Structural and functional analysis of the Vaccinia virus O1 virulence protein

by

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Poxviruses are double-stranded DNA viruses capable of causing disfiguring and deadly disease in a wide range of hosts, from insects to mammals. Orthopoxviruses (OPXV) encode many proteins that are not essential for viral replication, but are responsible for vast differences in pathogenesis. Of the >200 proteins in the prototypical OPXV vaccinia virus (VACV), many remain functionally cryptic. The objective of these studies was to understand how the VACV O1 protein functions by investigating cell-specific effects that may contribute to virulence.

The O1L gene is expressed early as the O1 protein, a 78 kDa protein that lacked N-linked glycosylation. These data are the first to demonstrate the reduced ability of an O1 deletion mutant (ΔO1) to induce cell migration compared to the parental VACV Western Reserve strain (VACV-WR). ΔO1-infected cell monolayers also exhibited reduced plaque diameter and clearance in plaque foci. These observations indicated that O1 is a significant contributor to VACV cytopathic effects (CPE) *in vitro,* in agreement with published reports. The results reported herein are the first to describe an altered immunological response with ΔO1, as levels of anti-VACV immunoglobulin significantly increased with ΔO1 infection at a time point (seven days post-infection) when VACV-WR induced VACV-specific antibody levels were comparable to sera from mock-infected mice. ΔO1 was more immunogenic in an *ex vivo* antigen presentation assay, although mitogen-induced $CD4^+$ T cell activation during ΔO1 infection was equivalent to VACV-WR infection. Surprisingly,

of all the immune cell types tested, ΔO1 significantly differed from VACV-WR infection in the metabolic readout of only one cell type – RAW 264.7 macrophages. VACV-WR infected RAW 264.7 macrophages were more metabolically active than ΔO1-infected cells at higher infectious doses, which may be indicative of a specialized niche for O1 function. Taken together, these data may provide clues into the mechanism of O1 virulence.

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by

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> "Every experience God gives us, every person He puts in our lives, is the perfect preparation for a future that only He can see."
>
> - Corrie ten Boom, *The Hiding Place* (1971)

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List of Abbreviations

1153 B	**antigen specific mouse B cell line**
2D2-FIG	**Transgenic 2D2 mice crossed with FoxP3-IRES-GFP (FIG) mice**
AD	***anno domini***
AIV	**avian influenza virus**
AKT	**Protein kinase B**
ANOVA	**analysis of variance**
AP	**alkaline phosphatase**
ATP	**Adenosine triphosphate**
B04	**antigen specific mouse T cell line**
BALB/c	**$H2^d$ haplotype mouse strain**
BCIP	**5-bromo-4-chloro-3indolyl phosphate**
BLAST	**Basic Local Alignment Search Tool**
BMDC	**bone marrow derived dendritic cells**
BS-C-1	**green monkey kidney epithelial cell line**
C57BL/6	**mouse strain**
CDK	**cyclin dependent kinase**
CEV	**cell-associated enveloped virus**
c-fos	**oncogene and transcriptional regulator**
Cip1	**CDK inhibitor protein-1**
cMEM	**complete Modified Eagle's Medium**
CMPV	**camelpoxvirus**
CMV	**Cytomegalovirus**
ConA	**concanavalin A**
CPE	**cytopathic effects**
CPXV	**cowpox virus**
cRPMI	**complete RPMI**
CTLL	**cytolytic T lymphocyte cell line**
CVA	**Chorioallantois Vaccinia Ankara strain**
CVA-ΔO1	**CVA parental strain with O1L gene deletion**
DCs	**dendritic cells**
DMEM	**Dulbecco's modified Eagle Medium**
DNA	**deoxyribonucleic acid**
dsDNA	**double-stranded DNA**
ECTV	**ectromelia virus**
EDTA	**ethylene diamine tetraacetic acid**
EEV	**extracellular enveloped virus**
EGF	**epidermal growth factor**

eGFP	**enhanced green fluorescent protein**
EGFR	**epidermal growth factor receptor**
EGR-1	**epidermal growth receptor 1**
EGTA	**ethylene glycol tetraacetic acid**
ELISA	**enzyme linked immunosorbant assay**
ELISpot	**enzyme linked immunospot assay**
EMT	**epithelial-to-mesenchymal transition**
Endo H	**endoglycosidase H enzyme**
ER	**endoplasmic reticulum**
ERK	**extracellular signal regulated kinase**
EV	**enveloped virus**
FAK	**focal adhesion kinase**
FasL	**Fas ligand**
FBS	**fetal bovine serum**
GAG	**glycosaminoglycan**
GDP	**guanosine diphosphate**
GEFs	**guanine exchange factors**
GP	**glycoprotein**
GP-VLP	**glycoprotein expressed by virus-like particle**
GTP	**guanosine triphosphate**
HBV	**Hepatitis B virus**
HCMV	**Human cytomegalovirus**
HCV	**Hepatitis C virus**
HEPES	**4-(2-hydroxyethyl)-1piperazineethanesulfonic acid**
HEL	**hen egg lysozyme antigenic peptide**
HIV	**Human immunodeficiency virus**
HPI	**hours post-infection**
HPV	**human papillomavirus**
HSPV	**horsepoxvirus**
IEV	**intracellular enveloped virus**
IFN	**interferon**
IFNB	**interferon-β**
IFNg	**interferon-γ**
Ig	**immunoglobulin**
IgA	**immunoglobulin A**
IgG	**immunoglobulin G**
IgM	**immunoglobulin M**
IL-2	**interleukin 2**
IMV	**intracellular mature virus**
IV	**immature virion**

