM. Cells were then washed once with 1xPBS and 200 μl of diluted inoculum was plated onto each well and incubated for 45mins at 37^{c} with 5% CO_2. Plates were rocked every 15 mins to ensure even virus distribution and to prevent the monolayers from drying. After incubation, 2mls of a methylcellulose overlay containing 5% FBS was added and plates were placed back into the incubator for 3 days. After 3 days, the overlay was removed and cells were fixed and stained with a methanol-crystal violet solution. Plates were then washed with tap water, clear plaques were then counted, and viral titers calculated.

2.4 Mice and Monitoring Pathology

Eight to ten-week-old female C57BL/6 (B6) mice were purchased from Jackson Laboratories and housed in micro-isolator cages in a biosafety level 2-equipped animal facility and kept under normal light conditions. All mice were housed at least one week prior to beginning any experiments. After primary and secondary vaccinia immunizations, all mice were monitored daily for 14 days for weight loss and changes

in body temperature (Physitemp rectal probe rodent thermometer; Physitemp Inc., Clifton, NJ) then weighed every other day until initial weight is reached. All mice losing 25% of initial (Day 0) body weight or in severe distress (ruffled fur, hunched posture, unresponsive) were euthanized in accordance with our protocol end points for humane euthanasia. The Duke University Institutional Animal Care and Use Committee (IACUC) approved all experiments and procedures.

2.5 Virus Inoculum Preparation and Immunizations

rVV Primary Immunization: This immunization was used to compare the priming effects between the different rVV vectors. At 12 weeks of age each mouse received a total of 10^5 pfu of each rVV diluted in 40μl total volume in sterile Dulbecco's Modified Eagle Medium (DMEM) and administered intranasally (IN). Virus was thawed on ice and pulse sonicated (Branson® 2510 pulse sonicator) for 10mins and diluted immediately prior to immunization. All virus preparations were warmed to room temperature just prior to inoculation. For all IN inoculations, all mice were lightly anesthetized with isoflurane using a vaporizer and quickly recovered after dosing.

VV-WR Primary Immunization: This immunization was used to induce a primary immune response to VV-WR. Twelve week old female B6 mice were given a primary immunization of 10^4 pfu of VV-WR by intramuscular (IM) injection. Each mouse

received a single 50µl injection in the right and left quadriceps (receiving a total of 100µl) using a 0.5ml insulin syringe (Becton-Dickinson).

Boosting Immunizations: All mice (groups) receiving a primary immunization of VV-WR received a boosting immunization of 1 x 10^3 pfu of one of the different rVVs diluted in 200µl total volume of sterile DMEM and administered by subcutaneous (SQ) injection in the nape of the neck between the shoulder blades. Inoculum was prepared immediately prior to immunization (same as above) and warmed to room temperature. For SQ immunizations, all mice were lightly anesthetized using an isoflurane vaporizer and quickly recovered after injection.

VV-WR lethal challenge: 8 weeks (56 days) after the rVV primary immunization, each mouse received an IN lethal dose of 10^6 pfu of VV-WR diluted in 40µl total volume in sterile DMEM.

2.6 Anti-B5R Antibody ELISA

All enzyme linked immunosorbent assays (ELISAs) were performed as described by Barefoot et al [32]. Briefly, 96 well polystyrene Immulon II ELISA plates (Fisher Scientific) were coated with purified recombinant CPXV B5R protein (200 ng per well) in 200µl of bicarbonate buffer (0.1 M $NaHCO_3$) overnight at 4 °C. The wells were washed with 1x PBS 0.1% Tween 20 (Sigma), blocked with Superblock [15% normal

goat serum, 0.04% whey protein (BiPro, Davisco Foods Inc) in PBS-Tween] for 2hrs, washed, and incubated with diluted serum samples at a starting dilution of 1:25. Anti-B5R IgG antibodies were detected with alkaline-phosphatase conjugated goat derived antibody specific for the murine gamma heavy chain (Sigma M8770). Antibody reactivity was detected by adding pNPP substrate (4-nitrophenyl phosphate (Sigma N2640) at 1 mg/ml as directed by the manufacturer by incubating for 45 min and then reading the optical density (OD) at 405 nm using a PerkinElmer™ Victor 3 plate reader. Serum IgG endpoint titers were reported as the reciprocal of the highest dilution that gave absorbance greater than 2× over background OD determined using pre-immune serum at the same dilution. All serum samples were assayed in duplicate and compared individually.

2.7 B5R Recombinant Protein

The ectodomain (amino acids 1–279) of the B5R protein of CPXV was expressed and purified as described by Barefoot et al [32]. Briefly, the forward primer 5-TTCAGAATTCGCTAGCGTCGACGACCATGAAGACCATCTC-3 and reverse primer 5-TTGTGGATCCGGTACCTTAGTGATGATGGTGGTGATGGTGGTAGGTGGCCTCCAGGGACTCGATCTCCTG-3 were used to PCR amplify the ectodomain (amino acids 1–279) of the B5R protein of CPXV, with the addition of 5’ NheI and 3’ KpnI restriction endonuclease sites as well as a C-terminal polyhistidine-tag. The amplified fragment was cloned into pcDNA3.1+ plasmid (Invitrogen). Human 293T cells were transfected with the His-B5R

expression construct, and recombinant B5R protein was purified from culture supernatant using a nickel column.

2.8 Lymphocyte Analysis and Flow Cytometry

To obtain peripheral blood mononuclear cells (PBMCs), blood was collected into 14ml polystyrene tubes containing 3mls of DMEM with 40units/ml of heparin sodium (APP Pharmaceuticals, cat# 504031). Mice were bled from the submandibular vein and the blood was mixed by immediately inverting the tubes prevents coagulation. The blood was underlayed with Lympholyte® cell separation media (Lympholyte® M, cat# CL5031) and spun at 2500rpm for 20 minutes in a tabletop centrifuge (Sorvall Legend RT Plus) at room temperature (RT), after which lymphocytes were collected from the interface using a 1ml serological pipet. The PBMCs were then washed in 1xPBS pH 7.4, spun down at 1500rpm for 10mins, and resuspended in 200μl of 1xPBS 0.1% BSA. Cells were then incubated with F_c receptor block (BD Biosciences 553141) for 20 minutes at RT and then incubated in a 96 well V-bottom plate with FITC-conjugated rat anti-CD62L (BD Biosciences 553150), APC-conjugated rat anti-CD8α (BD Biosciences 553032), and PE-conjugated tetramer for 30 minutes at RT. The tetramer was an MHC class I K^b tetramer containing the immunodominant K^b-restricted B8 epitope of vaccinia virus (N-TSYKFESV-C) as described by Moutaftsi et al [122]. The tetramer was obtained from the NIH Tetramer Facility. After incubation with the indicated antibodies and tetramer, cells were then resuspended and fixed in 3% paraformaldehyde for 5 mins, spun,

resuspended in buffer, and analyzed using a FACS Canto (BD Biosciences). A total of 100,000 CD8+ gated events were collected and analyzed for tetramer positive cells.

2.9 Live In Vivo Bioluminescent Imaging

All recombinant VVs express fLUC under the control of an early VV promoter (AAAAAATGAAAAAA/TA). All mice receiving secondary immunizations with VV expressing fLUC were imaged at the following time points: 4hrs, 12hrs, 24hrs, and every 24hrs until 120hrs after immunization (see **Table 4**). To detect bioluminescent signals, mice were lightly anesthetized with an isoflurane vaporizer and given a 200μl intraperitoneal (IP) injection of firefly D-luciferin (Caliper Life Sciences #22796). Production of fLUC will cleave the injected luciferin to its biologically active form, resulting in bioluminescence. Individual groups were then placed into the imaging chamber (Xenogen IVIS® Spectrum Imaging System) while still under anesthesia in the prone position to generate a dorsal image. Mice were imaged for one minute and a pseudocolor bioluminescent image was then overlaid on top of a gray scale image of the mouse. To ensure an even luciferin distribution and photon flux, mice were rested for 5 mins after luciferin injections. All images were then analyzed (Xenogen analysis software) and bioluminescence was then converted to a photon flux scale to compare individual mice and groups.

2.10 Immunoflourescent Confocal Microscopy

BSC-1 cells were grown to 50% confluency on glass coverslips in 30mm tissue culture dishes and infected with rVV+ATIs at an MOI of 10 pfu for 1 hour. Infection mixture was then removed and replaced with a transfection mixture containing a plasmid with the vaccinia A5 core protein fused to mGFP (A5-mGFP) under the control of its native promoter. Thirty-six hours post-infection (35 hrs post transfection) the cells were fixed in 3% paraformaldehyde-PBS, stained with indicated antibodies and nuclear stain, and analyzed by immunofluorescent confocal microscopy for VV ATIs with embedded MVs. Embedded MVs will have the A5-mGFP core protein incorporated and will fluoresce green at 395nm. To visualize the ATI protein, cells were permeated cells with a 0.5% Trition-X 100 detergent-PBS solution and stained with polyclonal mouse IgG specific for the ATIp followed by an anti-mouse IgG antibody (SouthernBiotech #1010-03) conjugated to phycoerythrin (PE) that will label the polyclonal mouse IgG and fluoresce red upon excitation. Hoechst stain (Invitrogen #H3569) was used to stain the nuclei of the infected cells that will fluoresce blue upon excitation. Vaccinia Virus ATIs should appear circular with a green interior and circumscribed by a red "halo-like" exterior. Both (A) and (B) in **Figure 1** are representative photos of infected/transfected cells that have ATIs in the cytoplasm. Arrows indicate cytosolic ATIs, virus factories (sites of replication). Images were captured using a Zeiss® LSM 510 inverted confocal microscope with z-sectioning at 100x magnification and were analyzed using Zeiss LSM 510 version 4.2 software.

2.11 Statistical Analysis

All statistical tests were performed using unpaired Student’s t-test with Graph Pad Prism statistical analysis software. Results were considered significant when the p value of <0.05 was reached. All p-values are rounded to three significant figures unless otherwise indicated. The standard error of the mean (SEM) is indicated by error bars in all graphs.