JAK	**Janus kinase**
kDa	**kilodalton**
L	**liter**
LPS	**lipopolysaccharide**
MAPK	**mitogen activated protein kinase**
MCV	**molluscum contagiosum virus**
MEK	**mitogen activated protein kinase kinase**
MEM	**modified Eagle medium**
MERS-CoV	**Middle Eastern Respiratory Syndrome coronavirus**
mL	**milliliter**
mm	**millimeter**
MOG	**myelin oligodendrocyte antigenic peptide**
MOI	**multiplicity of infection**
MPXV	**monkeypox virus**
mRNA	**messenger RNA**
MTS	**3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium**
MV	**mature virion**
MVA	**modified virus Ankara**
NaN_3	**sodium azide**
NFκB	**nuclear factor kappa-light-chain-enhancer of activated B cells**
NEFM	**neurofilament medium chain antigenic peptide**
nm	**nanometer**
OPXV	**orthopoxvirus**
OD	**spectrophotometric optical density**
ORF	**open reading frame**
PBS	**phosphate-buffered saline**
PCR	**polymerase chain reaction**
PDB	**Protein Database**
PFU	**plaque forming units**
PI3K	**phosphoinositide 3-kinase**
PKA	**Protein kinase A**
PMS	**phenazine methosulfate**
PNGase F	**peptide:N glycosidase F**
RAF	**mitogen activated kinase kinase kinase**
RAW 264.7	**mouse macrophage cell line**
RNA	**ribonucleic acid**
RPMI	**Roswell Park Memorial Institute medium**
SAMHD1	**Sterile Alpha Motif- and HD-domain containing protein 1**
SARS-CoV	**Severe Acute Respiratory Syndrome coronavirus**

SDS	**sodium dodecyl sulfate**
SDS-PAGE	**SDS-polyacrylamide gel electrophoresis**
Sf9	***Spodoptera frugiperda* insect ovarian epithelial cell line**
siRNAs	**small interfering RNA**
TEMED	**N,N,N=,N=tetramethylethylenediamine**
TGFB	**transforming growth factor β**
TNF	**tumor necrosis factor**
VACV	**vaccinia virus**
VACV-ΔO1	**VACV-WR parental strain with O1L gene deletion**
VACV-WR	**vaccinia virus strain Western Reserve**
VARV	**variola virus**
VGF	**vaccinia growth factor**
VLP	**virus-like particle**
WHO	**World Health Organization**
Y2H	**Yeast-two hybrid**

Chapter 1: Introduction and Literature Review

1.1 Research Overview

The overall purpose of the studies described in this thesis was to better understand how poxviruses cause disease in mammals. Poxviruses encode many proteins that promote virulence by manipulating the host immune system. The vaccinia virus O1 protein was hypothesized to be among a class of novel pox-specific immunoregulators; experiments performed to test this hypothesis are described herein. Identifying poxvirus proteins that manipulate the host immune response and determining their functional mechanisms provides knowledge of biological processes inherent to both poxviral pathogenesis and host immunity. Further appreciation of the poxviral-host systemic interactions generates new opportunities for a wide range of therapeutic interventions.

1.2 Poxvirus biology

1.2.1 Poxvirus classification

Poxviridae are large, enveloped, double-stranded DNA viruses with linear genomes that can encode over 200 proteins. Poxviruses are classified into two subfamilies: *Chordopoxvirinae* (poxviruses that infect vertebrates) and *Entemopoxvirinae* (poxviruses that infect insects). *Chordopoxvirinae* are divided among nine genera, including four genera that infect humans – orthopoxvirus (OPXV), parapoxvirus, yatapoxvirus, and molluscipoxvirus (MCV). MCV and the OPXV variola virus (VARV: the causative agent of smallpox) are strictly human pathogens; the remaining viruses cause zoonoses. While MCV infections are currently more widespread (1), it is the OPXV [*e.g.* VARV, monkeypox virus (MPXV), and vaccinia virus (VACV)] infections that are among the most pathogenic for humans.

1.2.2 Vaccinia virus

As the prototype OPXV, VACV is a valuable tool for investigating the biology of poxviruses and their mechanisms of pathogenicity. Structurally, VACV is composed of a dumbbell-shaped core flanked by two lateral bodies (**Figure 1 A**). The VACV core is enveloped by one or more lipid bilayers, resulting in two forms of infectious VACV. The most abundant form is the mature virus (MV), which has a single lipid bilayer studded with non-glycosylated viral proteins (2). The other infectious form is the enveloped virus (EV), which consists of MV particles with an additional viral membrane containing an alternate set of glycosylated viral and host proteins (3).

The linear VACV genome is covalently closed by hairpin loops at both ends, and inverted terminal repeats flank the open reading frames (ORF) (4). There is an overall genomic organization to the > 200 ORFs encoded by VACV (**Figure 1 B**), wherein the most highly conserved poxviral genes (encoding replication machinery and structural proteins) are located in the central part of the genome [49 are common to all sequenced *Poxviridae* (5, 6)]. In contrast, genes with greater genetic variation tend to be located in the flanking ends of the genome and encode for a large proportion of virulence and immunomodulatory accessory genes. These genes are not essential for replication in tissue culture and can vary widely among viral strains and species (7). Pox genes can also be classified as early, intermediate, or late genes based upon their expression kinetics following infection, and recently a new class of genes – intermediate early – has also been proposed. [**Figure 1C** (8)]. Genes are classically named by their location on a HindIII restriction digest map (9). The HindIII digest of VACV (Copenhagen strain) has 15 fragments (designated A-O in order of decreasing fragment size), with each ORF within a

fragment named using sequential numbers and relative orientation (10). Thus, the O1L gene is the first ORF in the HindIII "O" fragment, and the ORF reads to the left (L).

1.2.3 Replication, morphogenesis, and dissemination

The VACV infectious cycle (**Figure 2**) can be divided into seven phases (discussed as numbered): ***(1)*** entry, ***(2)*** uncoating, ***(3)*** DNA replication, ***(4)*** gene expression, ***(5)*** morphogenesis, ***(6)*** egress, and ***(7)*** spread. ***1)*** VACV entry into a host cell is complex and can occur *via* multiple routes. A single entry receptor for VACV has not been identified; the virus associates with various surface proteins prior to entry, including glycosaminoglycans (GAGs) and laminins (11). Entry into the cell is cell type and host dependent (12–14), and MV particles have been observed entering through macropinocytosis, endocytosis, apoptotic mimicry (12), and membrane fusion (15). Unlike most viruses, which may enter cells via a single protein-receptor interaction, VACV has highly complex entry/fusion machinery, with at least 12 separate viral proteins forming a complex to facilitate fusion independently of binding (15). Since the entry/fusion machinery of VACV is only on the MV membrane (16), the EV outer envelope must dissociate before EV entry can occur (17).

2) Upon entering the host cell, viral membranes are shed and the naked viral core is released into the cytoplasm. Microtubule machinery is hijacked by viral proteins to traffic the viral core further into the cell. Early mRNAs are transcribed within the cytoplasm, leading to uncoating of core and ***3)*** subsequent DNA replication. Unlike nearly all other DNA viruses, VACV encodes and packages its own enzymatic replication machinery, allowing for replication in a distinct cellular environment within the cytoplasm called the viral factory (2).

4) VACV gene expression is temporally separated into three classes: early, intermediate, and late. Prior to viral DNA replication, early genes are expressed to produce proteins necessary

for DNA replication, as well as proteins that combat the host immune response (18). VACV DNA initiates replication by self-priming in the inverted terminal repeats through a mechanism that is still incompletely understood. The resultant DNA concatamers are cleaved by a viral resolvase enzyme into single-genome units (19). Subsequently, intermediate genes are expressed, resulting in a small number of intermediate genes, several of which are transcription factors for late gene expression. The late genes generally encode structural proteins needed for viral morphogenesis and the transcription machinery that must be packaged into nascent virions.

5) During morphogenesis, MV particles are produced and processed within viral factories. A small proportion of MVs can be further transported through early endosomes and the trans-Golgi network, where the MV particles gain two additional membranes to form intracellular enveloped viruses (IEVs) (3). Again, VACV uses microtubules to transport itself within the cell, with IEVs being transported to the cell surface. ***6)*** For the majority of virions, morphogenesis ends when MVs escape from the cell *via* cell lysis. However, in the case of IEVs, the outermost membrane fuses with the plasma membrane to expose an EV particle, termed cell associated EV (CEV) (3). Actin tail polymerization beneath the CEV is used to drive the virus into adjacent cells, or the virus is released as an EV. ***7)*** EV particles are important for spread within the host, (20) and it is thought that the EV membrane functions to evade the host immune system, as it sequesters neutralizing antibody. Finally, cell-to-cell spread of VACV in tissue culture can occur in several ways. In cell culture, released EV can either infect adjacent cells or spread in a convection mediated, unidirectional manner to distal cells (21), forming the characteristic comet-shaped monolayer holes, with comet tails that are formed by secondary plaques.