3. RESULTS

3.1. Construction of rVVs Expressing Full-length A-type Inclusions

Cowpox virus A-type inclusions (ATIs) consist of multiple 160kDa individual ATI proteins (ATIp) that result in the formation of spherical matrix that encapsulates infectious MVs that have been directed to aggregate in the interior of the ATIp matrix by the native CPXV 60kDa P4c packaging protein that is also found in VV. There are two rVVs used in this study, rVV+ATIs and rVV-WT. The rVV+ATIs construct has a phenotype that expresses the full-length CPXV ATIp, thus giving the rVV the ability, in conjunction with the native P4c protein, to form ATIs with embedded MVs. The rVV-WT construct is essentially an rVV that retains all characteristics of the wild-type VV-WR strain from which the rVV was constructed. All rVVs used in this study express the selectable maker xanthine-guanine phosphoribosyltransferase (GTP), for rVV isolation, and fLUC for virus imaging. A description of the rVV construction is in the Materials and Methods and is diagrammed in **Figure 2**. Briefly, using similar methods described in McKelvey, T.A., et al (4), the first recombination event between the parent virus, VV-WR, and plasmid will replace the native 94kDa truncated ATIp gene and 58kDa P4c gene with the selectable marker beta-glucuronidase (GUS). This genetic recombination event will create a selectable rVV vector from which all of the rVVs will be constructed. A second recombination event will yield an rVV that expresses a full-length 160kDa CPXV ATIp, the native 60kDa P4c protein, selectable marker GTP, and fLUC for bioluminescent imaging. This virus, rVV+ATIs, will be able to produce ATIs

with embedded infectious MVs, in a manner similar to wild-type CPXV (CPXV-WT). An alternate recombination between virus and plasmid will yield the a virus, rVV-WT, that expresses the native truncated 94kDa ATIp and the native intact 60kDa P4c protein, along with GTP and fLUC. This virus will have essentially the same phenotype and pathological characteristics as the partner wild-type VV-WR, but now can produce fLUC for bioluminescent imaging. Expression of all inserted genes was confirmed, as described, by PCR amplification of the inserts, sequencing of the viral DNA, and the protein expression was checked by western blot analysis and (S^{35}) methionine radiolabeling. In this study we will also use a virus preparation that contains isolated ATIs with embedded infectious MVs that are harvested from cell cultures infected with rVV+ATIs. These ATI preparations will be enriched for just the ATIs with removal of any other form of virus (MVs, EEVs). This virus preparation will be referred to as enriched-ATIs (En-ATIs) throughout this study. The rVVs used in our studies have been named according to phenotype and are listed in **Table 1**.

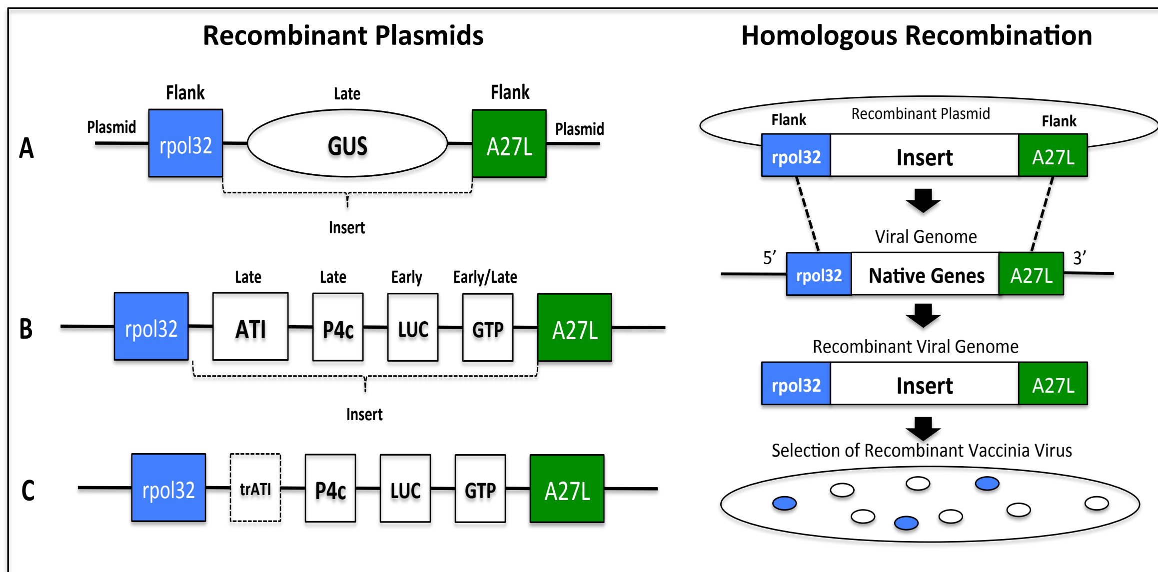


Figure 2. Recombinant Vaccinia Virus Construction. (A) Plasmid pGEM7zf+ containing the GUS insert flanked by homologous VV sequence from C-termi of rpol32 and the N-termi of A27L. By homologus recombination at the flanking sequences, the insert will replace the native gene sequences in wild-type VV-WR. The recovered rVV will contain the GUS insert (rVV-GUS). (B) Plasmid containing full-length ATI gene and P4c gene along with fLUC and GTP genes. Homologus recombination will yield rVV+ATI. (C) Plasmid containing the wild-type VV ATI gene that results in a truncated ATIp (trATIp) along with the native full-length P4c gene, along with fLUC, and GTP for virus selection and imaging. Homologus recombination will yield rVV-WT. **Table 1** contains the nomenclature and description of all viruses used. For a full description of rVV construction please see **Materials and Methods**.

Table 1. Properties and Names of Recombinant Viruses (rVVs) used in this study.

Virus	Full-length P4c	Full-length ATI	LUC	GTP	GUS	Virus Description
VV-WR	Yes	No	No	No	No	Vaccinia WR (Western Reserve) Wild-Type Virus
rVV-GUS	No	No	No	No	Yes	GUS has replaced native P4C and ATI genes
rVV-WT	Yes	No	Yes	Yes	No	Wild-type WR virus that expresses LUC and GTP.
rVV+ATIs	Yes	Yes	Yes	Yes	No	Virus that can produce ATIs with embedded MVs.
En-ATIs	Yes	Yes	Yes	Yes	No	Purified ATIs (by centrifugation) from rVV+ATIs cell lysate (ATIs only)

3.2. Recombinant VV Primary Immunization Followed by VV-WR Lethal Challenge

3.2.1. Pathological Response to rVV Primary Immunization and VV-WR Lethal Challenge

Improvement of safety is one of the main challenges that researchers face with the development of VV based vaccine vectors. (1). Genetically modified VV strains such as MVA and NYVAC are unable to replicate in many mammals, including humans, but have proven successful at generating immunity toward those VV strains [3, 123]. These replication deficient rVVs have a better safety profile that results in reduced side effects but fails to elicit optimal immune responses to foreign proteins expressed from other pathogens and ultimately requires boosting immunizations to generate optimal immunity [3, 123]. It is possible that a genetically modified rVV that has the ability to produce ATIs with embedded infectious MVs, similar to the viruses used in this study, could alter the resulting pathological consequences and immune responses in comparison to wild-type VV-WR. In this study, we compared the *in vivo* pathological consequences that result from immunization with either the wild-type vector (rVV-WR) or the rVV that results in the formation of ATIs with embedded virus (rVV+ATI). To better understand the effects of the ATIs with embedded virus, we administered a primary intranasal (IN) immunization to groups (n = 5) of 10-12 week old adult female C57BL/6 (B6) mice with 1 x 10^5 plaque forming units (PFUs) of each rVV. We then monitored clinical parameters of weight loss and temperature change until either the

humane endpoint was reached or until full recovery. Weight loss has been shown to be a strong correlate of VV infection and resulting disease [32, 68]. Upon administration of the different rVVs, weight loss was induced approximately 3 to 4 days post rVV immunization with maximal weight loss occurring at day 7, followed shortly there after by weight gain and recovery **(Figure 3a).** The group's mean body weights at day 7 are arranged in order from the group that had the least amount of weight loss to the group with the highest peak weight loss, measured as a percentage of body weight at day 0 (day of immunization): En-ATIs (91.91% ± 2.42) < rVV-WT (86.53% ± 2.17) < rVV+ATIs (82.87% ± 2.40) (**Table 2)**. The rVV+ATI immunized group lost significantly more weight than the group that received En-ATIs at day 5 (p = 0.001), day 6 (p = 0.001), and day 7 (p = 0.029) **(Table2)**. There were no significant differences in weight loss between the groups immunized with rVV+ATI or rVV-WT during the span of peak weight loss between days 5 and 9 post-immunization **(Table 2)**. The group immunized with En-ATIs lost significantly less weight than the group immunized with rVV-WT at day 5 (p = 0.040) and day 6 (p = 0.041) **(Table 2)**. All the groups that received the rVV primary IN immunization had a survival rate of 100 percent (data not shown) and fully recovered approximately 2 weeks after the immunization as indicated by body weights returning to their Day 0 weights prior to rVV immunization **(Figure 3a)**. Core body temperature loss as a result of viral infections, such as influenza, has been shown to be a consistent marker of the viral disease in mice [39]. A decrease in the core body temperature correlates with VV induced sickness and disease and conversely returns to

normal physiological levels during the recovery period [124]. Therefore, we monitored temperature change by use of a rodent rectal thermometer as an additional and secondary measureable parameter of adverse pathological effects of the rVV primary intranasal immunization. All groups that were immunized followed a very similar pattern of core body temperature change with no significant differences during sickness or recovery **(Figure 3b)**. All immunized groups lost less than 1 degree Celsius of core body temperature during the 8 days post immunization **(Figure 3b)**. Depending on the route of inoculation, *Ectromelia* virus (ECTV) can induce varying levels of disease depending on the dose of virus [125]. The induction of the inflammatory response that results in body temperature changes has been shown to be a dose dependent response to many PAMPs [70]. This could be a plausible explanation for the insignificant temperature changes we observed with the IN primary immunization, as previous studies that we have conducted resulted in significant temperature loss (>5 degrees Celsius) in mice given an IN lethal dose of VV-WR (unpublished data).

Overall, these results suggest that immunization with rVV+ATI results in a significant amount of sustained weight loss in mice when compared to En-ATIs, but not in comparison to rVV-WT. With the observed differences in weight loss and disease between the different rVV constructs we then asked the question, is the degree of protection that is generated from the different rVV IN primary immunizations different

between groups of mice challenged with a lethal dose of wild-type VV-WR? It has been shown that the adaptive immune response is crucial for protection against a second poxvirus infection (48). To assess if there were differences in the level of protection induced by the different rVV primary immunizations we challenged all the immunized groups with a lethal dose of wild-type VV-WR. If a primary immunization with rVV+ATIs and En-ATIs induces varying levels of protection in comparison to the rVV-WT immunization, we may be able to substantiate these differences by tracking differences in weight loss, body temperature recovery and the adaptive immune responses resulting from a lethal challenge.

Two months after the rVV primary immunization, all groups were challenged with an IN lethal dose, 1×10^6 pfu, of VV-WR and were then monitored for weight loss and temperature change. If protection induced by the rVV primary immunization varies between the groups, we reasoned that these differences would become apparent in weight loss, temperature loss, and survival after the VV-WR lethal challenge. There were no significant differences in weight loss ($p > 0.05$, Student's t test) or temperature change between the challenged groups **(Figure 3c)**. All mice that previously received the rVV primary IN immunization had a survival rate of 100 percent and fully recovered after the VV-WR lethal challenge **(Figure 3).** There were also no significant differences in temperature loss and all groups followed a similar temperature profile as the PBS control mock challenged group **(Figure 3d)**.

These results indicate that the rVV primary IN immunizations result in full protection against lethal challenge and therefore suggest that the different rVVs used for these immunizations induce similar adaptive immune responses. To determine whether or not this was true, we analyzed the cellular and humoral immune responses generated by rVV primary immunizations.