Figure 1

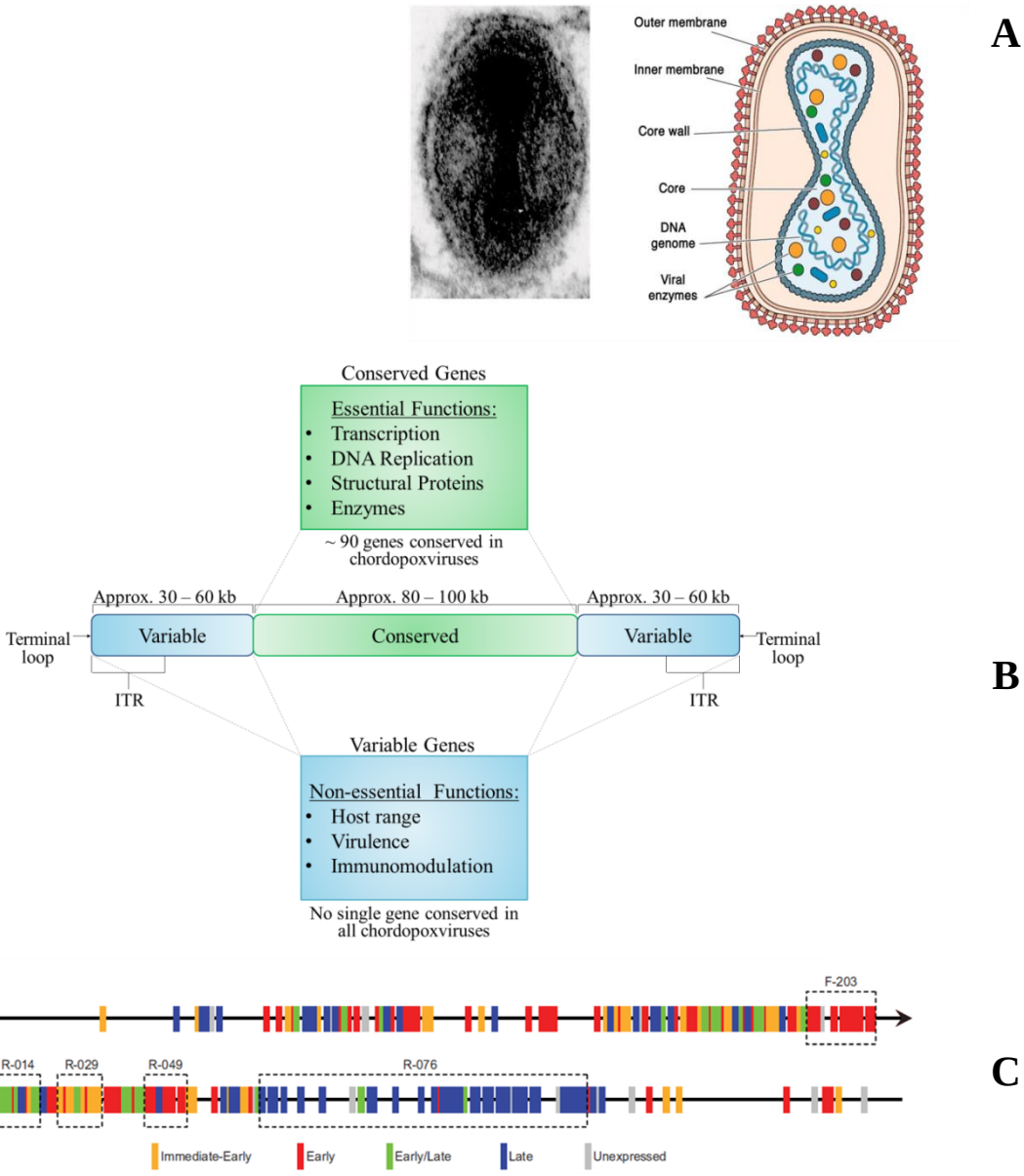


Figure 1. VACV structural and genomic organization

(A) VACV virion structure: Transmission electron micrograph of the double membrane-wrapped mature VACV virion (left); a cartoon depicts the virion structural components (right). Figure from (22) with permission from the publisher (Appendix B). **(B) VACV genomic organization:** The linear dsDNA VACV genome is ~200 kbp and flanked by terminal loops with inverted tandem repeats (ITR). Centrally located genes, generally essential for viral replication and morphogenesis, are more conserved, whereas termini-encoded ORFs are genetically variable and encode virulence factors. Adapted from (23) with permission from the publisher (Appendix B). **(C) Expression kinetics of VACV genome:** Genes are classified according to when they are expressed upon infection. Colored boxes represent genomic islands with similar gene expression profiles. Figure reproduced from (8) with permission from the publisher (Appendix B).

Figure 2

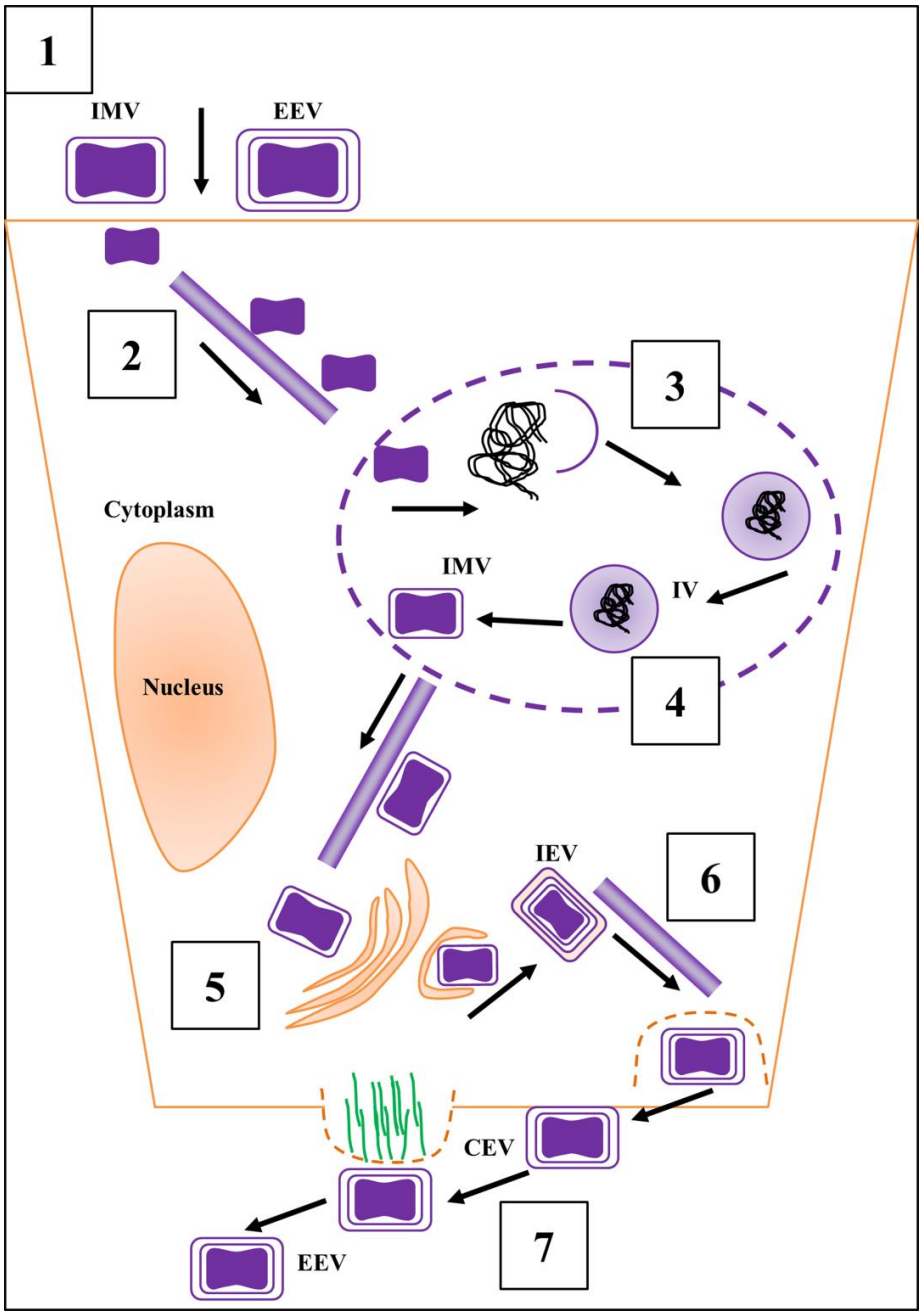


Figure 2. VACV morphogenesis.

(1) VACV virions attach to cells through varied mechanisms, and enter cells by membrane fusion. (2) Virus trafficking into cytoplasm is followed by virion uncoating and early gene transcription. (3) DNA replication occurs within cytoplasmic viral factories, with subsequent intermediate and late gene expression. (4) Immature virions (IV) are packaged into intracellular mature virions (IMV), which are either released upon cell lysis, or (5) traffic through Golgi to form intracellular enveloped virus (IEV). (6) IEV are transported by microtubules to the cell membrane, where (7) cell associated virions (CEV) egress further *via* polymerized actin protrusions (green), releasing the extracellular enveloped virus (EEV).

1.3 Smallpox, vaccination, and emerging poxviruses

1.3.1 A brief history of vaccination

The deliberate inoculation of smallpox (*i.e.* variolation) is thought to have been initiated by a Buddhist nun around 1000 AD. The practice of variolation spread from China to India and Turkey, but it was only in the late 1700s that this practice was implemented by European physicians. Several methods of variolation have been described, including the inhalation of powdered scabs or inoculation of pus into the skin. Lifelong immunity to smallpox was worth the slight chance of contracting smallpox (around 1%) from variolation. In 1796, Edward Jenner demonstrated that inoculation with infectious cowpox specimens also protected against smallpox. This form of inoculation, termed "vaccination," (from the Latin *vacca*, for cow) appeared to be safer than variolation. While complications from the cross-protective vaccination method were far less than those of variolation, the protective effects lasted only 5-10 years. Later vaccination regimens indicated a regional preferences for either horsepox (France) or cowpox (England) specimens (24); ultimately, it was discovered that the Jenner vaccine was replaced by inoculation with the live VACV (25, 26). In 1939, the smallpox vaccines being used at the time were recognized not as CPXV, as originally thought, but a distinct OPXV species that was later designated VACV (27). VACV is a virological enigma, as it is the only vaccine to have been used to eradicate a disease, while its origin and natural host remain unknown.

Although protective against OPXV, VACV can also be highly virulent in immunocompromised individuals, pregnant women, and individuals with inflammatory skin conditions like eczema, among others. If vaccinated with the currently stockpiled VACV vaccine, many individuals would have adverse and possibly fatal reactions, including: encephalitis, progressive vaccinia (uncontrolled spread of virus from vaccination site), eczema

vaccinatum (vaccinia lesions covering eczema prone regions) and generalized vaccinia (vaccinia lesions covering the body) (28, 29). VACV virulence makes its use contraindicated in up to 25% of the population (30–33), so vaccination is limited to military, first responders, and laboratory personnel. Research is ongoing to develop a more effective, safer VACV vaccine.

1.3.2 The pathogenesis and eradication of smallpox

Smallpox is one of the worst pandemics to decimate humanity, with an average fatality rate of ~30%. Indeed, in the 20th century alone, smallpox deaths are estimated to have been ~500 million (34). VARV infection begins when as few as 10 plaque-forming units (PFU) are absorbed into the respiratory or alimentary tracts (28, 35). VARV spreads from the initial site of infection to proximal draining lymph nodes. Over a 4 – 17 day latent period, VARV multiplies within the lymphatic phagocytic cells, primarily macrophages and monocytes. Following the conclusion of the latent period, VARV spreads from the lymph nodes into oropharyngeal mucous membranes and dermal capillaries. This brief viremia results in a prodrome stage, where symptoms include fever, malaise, and occasional vomiting. A rash appears 2 – 4 days after the prodromal symptoms, leading to the lesion formation in the mouth and subsequent centrifugal diffusion to the face, arms, and legs. The emerging lesions become pus filled and assume the distinctive "pock" mark: an opaque papule with a depressed center that eventually will burst, forming scab-covered lesions. Individuals are infectious from the onset of the rash until all scabs have fallen off and lesions heal. Approximately 30% of individuals exposed to VARV developed smallpox, with 5 – 40% succumbing to the disease (29). The major and minor strains of VARV accounted for the wide variance in morbidity, with the VARV major strain exhibiting more extensive morbidity and mortality rates from 10-40%. VARV minor strain mortality rates were usually <1% despite almost identical clinical presentation to the VARV major strain.

Historically, smallpox infections have been described as early as the 4th century AD in China and in the 7th century AD in India. Due to the World Health Organization (WHO) mass vaccination campaign, the last natural incidence of smallpox occurred in 1977, followed by a laboratory incident shortly thereafter. The WHO declared smallpox successfully eradicated from nature in 1980 (36). Currently, VARV only officially exists in two laboratories in the United States and in Russia; however, the US government and many scientists still consider smallpox a threat, due to its potential use as a biowarfare agent. Indeed, VARV use as a bioweapon would be more effective than *B. anthracis* (anthrax) due to the efficient anthroponotic (human-human) transmission via an aerosol route, a largely susceptible (unvaccinated) population, and a high fatality rate.