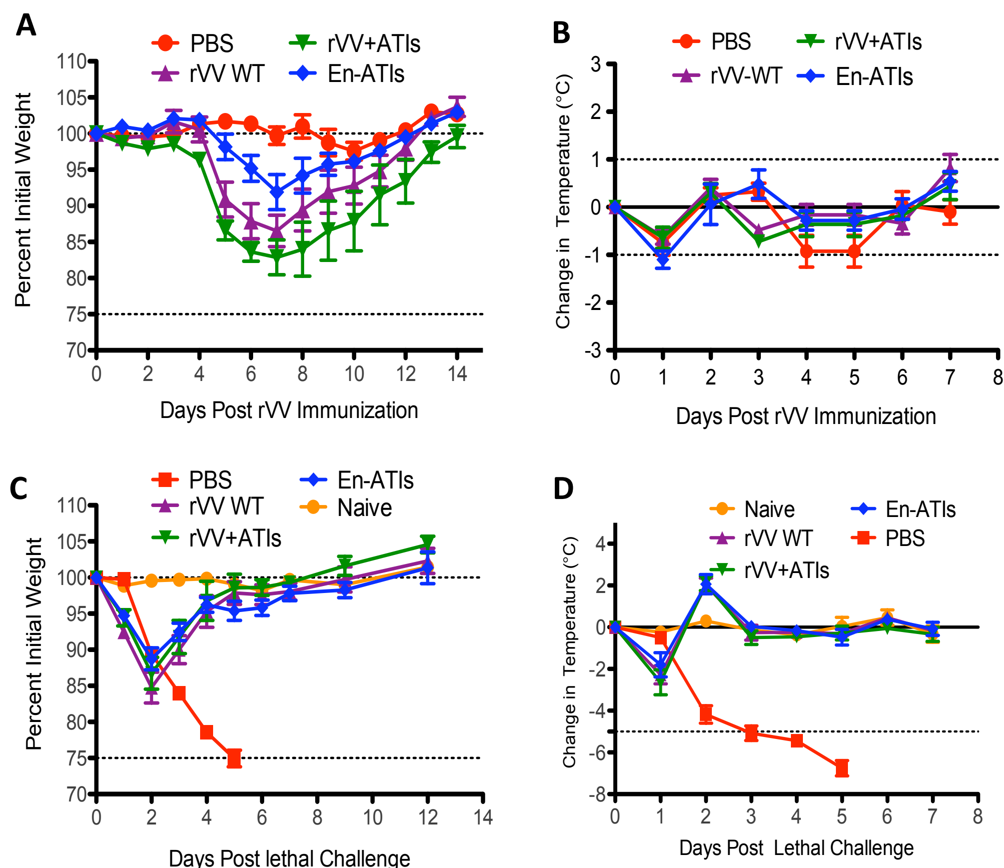


Figure 3. Weight and Temperature Change of rVV Primary Immunization and VV-WR Lethal Challenge. (A) and (B). Ten to twelve week old female B6 mice were given an IN primary immunization of 1 x 10^5 pfu of each rVV and monitored daily for weight and temperature change. Graphs represent the mean percent of the day 0 (day of immunization) initial weight ± SEM. n = 5 (C) and (D). Fifty-six days after the rVV primary immunization, the groups were given a lethal dose of VV-WR, 1 x 10^6 pfu, IN and monitored for weight and temperature change. Graphs show change (±) in degrees Celsius from the mean initial temperature ± SEM. All mice receiving the rVV primary immunization survived the VV-WR lethal dose challenge with none of the naïve mice surviving the challenge.

Table 2. Statistical Analysis of rVV Primary Immunization Induced Weight Loss. The mean (± SEM) weight loss from days 5 through 9 for each of the groups is shown and P values along with group-by-group comparisons. All comparisons were made using Student's t test with $p < 0.05$ indicating significance. Significant differences are highlighted in yellow.

	Primary Immunization	Mean ± SEM	Day 5 P-val rVV+ATIs	Day 5 P-val En-ATIs	Day 6 P-val rVV+ATIs	Day 6 P-val En-ATIs	Day 7 P-val rVV+ATIs	Day 7 P-val En-ATIs	Day 8 P-val rVV+ATIs	Day 8 P-val En-ATIs	Day 9 P-val rVV+ATIs	Day 9 P-val En-ATIs
Day 5	rVV-WT	90.86 ± 2.42	0.160	0.040								
	rVV+ATIs	86.60 ± 1.31	x	0.001								
	En-ATIs	98.16 ± 1.76	x	x								
Day 6	rVV-WT	87.88 ± 2.42			0.16	0.041						
	rVV+ATIs	83.63 ± 1.30			x	0.001						
	En-ATIs	95.18 ± 1.79			x	x						
Day 7	rVV-WT	86.53 ± 2.17					0.291	0.177				
	rVV+ATIs	82.87 ± 2.40					x	0.029				
	En-ATIs	91.91 ± 2.42					x	x				
Day 8	rVV-WT	89.42 ± 2.88							0.283	0.242		
	rVV+ATIs	84.00 ± 3.73							x	0.051		
	En-ATIs	94.19 ± 2.42							x	x		
Day 9	rVV-WT	91.97 ± 2.98									0.317	0.292
	rVV+ATIs	86.57 ± 4.08									x	0.069
	En-ATIs	95.00 ± 1.53									x	x

3.2.2. B8R-specific CD8 T Cell Response after rVV Primary Immunization and VV-WR Lethal Challenge

The CD8 T cell response plays a very important role in the clearance of poxvirus infections but the impact of the vaccinia specific CD8 T cell response on protection is secondary to the importance of the CD4 T cell and B cell driven antibody responses (48). Though not as effective as the humoral response, the VV-specific CD8 response can still provide protection and assist with clearance of both primary and secondary poxvirus infections in the absence of the B cell response (55). To determine whether the induction and duration of the vaccinia specific CD8 T response is altered by the different rVV primary immunizations, we used an MHC Class I H-2K^b tetramer containing the epitope $B8R_{20-27}$ (N-TSYKFESV-C) to quantitate the number of anti-B8R-specific CD8 T cells in the peripheral blood. The B8R gene, native to both VV and CPXV, encodes a secreted IFNγ receptor that contains one of the most immunodominant components of both CPXV and VV in mice [126]. B8R has also been shown to be an important virulence factor in VV and CPXV infections, as it is able to bind and sequester IFNγ and inhibit its antiviral properties [126].

In this study, the groups that previously received the rVV primary immunization were bled via the submandibular vein at various time points to analyze peripheral blood anti-B8R-specific CD8 T cell numbers **(Figure 4).** A representative flow cytometric analysis of anti-B8R-specific CD8 T cells from the peripheral blood of challenged mice is shown

in **Figure 4a**. Lymphocytes were first gated on forward scatter (FSC) and side scatter (SSC) parameters and then gated for CD8+ T cells (CD8+). From the gated CD8+ T cell population, cells negative for expression of CD62L and cells positive for the B8R tetramer (Tet+) cells were enumerated **(Figure 4a)**. We analyzed CD8 T cells that do not express CD62L so that we could specifically follow cells that are most capable of cytotoxic T lymphocyte (CTL) effector functions [127]. The transmembrane protein CD62L (L-selectin) is an adhesion molecule expressed on leukocytes. T cells that express CD62L are able to migrate into secondary lymphoid tissues. Upon antigen stimulation, CD62L is rapidly cleaved from the cell surface, which renders these antigen specific cells able to leave lymph nodes and circulate to virus-infected peripheral tissue [127]. We specifically tracked CD62L-negative (CD62L-) cells so that we could monitor virus stimulated CD8+ lymphocytes [128].

Overall, the rVV primary immunization resulted in robust numbers of anti-B8R-specific CD8 T (Tet+ cells) cells at day 7 in all groups followed by a gradual decline in Tet+ cells until administration of the lethal challenge at day 56 **(Figure 4b and Table 3a)**. Day 7 Tet+ cell proportions are listed as the percentage of Tet+ cells (naïve Tet+ background subtracted) and follows from least to greatest: En-ATIs (8.00% ± 1.13) < rVV+ATIs (8.82% ± 2.08) < rVV-WT (11.50% ± 1.38) **(Figure 4b and Table 3a)**. Upon statistical analysis no significant differences in Tet+ cells at day 7 were found among the rVV immunized groups 7 **(Table 3a)**. After day 7, the number of Tet+ cells declined to

approximately 1 percent (after subtracting the naïve Tet+ background) for all groups at day 55 (1 day prior to lethal challenge) after the rVV primary immunization **(Figure 4b and Table 3a).** These results suggest that the induction, expansion, and longevity of the anti-B8R-specific CD8 T cell response is not significantly altered by the production of ATIs with embedded MVs (rVV+ATIs) or by direct inoculation with En-ATIs. Even though these results may suggest that the percentage of antigen specific CD8 T cells is not affected by the rVVs (rVV+ATIs, En-ATIs), we do not know; 1), if the CTL function is affected 2), if the protective capacity of these anti-B8R-specific CD8 T cells is altered in response to the different rVV constructs or 3), how proportions of peripheral blood Tet+ cells correlate with disease and virus clearance.

To analyze reactivation of the anti-B8R-specific CD8 T cell memory response we challenged each group with a lethal IN dose of VV-WR at 56 days after the rVV IN primary immunization. Each group was bled at day 55 to analyze the level of peripheral blood Tet+ cells and was found to be approximately 1 percent for all groups prior to challenge **(Figure 4b and Table 3a)**. At 5 days post lethal VV-WR challenge (day 61) the rVV-WT immunized group had significantly more Tet+ cells (12.06% ± 1.72, $p = 0.047$) than the En-ATI group (7.04% ± 1.04) **(Figure 4b and Table 3b)**. At 14 days (day 70) post lethal challenge the rVV-WT group had a significantly higher level of Tet+ cells (9.27% ± 1.63, $p = 0.050$) than the rVV+ATIs immunized group (3.86% ± 1.50) **(Figure 4b and Table 3b)**. There were no significant differences in

the induction, expansion, or decline of Tet+ cells between other groups at any other time point measured through 21 days (day 77) post lethal challenge **(Figure 4b and Table 3b).** The significant difference between the induction of Tet+ cells at day 7 in mice receiving the rVV primary immunization compared to rVV-WT or En-ATIs seems to play a minimal role in protection upon lethal VV-WR challenge because there were no observed differences in weight loss, temperature change, or survival among these groups.

Although there were no differences in weight loss, body temperature, recovery, and survival among the rVV immunized groups after lethal challenge, we cannot exclude the possibility that the CTL function of the anti-B8R-specific CD8 T cells and their ability to clear infected cells could vary among the different rVV immunized groups.