1.3.3 Studying an eradicated virus

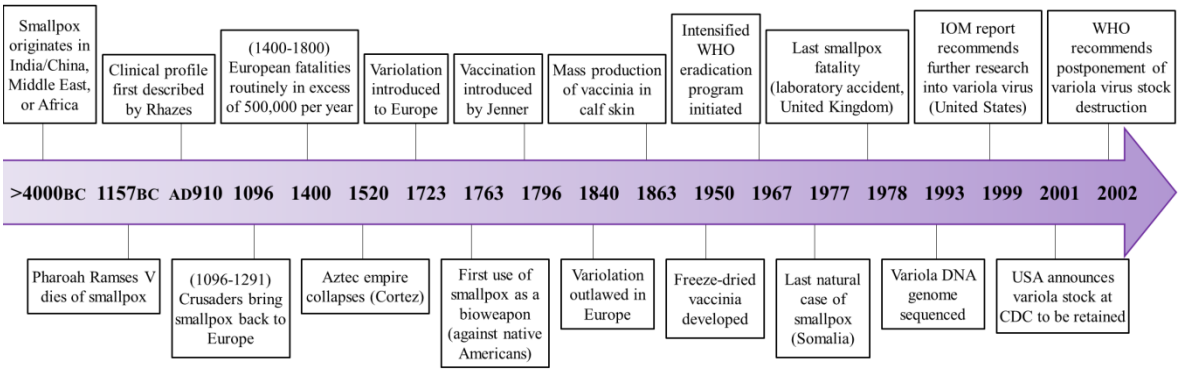
Several poxviruses currently present a credible threat to human health. MCV infections are common in young children, and recently MCV has also become a common sexually transmitted infection worldwide (1). The emergence of MPXV has dramatically increased in Africa, with one outbreak spreading to the USA in 2003 (37, 38). Although MCV infections are more prevalent, MPXV is currently the most significant threat to public health by a member of the Orthopoxvirus genus (39), because it is a viral zoonotic disease with an unverified reservoir (likely African rodents), has an indistinguishable clinical presentation to VARV infection, and a high case-fatality rate (37). Additionally, cowpox transmitted by rats recently caused an outbreak in a primate facility, killing 40% of animals, underscoring how dangerous zoonotic pox can be (40–42). Other pox zoonoses [buffalopox (43), tanapox (44), Cantagalo (45)] are also emerging, and it is thought by some to be occurring due to the decline in smallpox vaccinations (25, 38, 46, 47). Current literature on the phylogenetic relationships, ecology, and host range of OPXV

suggests that a VARV-like virus could emerge in the course of the natural adaptation of modern zoonotic OPXV, so continuing pox research is necessary.

Poxviruses can also be used to treat diseases, as there are numerous biological properties that make them ideal for use as a vaccine vector (48–51). Although there are still a number of poxvirus proteins of unknown function, there are a large number of well characterized poxvirus vectors that are in use or are being developed. These pox vectors are stable in both the physical particle and the genome, so they are suitable for accommodating large insertions of DNA for recombinant vaccines. Poxvirus vectors are also immunogenic (*i.e.* induce B cell and T cell responses), and are relatively easy to produce. Poxviruses are currently used as, or are being developed into, vaccines to treat malaria (52), rabies (48, 53), severe acute respiratory syndrome coronavirus (SARS-CoV) (34), Middle Eastern respiratory syndrome coronavirus (MERS-CoV) (54, 55), flu (52, 56), human immunodeficiency virus (HIV) (50, 57, 58), as well as cancer (59–62). A more thorough characterization of the pox virulence proteins and host immune responses that promote adverse and fatal reactions to VACV vaccination could enhance development of VACV into more effective vectors and oncolytic agents.

Figure 3

A



B

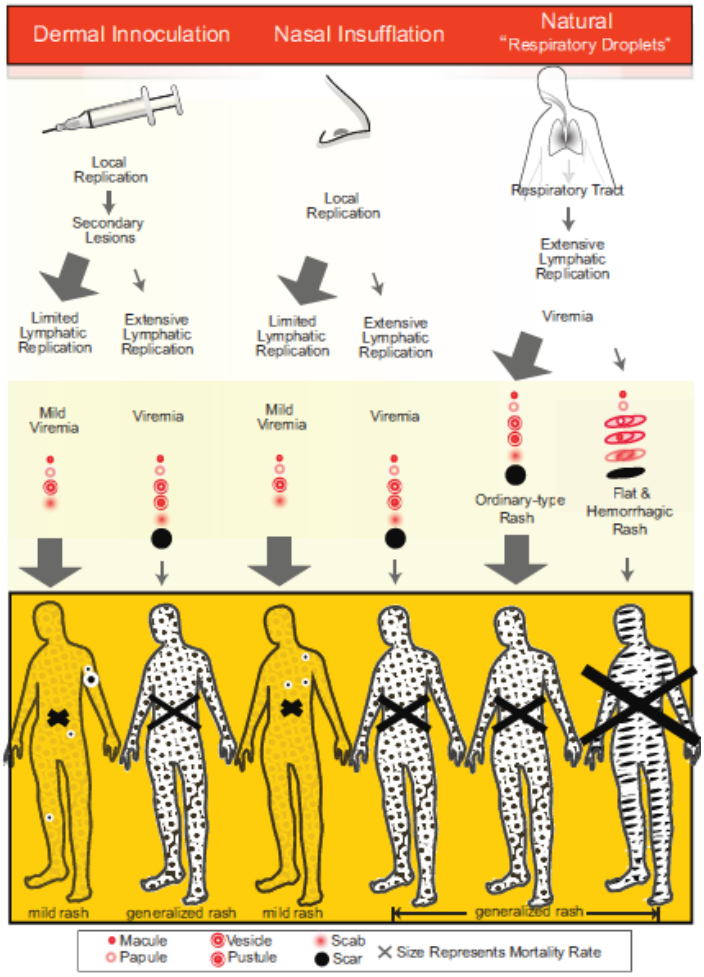


Figure 3. History of smallpox vaccination and pathogenesis

Figure 3 A depicts the timeline of human smallpox infections and highlights the history of VACV vaccination. Reproduced from (23), with permission (see Appendix B). **Figure 3 B**) Smallpox morbidity (characteristic rash) and mortality rate (**X**) differ with inoculation route (respiratory, nasal, and dermal) and the ability of a host to control viral replication (lymphatic) and spread (viremia). Sourced from (63), with permission (Appendix B).

1.4 Viral manipulation of the mammalian immune system

1.4.1 Poxvirus-encoded mammalian homologs

Mammalian hosts have developed complex defense mechanisms to control and clear virus infection, and protect against subsequent infections (**Figure 4**). Within the host environment, poxviruses are under selective pressure to evade the immune response and actively manipulate the host immune system in order to survive and replicate. Many poxviral genes that encode immunomodulatory proteins have an origin in the host genome (18, 64–66), having been acquired as necessary defenses from their host environments over time. These genes were identified through their homology to host proteins; however, poxviruses have also developed unique mechanisms for suppressing host immunity (18). Poxviral immunomodulatory proteins facilitate infection by preventing, evading, and diminishing host immune responses. A number of strategies are employed (**Figure 5**), including secreting decoy cytokine or chemokine receptors (66, 67), regulating apoptosis (68–72), and preventing complement-mediated lysis (73, 74). These effective immune evasion strategies also contribute to viral host specificity (18, 68). The majority of immunomodulatory genes are expressed early during infection to provide the virus with a substantial defense against the host immune system (reviewed in 19). With few exceptions, the removal of one or more of these immunomodulatory genes from VACV results in a mutant virus that causes attenuated disease in animals (44, 51).

1.4.2 Poxviral modulators of antibody production

Both the innate (75–78) and the adaptive (18, 79–81) immune responses are critical for controlling primary poxvirus infection (**Figure 4**) (18, 64, 69, 78). Furthermore, the successful use of poxvirus vaccines highlights the critical role of the adaptive immune response in controlling subsequent pox exposure events. The production of antibody by B cells is essential

for survival during primary pox infection, as it is one of the principal means of clearing poxviruses and inhibiting viral replication (80–82). The humoral response is the longest lasting, most effective means of preventing a secondary poxvirus infection (81). Thus, VACV may employ several direct approaches that reduce antibody production, including infecting B cells (82–84), manipulating intracellular signal transduction (85–91), and inhibiting regulatory cytokine production (77, 78, 85, 88, 92).

$CD4^+$ T cells also play a critical role during acute poxvirus infection by stimulating cytolytic T cells to clear virus (76, 82, 93) and helping B cells produce virus neutralizing antibody (76, 80, 94). Furthermore, $CD4^+$ T cell help is necessary for B cell affinity maturation to produce high-affinity antibodies (25, 93). When $CD4^+$ T cells are ablated during a poxvirus infection, there is a marked reduction in memory B cells, and a subsequent reduction in virus-specific IgG and neutralizing antibody titers (76, 93, 95). However, it is not well understood whether VACV can act directly within $CD4^+$ T cells to reduce their ability to help B cells generate high antibody titers during VACV infection. Suppressing the humoral immune response would be advantageous for virus survival, so identifying and understanding any contributing virulence factors is important.

1.4.3 Inhibiting antigen presentation

Poxviruses encode immunosuppressive proteins that are able to suppress T cell activation (77, 96), major histocompatibility complex I and II (MHC I and MHC II) molecules (97, 98) and anti-viral cytokines (IFN, TNF)(18) produced by $CD4^+$ T cells in the early stages of natural pox infection. Prior studies have employed antigen presentation assays to understand basic biological interactions of the host immune responses in the context of viral pathogenesis during VACV infection (84, 97–99). Antigen presentation can be studied using splenocytes, which contain T

cells and a variety of antigen presentation cells (APCs: macrophages, dendritic cells, B cells). T cells become activated when the T cell receptor (TCR) recognizes its cognate antigen by interacting with MHC II on the surface of APCs. The activation state of T cells can be further modulated by APC co-stimulatory molecules. When T cells are activated they produce various lymphokines, including interleukin-2 (IL-2). Since production of IL-2 changes depending on the activation state of T cells, IL-2 is one measure of antigen-dependent T cell activation (84, 98, 99). Thus, splenocytes from mice with TCR specific for a single cognate peptide can be used as a source of antigen-specific T cells to measure T cell activation, which is assessed by a cell proliferation bioassay using IL-2 dependent T cell lines. By employing antigen presentation assays, it is possible to then dissect intra- and intercellular mechanisms involved in adaptive immunity. Since a number of viral immunomodulatory strategies have previously been determined using antigen presentation assays (77, 84, 98–100), it may be possible to uncover novel cell-specific mechanism(s) that VACV employs to regulate host immunity.