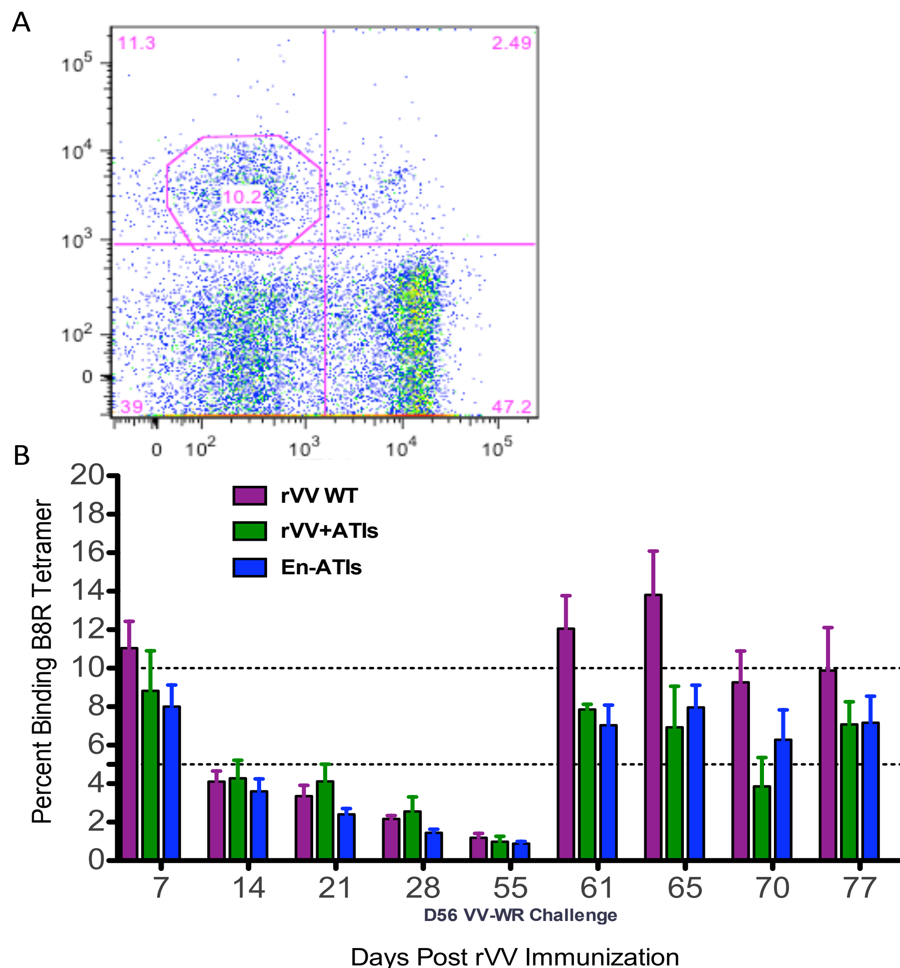


Figure 4. Anti-B8R CD8 T Cells Detected after Primary Immunization and VV-WR Lethal Challenge. Groups of mice (n = 5) were given an IN primary immunizing dose of 1 x 10^5 pfu of each of the rVVs and followed with a lethal VV-WR challenge dose at 56 days (2 months) after the rVV primary immunization. (A) Representative dot plot of peripheral blood CD8+ gated CD62L- B8R specific T cells 7 days post rVV-WT immunization. Inset gate has been drawn around B8R specific cells. (B) Proportions of B8R-specific CD8+ and CD62L- cells detected at various time points after primary immunization and after lethal challenge. Mice were lethally challenged 56 days after the primary rVV immunization. Error bars represent the SEM.

Table 3. Statistical Analysis of the Anti-B8R CD8 T Cell Response to the rVV Primary Immunization and Lethal Challenge. Proportions of peripheral blood B8R-specific CD8 T cells of the immunized and challenged groups were compared to determine statistical significance. (A) Comparison of anti-B8R CD8 T cell response between different rVV primary immunizations. (B) Comparison of anti-B8R CD8 T cell response after VV WR lethal challenge. The mean ± SEM is listed for each group at each day. P values corresponding to group comparisons are listed with significant P values<0.05 highlighted in yellow. All comparisons were made using Student's t test.

A — rVV Primary Immunization

			Day 7 P-val		Day 14 P-val		Day 21 P-val		Day 28 P-val		Day 55 P-val	
		Mean ± SEM	rVV+ATIs	En-ATIs	rVV+ATIs	En-ATIs	rVV+ATIs	En-ATIs	rVV+ATIs	En-ATIs	rVV+ATIs	En-ATIs
Day 7	rVV-WT	11.05 ± 1.38	0.408	0.139								
	rVV+ATIs	8.82 ± 2.09	x	0.742								
	En-ATIs	8.00 ± 1.13	x	x								
Day 14	rVV-WT	4.11 ± 0.55			0.879	0.575						
	rVV+ATIs	4.28 ± 0.93			x	0.572						
	En-ATIs	3.61 ± 0.65			x	x						
Day 21	rVV-WT	3.35 ± 0.57					0.494	0.191				
	rVV+ATIs	4.12 ± 0.89					x	0.119				
	En-ATIs	0.95 ± 0.31					x	x				
Day 28	rVV-WT	2.17 ± 0.17							0.628	0.028		
	rVV+ATIs	2.56 ± 0.74							x	0.195		
	En-ATIs	1.45 ± 0.18							x	x		
Day 55	rVV-WT	1.20 ± 0.22									0.587	0.256
	rVV+ATIs	0.99 ± 0.28									x	0.739
	En-ATIs	0.89 ± 0.11									x	x

B — VV WR Lethal Challenge

			Day 61 P-val		Day 65 P-val		Day 70 P-val		Day 77 P-val	
		Mean ± SEM	rVV+ATIs	En-ATIs	rVV+ATIs	En-ATIs	rVV+ATIs	En-ATIs	rVV+ATIs	En-ATIs
Day 61	rVV-WT	12.06 ± 1.72	0.052	0.047						
	rVV+ATIs	7.86 ± 1.74	x	0.475						
	En-ATIs	7.04 ± 1.04	x	x						
Day 65	rVV-WT	13.82 ± 2.26			0.068	0.061				
	rVV+ATIs	6.93 ± 2.13			x	0.683				
	En-ATIs	7.97 ± 1.15			x	x				
Day 70	rVV-WT	9.27 ± 1.63					0.050	0.233		
	rVV+ATIs	3.83 ± 1.50					x	0.303		
	En-ATIs	6.29 ± 1.55					x	x		
Day 77	rVV-WT	9.89 ± 2.21							0.307	0.338
	rVV+ATIs	7.08 ± 1.17							x	0.965
	En-ATIs	7.13 ± 1.38							x	x

3.2.3. B cell Response to rVV Primary Immunization

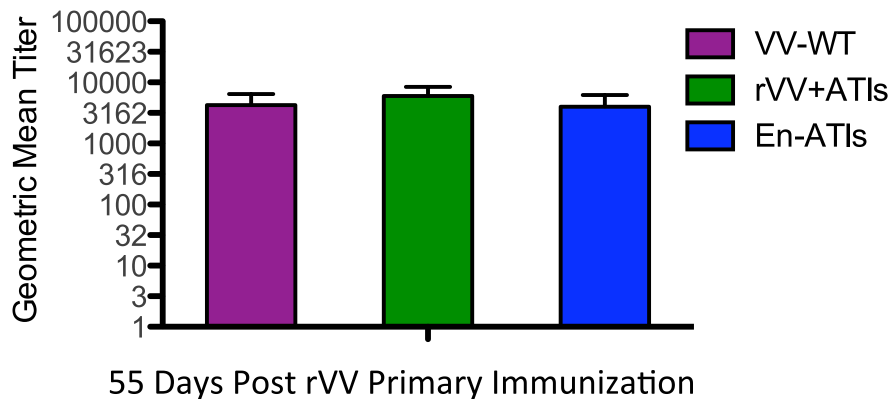
Orthopoxvirus infections result in potent humoral responses that are crucial in the clearance of both primary infections and secondary reinfections [56, 129]. Defects in antibody production result in either death or prolonged sickness in B cell deficient mice, even in the presence of anti-viral CD8 T cells [59].

To quantitatively measure the antibody response induced by the rVV primary immunization we used an ELISA that detects antibodies specific for the ectodomain of the B5R envelope protein. B5R is 42kDa glycoprotein protein expressed on surface of wrapped EVs and CEVs of *Orthopoxviruses* and has been shown to be an important protein involved in virus dissemination and spread of infection [62, 130]. It has been shown that the large extracellular domain of B5R is targeted by key neutralizing antibodies that prevent cell-to-cell spread and virus dissemination upon reinfection [62].

The rVV primary immunization induced quantitatively similar anti-B5R IgG antibody titers at day 55 ($p > 0.05$, Student's t test), one day prior to the VV-WR lethal challenge **(Figure 5a and b)**. Although each group has very similar amounts of anti-B5R IgG antibodies at day 55 prior to the lethal challenge, it is possible that the different types of rVV immunizations could differ in time of induction, peak production, and contraction of serum anti-B5R IgG antibody levels. However, we are unable to address

these issues because we collected serum at only one time point, day 55. The fact that these mice have strikingly similar levels of anti-B5R serum IgG antibodies approximately two months after the primary rVV immunization combined with what seems to be a very similar degree of protection from lethal VV-WR challenge in all groups **(Figure 3c and d)** suggests that ATIs with embedded MVs do not induce any subtle differences (in IgG isotype or specificity for example) in the humoral response between groups. This result could be promising, from a vaccine safety perspective, considering that mice immunized with En-ATIs exhibit an overall reduced weight loss upon the primary immunization when compared to the other rVV vectors (rVV-WT and rVV+ATIs).

A



B

	rVV Primary Immunization	Mean ± SEM	Day 55 rVV+ATIs (Pval)	Day 55 En-ATIs (Pval)
Day 55	rVV-WT	4240 ± 2204	0.612	0.941
	rVV+ATIs	6000 ± 2477	x	0.568
	En-ATIs	4000 ± 2234	x	x

Figure 5. Anti-B5R IgG Antibody Response in Mice Immunized with rVV. Mice receiving the rVV primary immunization were bled for serum at day 55. Anti-B5R IgG serum antibodies were measured using a B5R specific ELISA. (A) Titers are represented as the reciprocal of the last positive dilution (2-fold over naïve background). Error bars represent SEM. (B) Groups were statistically compared using Student's t test ($p < 0.05$).

3.3. Pre-existing Immunity: VV-WR Prime and rVV Boost Efficacy

3.3.1. Addressing The Issue of Poxvirus Pre-existing Immunity

Pre-existing immunity to *Orthopoxviruses* can provide substantial protection against subsequent re-infection with *Orthopoxviruses*. Recombinant vaccinia virus vectors expressing foreign genes of pathogenic microorganisms might provide a promising strategy for vaccination against many infectious pathogens [131-133]. A problem associated with the use of rVVs as potential vaccine vectors is that currently, a large percentage of the worldwide human population already has pre-existing immunity against *Orthopoxviruses* due to the vaccination against the smallpox. Global vaccination against smallpox ended in the early 1980s with only high-risk individuals receiving the vaccine since, thus leaving a large population with pre-existing poxviral immunity. The DRYVAX® vaccine used to vaccinate against smallpox was based on live VV and has been shown to induce long-lasting protective immunity against *Variola* that can span the lifetime of a healthy individual [134]. The persistence of long-lasting immunity in individuals that received a smallpox vaccination could impair the efficacy of immune responses against not only the vaccinia vectors themselves, but to the foreign antigens expressed within those vectors. The impact of pre-existing immunity to rVV vaccine vectors has been poorly studied. However, the results of one study showed that mice receiving an empty vector VV-WR immunization and boosted with recombinant modified vaccinia Ankara (rMVA) expressing a foreign protein exhibited a considerably decreased cellular and humoral response to both the vector and the foreign antigens

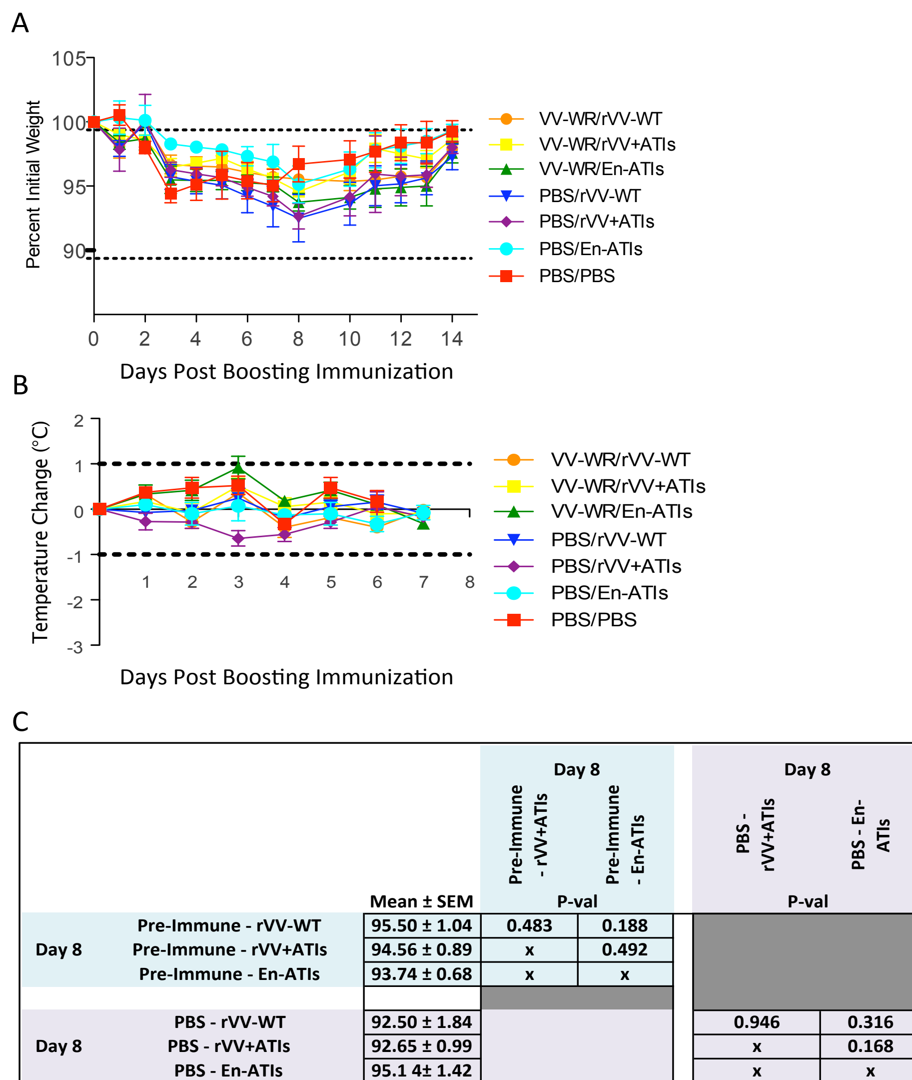
[135]. It is possible that the ATI protein could alter the recognition of proteins that are on the surface of the MVs and shield the encased virus from pre-existing neutralizing antibodies. Therefore, our En-ATIs or rVV+ATIs virus vectors could avoid the problem of pre-existing immunity. To investigate this hypothesis we performed an experiment in which mice were given an intramuscular (IM) primary immunization of 1 x 10^4 pfu of VV-WR at 12 weeks of age and were boosted subcutaneously (SQ) with 1 x 10^3 pfu of one of the different rVVs **(Table 4)**.

Table 4. Strategy for rVV Boosting Immunization in the Presence of Pre-existing Immunity. Twelve week old female B6 mice were given a IM primary immunization of 1 x 10^4 pfu of VV-WR in a total volume of 100 μl (50 μl per quadricep) of sterile PBS. At approximately 34 weeks of age (155 days post VV-WR priming immunization), the immunized mice were given a SQ boosting dose of 1 x 10^3 pfu (200 μl total volume) of the different rVVs in the nape of the neck. In vivo imaging was performed at the indicated time points and described in the materials and methods.

Group	Prime (w/10^4 pfu IM) at 12wks		Boost (w/10^3 pfu IP) at 34wks	Mice (n)
VV-WR/rVV-WT	VV-WR	-	rVV-WT	7
VV-WR/rVV+ATIs	VV-WR	-	rVV+ATIs	7
VV-WR/En-ATIs	VV-WR	-	EN-ATIs	6
PBS/rVV-WT	PBS	-	rVV-WT	7
PBS/rVV+ATIs	PBS	-	rVV+ATIs	7
PBS/En-ATIs	PBS	-	En-ATIs	6
PBS/PBS	PBS	-	PBS	5

If the presence of ATIs improves the outcome of vaccination in the individuals with pre-existing immunity to VV-WR, we predict less weight loss and lower temperatures in mice with VV immunity when boosted with ATI producing vectors compared to mice boosted with the rVV vectors that cannot produce ATIs. However, our results show no significant differences in weight loss among the mice that received rVV boosting with or without ATIs **(Figure 6a and c)**. There were also no significant changes in temperature between any of the rVV-boosted groups **(Figure 6b)**.

Also, our results show no significant differences in weight or temperature between groups of mice treated with PBS instead of VV primary immunization and subsequently boosted with or without ATIs **(Figure 6a, b, and c)**. It is important to note that there was an unusual amount of weight loss in the control group that received PBS only **(Figure 6a)**. This could be a result of possible adverse reactions or induced stress from the isoflurane anesthesia and luciferin injections during the in vivo imaging regimen. Therefore, it is also possible that the weight loss in the groups that received an rVV boosting immunization could be due to the imaging regimen. This is supported by the fact that we have shown previously that an IM priming dose of 1 x 10^4 pfu of VV-WR does not induce weight loss in adult B6 mice (data not shown).



			Day 8		Day 8	
		Mean ± SEM	Pre-Immune - rVV+ATIs (P-val)	Pre-Immune - En-ATIs (P-val)	PBS - rVV+ATIs (P-val)	PBS - En-ATIs (P-val)
Day 8	Pre-Immune - rVV-WT	95.50 ± 1.04	0.483	0.188		
	Pre-Immune - rVV+ATIs	94.56 ± 0.89	x	0.492		
	Pre-Immune - En-ATIs	93.74 ± 0.68	x	x		
Day 8	PBS - rVV-WT	92.50 ± 1.84			0.946	0.316
	PBS - rVV+ATIs	92.65 ± 0.99			x	0.168
	PBS - En-ATIs	95.1 4± 1.42			x	x

Figure 6. Weight and Temperature Change after Boosting with rVV. Ten to twelve week old female B6 mice were given an IM primary immunization of 1 x 10^4 pfu of VV-WR. (A and B) Approximately 22 weeks (155 days) after the VV-WR primary immunization the groups were given a SQ boosting immunization of 1 x 10^3 pfu of the indicated rVV and monitored for weight and temperature change. The x-axis represents post rVV boosting immunization. (C) Groups were statistically compared at the peak weight loss, day 8, by Student's t test.

3.3.2. The T Cell Response in the Presence of Pre-existing Immunity to VV

We next asked the question, does boosting with either En-ATIs or rVV+ATIs compared to rVV-WT alter the proportion of detectable anti-viral CD8 T cells in the presence of pre-existing immunity to VV? We observed no significant differences ($P > 0.05$, Student's t test) with the recall and expansion of anti-B8R-specific CD8 T cells in either of the rVV boosted groups at any time point through the course of infection or recovery. Although the group that received the VV-WR priming and rVV+ATIs boosting immunization developed the highest percentage of tet+ CD8 T cells in the peripheral blood, this response was not significantly different from the response of the other groups at any time point **(Figure 7 and Table 5a)**. The minimal, but not statistically significant differences in the anti-B8R-specific CD8 T cell response do not seem to have an effect on either weight loss or recovery between the groups that received the VV-WR prime and rVV boost **(Figure 6a, b, and c).** These results suggest that ATIs with embedded MVs do not make a significant difference in the recall and expansion of anti-B8R-specific CD8 T cell memory response in the presence of pre-existing immunity from the wild-type virus VV-WR. At approximately 22 weeks (or 155 days) post-VV-WR primary immunization, the control (PBS) treatment followed by the rVV boosting immunization resulted in the induction of relatively high percentages of anti-B8R-specific CD8 T cells. At day 164 (day 9 after the rVV boost), peripheral blood of the control group given PBS, and then immunized with rVV+ATIs contained a significantly higher proportion (5.98% ± 0.46) of anti-B8R-specific CD8 T cells

compared to blood of the group immunized with rVV-WT (4.15 ± 0.44, p = 0.029) or with En-ATIs boosted group (4.45 ± 0.41, p = 0.048) **(Figure 7 and Table 5b)**. There were no significant differences in the percentage of peripheral blood anti-B8R-specific CD8 T cells at any of the other time points after boosting with the rVVs. These results suggest that ATIs do not significantly alter the CD8 T cell response in the presence of preexisting immunity to wild-type VV-WR. .

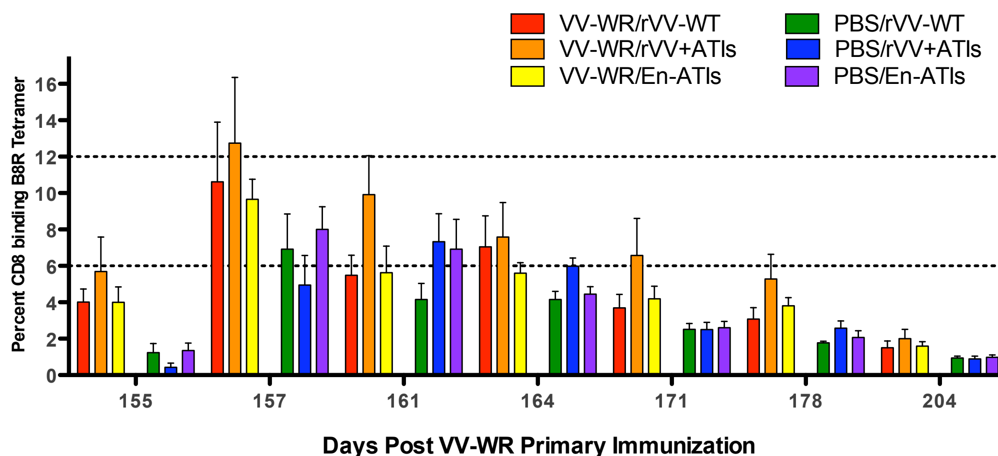


Figure 7. VV-WR Primed and rVV Boosted Anti-B8R-specific CD8 T Cell Response. Twelve-week-old female B6 mice were given an IM primary immunization with 1 x 10^4 pfu of VV-WR. Mice were bled on day 155, 8 hrs prior to the rVV boosting immunization to measure anti-B8R-specific CD8 T cell proportions prior to boosting. At 155 days post VV-WR priming (approximately 22 weeks) individual groups of mice (see Table 4 for total number of mice per group) were given a SQ boosting immunization of 1 x 10^3 pfu of the different rVVs. Anti-B8R-specific CD8 T cell proportions were measured at various time points . The first time point at which proportions of anti-B8R-specific CD8 T cells were analyzed after the boost is day 157 (2 days after the boosting immunization). Control groups (green, blue, and purple bars) were given PBS instead of the priming immunization. . Error bars represent the SEM.

Table 5. VV-WR Primed and rVV Boosted Anti-B8R-specific CD8 T Cell Response - Statistical Analysis. Peripheral blood B8R-specific CD8 T cell percentages were compared to determine statistical significance at various time points (A) Mice were primed with VV-WR and boosted and anti-B8R-specific CD8 T cell percentages were measured at various time points. The immunized groups were boosted 155 days later with rVV-WT, rVV+ATIs, or En-ATIs. Mice were bled on day 155 8hrs prior to the rVV boosting immunization to measure anti-B8R-specific CD8 T cell percentages prior to boosting (B) Control mice were treated with PBS and then immunized on day 155 with one of the rVV constructs Means ±SEM are listed for each group at each day (y axis). P values corresponding the group comparisons (x axis vs. y axis) are listed with significant values highlighted in yellow. All comparisons were made using Student's t test being significant at $p < 0.05$.

A

		Mean ± SEM	Day 155 P-val rVV+ATIs	Day 155 P-val En-ATIs	Day 157 P-val rVV+ATIs	Day 157 P-val En-ATIs	Day 161 P-val rVV+ATIs	Day 161 P-val En-ATIs	Day 164 P-val rVV+ATIs	Day 164 P-val En-ATIs	Day 171 P-val rVV+ATIs	Day 171 P-val En-ATIs	Day 178 P-val rVV+ATIs	Day 178 P-val En-ATIs	Day 204 P-val rVV+ATIs	Day 204 P-val En-ATIs
Day 155	rVV-WT	4.008±0.725	0.4408	0.995												
	rVV+ATIs	5.685±1.899	x	0.449												
	En-ATIs	4.0±0.8481	x	x												
Day 157	rVV-WT	10.62±3.282			0.678	0.792										
	rVV+ATIs	12.75±3.61			x	0.445										
	En-ATIs	9.663±3.46			x	x										
Day 161	rVV-WT	5.48±1.107					0.114	0.942								
	rVV+ATIs	9.918±2.131					x	0.148								
	En-ATIs	5.62±1.469					x	x								
Day 164	rVV-WT	7.050±1.694							0.89	0.448						
	rVV+ATIs	7.588±1.894							x	0.354						
	En-ATIs	5.598±0.5782							x	x						
Day 171	rVV-WT	3.698±0.735									0.23	0.637				
	rVV+ATIs	6.578±2.028									x	0.309				
	En-ATIs	4.198±0.685									x	x				
Day 178	rVV-WT	3.078±0.631											0.19	0.386		
	rVV+ATIs	5.275±1.360											x	0.344		
	En-ATIs	3.803±0.4512											x	x		
Day 204	rVV-WT	1.505±0.370													0.467	0.854
	rVV+ATIs	2.000±0.519													x	0.501
	En-ATIs	1.590±0.2427													x	x

B

		Mean ± SEM	Day 155 P-val rVV+ATIs	Day 155 P-val En-ATIs	Day 157 P-val rVV+ATIs	Day 157 P-val En-ATIs	Day 161 P-val rVV+ATIs	Day 161 P-val En-ATIs	Day 164 P-val rVV+ATIs	Day 164 P-val En-ATIs	Day 171 P-val rVV+ATIs	Day 171 P-val En-ATIs	Day 178 P-val rVV+ATIs	Day 178 P-val En-ATIs	Day 204 P-val rVV+ATIs	Day 204 P-val En-ATIs
Day 155	rVV-WT	1.238±0.4914	0.1855	0.869												
	rVV+ATIs	0.4295±0.2248	x	0.097												
	En-ATIs	1.348±0.4092	x	x												
Day 157	rVV-WT	6.923±1.934			0.465	0.655										
	rVV+ATIs	4.95±1.627			x	0.187										
	En-ATIs	8.005±1.248			x	x										
Day 161	rVV-WT	4.15±0.8924					0.123	0.187								
	rVV+ATIs	7.33±1.531					x	0.862								
	En-ATIs	6.923±1.631					x	x								
Day 164	rVV-WT	4.158±0.4435							0.029	0.647						
	rVV+ATIs	5.980±0.4571							x	0.048						
	En-ATIs	4.450±0.414							x	x						
Day 171	rVV-WT	2.52±0.3189									0.977	0.855				
	rVV+ATIs	2.505±0.397									x	0.861				
	En-ATIs	2.605±0.3398									x	x				
Day 178	rVV-WT	1.773±0.0907											0.09	0.487		
	rVV+ATIs	2.583±0.3959											x	0.38		
	En-ATIs	2.063±0.3806											x	x		
Day 204	rVV-WT	0.940±0.0987													0.796	0.839
	rVV+ATIs	0.890±0.1563													x	0.693
	En-ATIs	0.975±0.1325													x	x

3.3.3. The Anti-B5R Antibody Response to Boosting with **rVV-WT, rVV+ATIs, or En-ATIs**

People that have anti-B5R antibodies, along with other viral antigen specific antibodies, may not be able to mount a robust secondary immune response to boosting with a vaccinia based vaccine as well as to any foreign antigen(s) expressed in that boosting vaccine. It has been shown that an rVV vaccine expressing a foreign antigen may fail to induce an adequate B cell or T cell response to that foreign antigen in an individual with pre-existing *Orthopoxvirus* immunity [136]. Therefore, we asked the question, does the ATI protein affect the recognition by antibodies that were induced by wild-type VV-WR that lacks ATI embedded MVs and how would this affect a boosting immunization using a virus that either consists of ATIs or can competently produce ATIs? We postulate that the ATI protein matrix is able to shield the embedded infectious MVs from pre-existing immunity such as neutralizing antibodies that recognize MV surface proteins. In order to understand how ATIs with embedded MVs affect the boosting response in mice with pre-existing immunity to VV-WR, we measured anti-B5R serum IgG antibodies after boosting with the different rVVs **(Figure 8)**. Groups of mice were given an IM primary immunization of 1 x 10^4 pfu of VV-WR to generate humoral immunity toward VV-WR. We then confirmed the B cell response by measuring levels of anti-B5R serum IgG. We observed a peak antibody titer at approximately day 28 followed by a gradual decline to minimal detectable levels until the rVV boost on day 155 **(Figure 8a and Table 6 and data not shown)**. At day 155 (approximately 22 weeks post VV-WR

priming) the mice were given a SQ boosting immunization of 1 x10^3 pfu of each of the rVVs and were bled at several time points after the rVV boost to assess the secondary anti-B5R serum IgG response to each of the rVVs. Although boosting the VV-WR immune mice with En-ATIs induced the highest anti-B5R serum IgG response of all the groups, the mean antibody titers were not significantly different from the antibody titers in the rVV+ATIs or rVV-WT boosted groups **(Figure 8b and Table 6)**. In order to compare an age-specific immune response we also gave naive mice PBS as a placebo at the same time that the other groups received the VV-WR prime. The PBS-treated mice were then immunized with the different rVVs at the same time as the mice that had been primed with VV-WR. The groups that first received PBS and were then immunized with the different rVVs had very little or no anti-B5R serum IgG at any time point after the rVV immunization **(Figure 8b)**. This low antibody response could be a result of the low dose of the rVV given, 1 x 10^3 pfu, which is ten-fold less than the VV-WR priming dose given to the other groups. The rVV boost was also administered by route of SQ vs IM for the VV-WR prime, which could also impact the overall antibody response to the rVVs. The age of the mice, approximately 34 weeks, could also have affected the antibody response in combination with the lower dose of the rVV as it has been shown that older mice can have a diminished antibody response to viruses compared to younger mice [137]. Collectively, these data suggest that the rVVs all induced similar secondary humoral immune responses as measured by anti-B5R serum IgG levels at different time points after the boost.

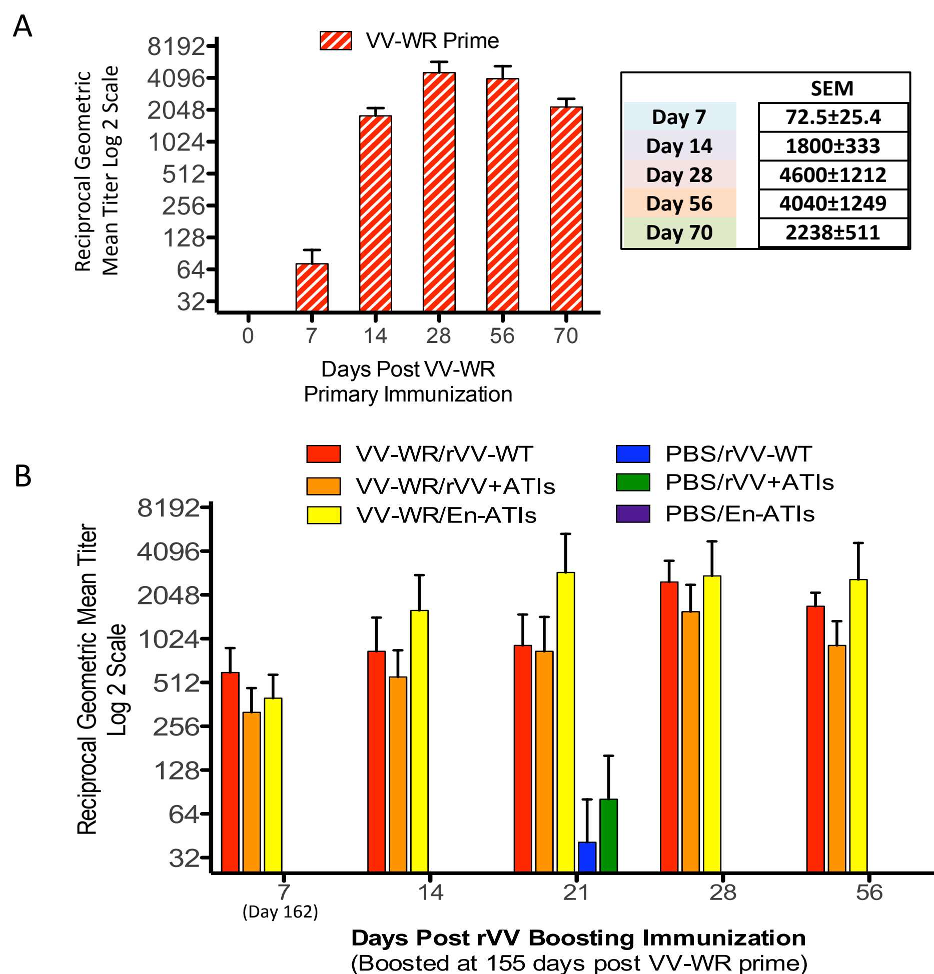


Figure 8. Anti-B5R Antibody Response in Mice Primed with VV-WR and Boosted with rVVs. Mice were bled for serum at various time points to measure anti-B5R IgG antibodies in mice primed with VV-WR and boosted with the rVVs. (A) Anti-B5R serum IgG antibody titers at various time points after VV-WR primary (IM) Immunization. Table to the right of the graph contains the reciprocal geometric mean titer (±SEM) of the mice receiving the VV-WR primary immunization. (B) At 155 days post VV-WR prime, groups (see **Table 4** for number of mice per group) of the VV-WR primed mice received an rVV (SQ) boosting immunization with the anti-B5R antibodies measured at various time points after the boost. Control groups received PBS instead of VV-WR priming immunization. The titers for the boosted antibody responses are listed in **Table 6**.

Table 6. Statistical Analysis of the VV-WR Prime and rVV Boost Anti-B5R IgG Serum Antibody Response. Mice were bled for serum at various time points after the boosting immunization and anti-B5R IgG antibodies were measured. Statistical inferences were made using Student's t test and were significantly different at $p < 0.05$. The mean and ±SEM of the antibody titers are listed for each group at each day (y axis). P values corresponding to group comparisons (x axis vs. y axis) are listed.

			Day 7		Day 14		Day 21		Day 28		Day 56	
			rVV+ATIs	En-ATIs	rVV+ATIs	En-ATIs	rVV+ATIs	En-ATIs	rVV+ATIs	En-ATIs	rVV+ATIs	En-ATIs
		± SEM	Pval		Pval		Pval		Pval		Pval	
Day 7	rVV-WT	600 ± 282	0.407	0.566								
	rVV+ATIs	320 ± 149	x	0.740								
	En-ATIs	400 ± 178	x	x								
Day 14	rVV-WT	840 ± 595			0.684	0.587						
	rVV+ATIs	560 ± 293			x	0.426						
	En-ATIs	1600 ± 1205			x	x						
Day 21	rVV-WT	920 ± 585					0.927	0.454				
	rVV+ATIs	840 ± 608					x	0.438				
	En-ATIs	2920 ± 2472					x	x				
Day 28	rVV-WT	2514 ± 1009							0.486	0.909		
	rVV+ATIs	1571 ± 835							x	0.574		
	En-ATIs	2767 ± 2016							x	x		
Day 56	rVV-WT	1714 ± 407									0.205	0.650
	rVV+ATIs	921 ± 429									x	0.401
	En-ATIs	2617 ± 2048									x	x

3.3.4. In Vivo Imaging of rVV Replication and Dissemination

For in vivo imaging, we engineered rVVs to express firefly luciferase (fLUC) without deleting or affecting any of the viral genes or attributes of the rVV vectors. This technology gives us the ability to track dissemination and replication of viruses expressing fLUC using *in vivo* live-imaging technology [138, 139].

Using these novel rVVs expressing fLUC we addressed the question, does the ATI protein impact both replication and dissemination of VV-WR? After the SQ boosting with the rVVs we quantified light emission within several regions of interest (ROI) including the head, chest, and abdomen at multiple time points. Unfortunately, we detected only very minimal amount of light emission from both the groups that received the rVV boosting immunizations as measured by photon flux. Although there was a small amount of light emission, none of the groups displayed levels that were consistently above background of the control group that received PBS instead of the priming and boosting immunizations **(Figure 9)**. It is possible that we did not detect consistent levels of light emission above background because we have not properly titrated the lower limit of luciferase production from these viruses that is detectable by the IVIS imaging system or we have not properly optimized other aspects of the imaging process. It is also possible that the dark coat color of B6 mice may have interfered with light emission. If we can optimize imaging and overcome the limitations, such as autoflourescence and high background, this imaging strategy could still be a powerful tool for future studies.

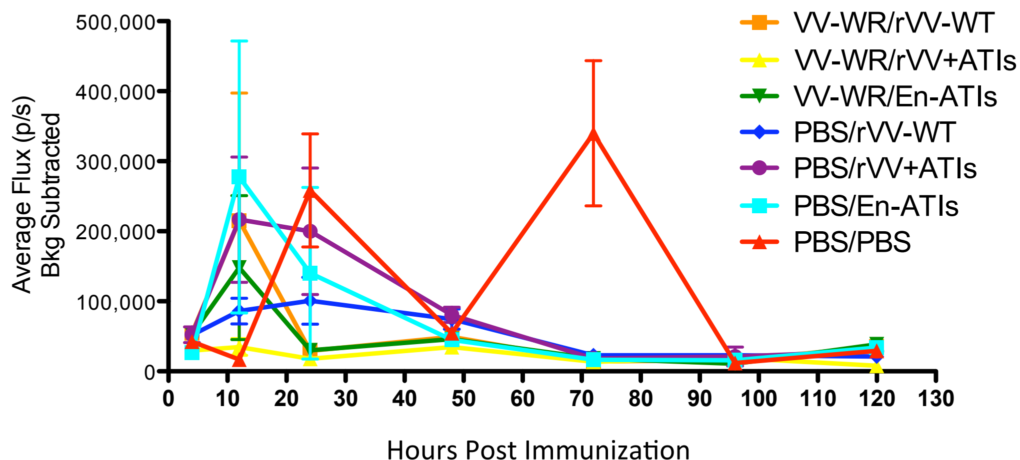


Figure 9. Luciferase Activity in Mice Receiving either an rVV Primary or Boosting Immunization. B6 mice ($n = 4$) were immunized IM with 1 x 10^4 pfu VV-WR to generate pre-existing immunity and then received an rVV SQ boosting immunization approximately 155 days later with 1×10^3 pfu of each of the rVVs. Naïve groups of mice also received an rVV priming immunization in parallel with the groups receiving the rVV boosting immunization. Bioluminescence imaging of the immunized mice was performed at 0, 12, 24, 48, 72, 96, 120 hours post-boosting or post-priming. The emission of light is expressed as photon flux vs. time (hrs.). Mean values ± SEM for photon flux are plotted.

4. DISCUSSION

The potential of VV based vaccines to induce multivalent protection by expression of multiple foreign proteins from multiple pathogens has brought its potential use back to the forefront of prophylactic vaccine research [140]. As discussed in the introduction, wild-type CPXV produces the full-length ATI protein (ATIp) that encapsulates multiple infectious MVs. The ATIp in many wild-type strains of VV, as well as other *Orthopoxviruses*, is truncated and results in the loss of its ability to form ATIs. We propose that restoring the ability of VV strain, Western Reserve (VV-WR), to produce ATIs with embedded MVs could possibly impact resulting disease and the immune response to VV-WR. In this study we designed a series of experiments to determine; 1) if an rVV that produces ATIs during replication or if purified ATIs (En-ATIs) results in disease and immune response that differs in comparison to wild-type VV-WR, and 2) if these novel rVVs result in different immune responses in mice that have pre-existing immunity to wild-type VV-WR. By addressing these questions, we can begin to define the role of *Orthopoxvirus* ATIs in activation of the immune response. Results of these experiments may ultimately contribute to achieving our long-term goal of developing safe multivalent vaccine platforms with improved efficacy.

In our first set of experiments, we administered a primary IN immunization of 1×10^5 PFU of each of the virus preparations (rVV+ATIs, En-ATIs, rVV-WT) so that we could examine and differentiate the effects of these rVVs on virus related disease in

comparison to the wild-type vector, rVV-WT. The results from these experiments show that the group immunized with the En-ATI virus preparation lost significantly less weight during the peak period of weight loss than the groups that received an rVV+ATI or rVV-WT immunization. Interestingly, the group receiving the rVV+ATI primary immunization lost the most weight of all the groups, which was significantly different from the group receiving En-ATIs but not from the group receiving rVV-WT. We also found no significant differences in the percentages of anti-B8R-specific CD8 T cells during primary immunization with the different virus constructs**.** We observed no illness or disease in any of the groups after VV-WR lethal challenge. We also observed minimal differences in the reinduction, expansion, or contraction of the anti-B8R-specific CD8 T cell response, although the group that received the rVV-ATI immunization did have a consistently higher proportion of anti-B8R-specific CD8 T cells upon the VV-WR challenge. Increasing the dose of VV correlates with increased disease and illness, along with an increased immune response [141]. Wild-type VV produces a virion that corresponds to a single, independent infectious unit that can remain as an MV or acquire additional envelopes during the replication cycle to become either a CEV or EEV. However, a single ATI infectious particle, as determined by plaque assay, corresponds to multiple embedded infectious MVs aggregated together in the ATI. This would suggest that we are essentially multiplying the amount of virus administered when the dose of the En-ATI preparation is based on the number of PFUs the ATI produces in a cell-based virus titration assay, meaning that one ATI virus plaque could

be a result of multiple MVs released from that single ATI. We hypothesized that an immunizing dose of En-ATIs would result in increased disease along with heightened immune responses in comparison to immunization with the same dose (as determined by number of PFUs) of the rVV+ATI or rVV-WT virus preparations. It is possible that the ability of the rVV+ATI to replicate and embed infectious MVs into ATIs will increase both disease and the resulting immune responses, but to a lesser degree in comparison to En-ATIs. This is because the rVV+ATI virus preparation corresponds to single infectious virions as determined by plaque assay, but the En-ATI preparation corresponds to multiple embedded virions that are not accounted for by the plaque assay. Therefore, we are administering more infectious virus with the En-ATI preparation compared to the other preparations.

The study using rVV primary immunization and VV-WR lethal challenge, as described above, yields three evident results; 1) the rVV+ATI primary immunization resulted in the most weight loss with En-ATIs inducing the least amount of weight loss in comparison the wild-type vector, rVV-WT, 2) the immune responses did not correlate with the weight loss results; in fact, the different rVV immunizations all induced similar humoral responses while rVV-WT induced the highest proportions of peripheral blood anti-B8R-specific CD8 T cells, 3) the different rVV primary immunizations resulted in a similar degree of protection upon VV-WR lethal challenge as indicated by weight loss and survival. Therefore, these results do not support our hypothesis because we did

not observe any significant differences in either disease or the immune response induced by the different rVV preparations.

Almost all *Orthopoxvirus* infections result in cross-protective immunity against each other [3]. Genetic alterations that reduce expression of cross-reactive antigens of these viruses can result in limited secondary immune responses that are less effective at clearing reinfections in hosts that were previously infected with wild-type virus. This has been demonstrated by the insertion and deletion of many *Orthopoxvirus* immunomodulators, host range genes, and envelope proteins [24, 58, 142, 143]. We hypothesized that an immunization with either En-ATIs or rVV-ATIs would result in an immune response to those rVVs that is different from the wild-type counterpart, VV-WR. Our reasoning is that the ATIp matrix could possibly shield the embedded infectious MVs from pre-existing immunity against the MV surface proteins generated during a previous wild-type VV infection, such as pre-existing immunity resulting from the VV based smallpox vaccine. Our results show that a primary immunization with the rVV preparations (rVV+ATIs or En-ATIs) does not seem to alter the proportions of anti-B5R serum IgG or anit-B8R-specific CD8 T cells in comparison to a primary immunization with the wild-type virus, rVV-WT, nor does the degree of protection that these virus preparations induce seem to differ from rVV-WT induced protection. In contrast to the similar immune responses that we observed as a result of the rVV primary immunizations, there were differences in disease manifestations. As indicated above,

the rVV+ATI immunization resulted in the highest proportions of weight loss in comparison to rVV-WT while the En-ATI immunization resulted in the least amount of weight loss of all the groups.

How can we explain these results? The mechanism of virus release from the ATIp matrix is poorly characterized. It is possible that the IN route of inoculation that we used for the primary immunization does not optimally facilitate the release of the virus from the ATI. It has been shown that the route of VV inoculation affects not only the pathological consequences of infection but also the overall immune response to the virus [144]. It is possible that different host cell proteases found in different compartments of the body could play a role in proteolytic cleavage or processing of the ATIp matrix and result in release of the virions from the ATI. This is a possibility as it has been shown that several viruses, such as Sendai virus, influenza virus, Newcastle disease virus, and herpes virus all depend on post-translational extracellular proteolytic cleavage of the surface glycoproteins, which is catalyzed by tissue specific host proteases [145]. Trypsin, a serine protease commonly found in the digestive tract of mammals, has been shown, *in vitro*, to cleave the ATIp into several fragments [103], which could possibly result in the release of the embedded MVs from the ATI aggregate, but this has yet to be proven. To the contrary, there are also protease inhibitors that can bind and inhibit these critical proteases in certain tissues [145] that could possibly inhibit the release of the MVs from the ATI. Thus, while we are administering a larger

dose of virus, the efficiency of virus release from the intranasally delivered ATIs is in question. *In vitro* experiments in which we alter the environment by adding proteases or protease inhibitors may help determine if key proteases or protease inhibitors affect the release of MVs from the ATI. Evaluating different immunizing routes will also help us determine if there are specific tissues that better facilitate the release of the MVs from the ATI. It is possible that the ATIs could be endocytosed by phagocytic cells (such as dendritic cells, macrophages, neutrophils, or mast cells) and be trafficked to lymphoid tissues, thus potentially influencing the innate immune response and antigen presentation thus affecting the adaptive immune response. It has been shown that spleen cells can produce large amounts of pro-inflammatory cytokines such as IL-1, IL-6, TNF, along with IFNγ, in response to VV infections [146]. Such innate responses can not only reduce viral replication but also affect lymphocyte activation and proliferation [146]. It is possible that endocytosis of VV ATIs and trafficking of the ATIs to lymphoid tissues could decrease infection of other cells such as myocytes, epithelial and endothelial cells. This could possibly reduce infection and resulting disease in body compartments that are comprised of such cells but still result in an adequate innate and adaptive immune response generated in the lymphoid tissues that results anti-viral immunity.

How can we explain our observations that En-ATI primary immunization induced the lowest amount of weight loss while rVV+ATI immunization result in significantly more

loss of weight? It is possible that a VV infection needs to be initiated by readily infectious virus (MVs and EEVs) to induce host cell protease production that will facilitate the release of the embedded virus from the ATIs. This is plausible as the rVV+ATI preparation contains readily infectious virus that does not need to be released from the ATIp matrix to be infectious. Thus, immunizing with rVV+ATI can initiate infection, which will induce physiological and biological changes in the host that could lead to optimal conditions that facilitate the release of the ATI embedded MVs that are produced after replication begins. This reasoning would explain why the group of mice that received the rVV+ATI immunization lost more weight than the group that received the En-ATI immunization. Understanding how the ATI releases the embedded infectious virus is critical for future experiments designed to determine whether or not ATIs potentiate VV induced immunity.

Although the different rVV immunizations result in similar numbers of anti-B5R IgG serum antibodies and anti-B8R-specific CD8 T cells, it is possible that there are differences in the specificity, function, and overall quality of these humoral and cellular responses. While the results of this study suggest that the immune system recognizes the different constructs equivalently, we will need to analyze the specificity and function of the cellular and humoral response to each of the different rVV constructs. Using *in vitro* assays to measure differences in effector cell function or cytokine production may provide us with a better understanding of the overall function of the

anti-VV responses to the different rVVs. We can develop assays to test antibody neutralization to better define the quality or specificity of the humoral response. In addition, we could transfer VV antigen-specific B cells and T cells, or passively transfer post-immune serum from rVV immunized mice into naïve mice and then challenge them with the different rVV constructs, including the VV-WR wild-type virus, to assess the degree of protection provided by the individual humoral or cellular components of the adaptive responses induced by the different rVV preparations.

Many of the factors mentioned above could help to explain the differences in weight loss after a primary IN immunization with rVV+ATIs, En-ATIs, or rVV-WT. The collective data from the rVV primary immunization and the VV-WR lethal challenge suggest that neither the cell mediated response nor the humoral immune response is greatly impacted by ATIs. The degree of protection induced by the rVVs seems to be very similar upon lethal challenge and suggests that the function of antibody and cellular responses are similar, although we will have to confirm this through further experimentation. We will also have to conduct additional experiments to support our observation that En-ATIs are less pathogenic than the wild type vector, rVV-WT. Decreasing the adverse effects that result from VV vaccination will be an important goal of future studies designed to improve VV based vaccines. If we can further substantiate that En-ATIs reduce the pathological consequences of VV-WR while retaining the

immune efficacy, as our results suggest, we may be able improve current rVV platforms by employing ATIs.

Ideally, viral vaccine vectors should be able to generate a potent immune response to the incorporated antigen(s) in the presence of pre-existing immunity to the vector. To investigate whether the rVV+ATIs or En-ATIs can generate a successful boosting immunization in the presence of pre-existing immunity to VV-WR, we administered a primary VV-WR immunization and then gave a boosting immunization with the different rVVs. The logic behind this experiment is based on the hypothesis that the ATIp matrix may be able to shield the embedded MVs from both pre-existing humoral and cellular immunity and thus be able to effectively deliver an infectious dose of virus that will result in increased boosting immune response in comparison to the wild-type vector, rVV-WT. If the ATI can increase the response, in comparison to rVV-WT, we may be able to design VV vaccines that are able to generate successful immune responses to foreign antigens expressed in VV vectors, even in the presence of pre-existing immunity toward the vector. After priming with VV-WR and boosting with the rVV preparations, we did not observe any differences in rVV induced pathological consequences as indicated by weight and temperature change, and there were no significant differences in humoral antibody or peripheral blood CD8 T cell response in comparison to the wild-type virus, rVV-WT. These results suggest that a boosting

immunization with either rVV+ATIs or En-ATIs does not alter the secondary immune response in comparison to boosting with the wild-type vector, rVV-WT.

There are several factors to consider that may affect the response to rVV in the presence of pre-existing immunity. It is possible that the truncated 92kDa ATIp, native to VV-WR, contains the same immunogenic epitopes as the full-length 160kDa CPXV ATI protein. In the presence of immunity to VV-WR, it is possible that pre-existing humoral and cellular immunity against the ATI protein may interfere with virus release or facilitate clearance of the ATIs, thus preventing an increased rate of infection in comparison to boosting with the rVV-WT vector. There are several ways to address this issue, such as: 1) immunize with an rVV that has the ATIp deleted and then boost with either the rVV+ATI or EN-ATI vector. Results of this prime and boost experiment could help determine if the truncated ATI protein produced by VV-WR induces T cells or antibodies that recognize the recombinant ATI vectors (rVV+ATIs and En-ATIs); 2) determine if humoral immunity against truncated ATIp can inhibit either the infection or replication of rVV+ATIs and En-ATIs in comparison to humoral immunity induced by VV-WR infection; 3) immunize with purified ATIp to generate polyclonal anti-ATIp antibodies. Then determine whether or not these antibodies inhibit infection and replication of rVV-ATIs and En-ATIs in a cell-based neutralization assay.

It is possible that the SQ route of immunization that we used might not be optimal for boosting with an rVV that consists of ATIs (En-ATIs) or that can produce ATIs (rVV+ATIs). Perhaps the pre-existing anti-VV-WR humoral immunity efficiently cleared the rVV boosting immunization. It has been shown that adaptive immunity, particularly the humoral response, against can be compartmentalized to specific tissues, such as the lungs [147]. Based on the concept of compartmentalized immunity we may be able to delay clearance of a boosting immunization by use of different routes from which the primary immunization was administered. This may enable the virus to more efficiently infect and replicate enough to induce a more heightened secondary immune response in comparison to using the same route that was used for the primary immunization. By utilizing different routes for the rVV primary and boosting immunizations we may be able to evade tissue specific pre-existing immunity and ultimately induce a potent immune response to the vector and also to foreign proteins expressed in that vector. The boosting immunizations with En-ATI and rVV+ATI resulted in both humoral and cellular responses that were similar to responses generated after boosting with the wild-type vector, rVV-WT. This suggests that the primary immune response induced by VV-WR is effectively boosted by all three rVV preparations. Even though we can effectively boost the VV-WR immune response with the different rVVs, we do not know how pre-existing immunity will affect the immune response to a foreign antigen expressed in those rVV vectors, which is a concern in developing VV based vaccines that are used to vaccinate against other pathogens.

The long-term goal of this study is to develop a better understanding of the role of the ATI and how it alters the resulting immunogenicity and disease that develops in immunized individuals. By defining the host interactions with the ATI, we will be better able to design future studies aimed towards improving the efficacy of VV vaccine vectors. Our results using the novel rVV, rVV+ATIs, suggest that ATIp neither increases nor decreases the humoral or cellular response upon immunization in mice when compared to the wild-type vector VV-WR, nor does it alter the secondary immune response in the presence of pre-existing immunity to VV-WR. When we immunized mice with ATIs purified from rVV+ATI cultures (En-ATIs) we observed similar results in comparison to the immunization with rVV+ATI vector or with the wild-type vector rVV-WT. When we compared the disease induced by these rVVs, we found that En-ATI immunization resulted in the least amount of weight loss. Therefore, our future experiments will focus on the En-ATI preparation as a vaccine vector.

4. FUTURE DIRECTIONS

While addressing many of the variables and factors that may affect the results that we observed from these studies, we have identified the following additional questions regarding the ATI protein: 1) Do ATIs alter the innate cytokine responses in comparison to other VV vectors, such as VV-WR? 2) How do ATIs alter foreign gene(s) expression? It has been shown that the ATI protein is the most abundantly transcribed protein under its native promoter. Would proteins encoded by foreign genes expressed under the control of this promoter elicit a robust immune response? 3) It has been show that entomopoxvirus ATIs provide protection from acidic environments [13]. Do VV ATIs provide similar protection for VV either in the host or during host-to-host transmission? 4) Does the ATI protein provide protection from non-specific anti-viral host components such as complement and defensins? 5) How is the infectious virus released from the ATIs and does this release extend over a period of time or are the entire contents of the ATI matrix released at once? 6) Could the ATI protein possess beneficial adjuvant properties? 7) Is it possible that the incorporation of the ATIp into replication deficient VV strains such as NYVAC and MVA VV would increase the efficacy of these VV vaccine platforms? As we address these questions and further elucidate the properties of ATI, we will gain an understanding of the purpose of ATIs and their potential to improve the efficacy of VV vaccine vectors.

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