1.4.4 Other immunomodulatory strategies

VACV primarily targets monocytes (101) and the innate APCs [*e.g.* dendritic cells (DCs) and macrophages] that are necessary for initiating adaptive immune responses. VACV has recently been shown to productively infect M1 and M2 primary human monocyte derived macrophages (MDM) polarized in culture (102). This is surprising, given earlier reports that VACV infection of macrophages is abortive (75, 103). Byrd *et al.* demonstrated that VACV productively infects primary macrophages, with higher viral loads produced by infection of anti-inflammatory M2 polarized macrophages (102). VACV replication within M2 macrophages resulted mainly in EV, the virion subset that mediates long-range dissemination in hosts. In contrast to the detection of EV in supernatants by Byrd, *et al.* (102), prior reports only assessed VACV CEV from

macrophage lysates, so the lack of EV detection could be construed as an abortive infection (103). VACV replication was also enhanced with upregulated ERK and AKT activity and was susceptible to IL-10 mediated suppression. Furthermore, VACV-infected macrophages exhibited characteristic cellular protrusions and branching reminiscent of VACV-induced migration of BS-C-1 cells (discussed below), supporting theories that VACV-induced migration may be a mechanism of viral dissemination. The apparent tropism for the M2 subset is interesting, as M2 macrophages are found within tissues with high replicative capacity (e.g. ovaries and tumors) for which VACV is highly tropic. It will be important to determine if macrophages with a lower innate antiviral response are specifically targeted by VACV, and if production of EV within macrophages promotes dissemination to other tissues where VACV can replicate unimpeded by the immune system. Migration of VACV-infected macrophages would promote VACV dissemination as well. Finally, the influence of VACV infection on tumor resident macrophage behavior is an important consideration for the development of more effective oncolytic vectors.

DCs are required to survive primary poxvirus infection (104), so it is interesting that VACV infection induces differentiation of monocytes into DCs (105). VACV also directly infects immature and mature DCs; however, VACV is only able to produce early genes in those abortive infections (83). Still, these early genes are sufficient to suppress many DC functions, including antigen uptake (106) and presentation (98), migration (107), maturation (98, 108, 109), and cytokine secretion (77). Furthermore, VACV inhibition of APC function results indirectly in impaired antiviral cytokine secretion by T cells (98), as well as reduced antibody production (84). In an evolutionary context, it is possible that poxviruses acquired mechanisms to induce monocyte differentiation into DCs in order to spread within the host. With the advent of the

adaptive immune response, poxviruses may have subsequently acquired separate mechanism(s) to prevent further maturation of immunostimulatory DCs. Determining which specific pox protein(s) mediate immunosuppressive functions could be important for the future development of effective DC-delivered tumor immunotherapies, as well as safer vaccine vectors (66).

Figure 4

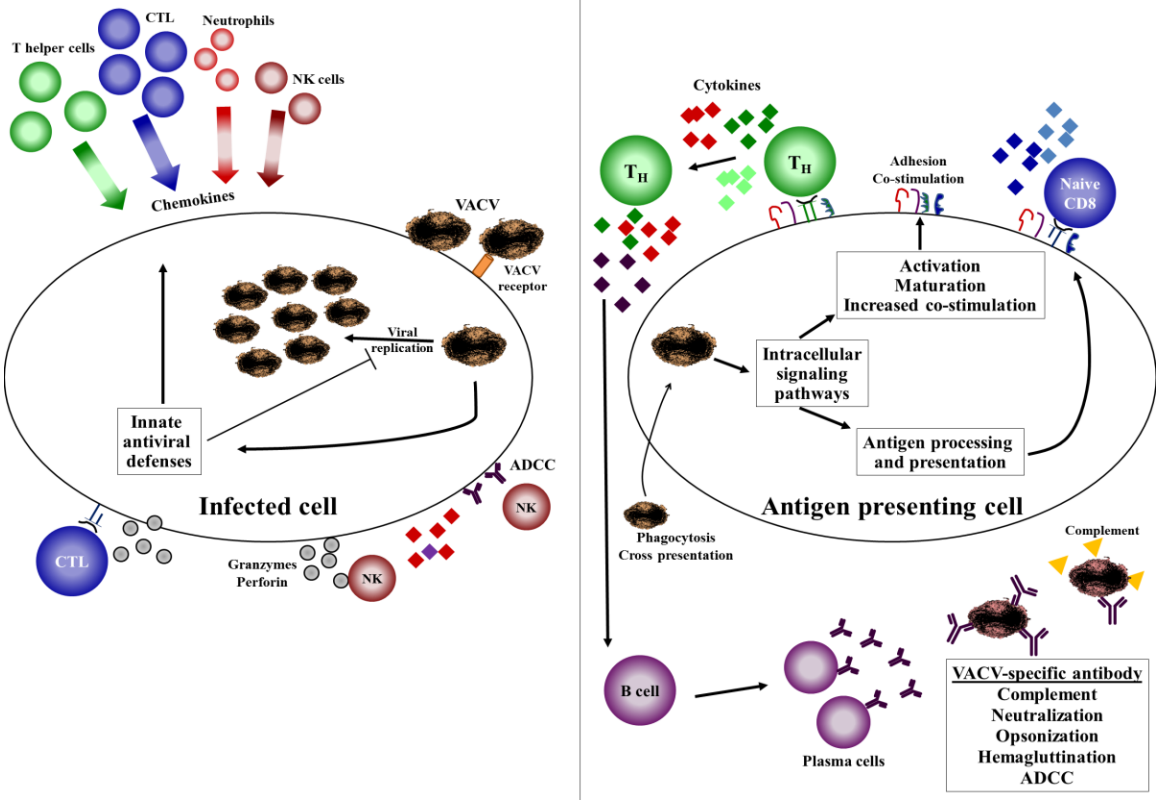


Figure 4. Anti-VACV host immune response.
Antiviral innate immune defenses activated by pattern recognition receptors inhibit viral replication and activate antigen presenting cells (APCs) to initiate adaptive immunity. APC secrete cytokines and chemokines to attract effector lymphocytes into infected tissues. APC acquire and present endogenous (*via* MHC I), or exogenous antigens (*via* MHC II), thus activating $CD8^+$ and $CD4^+$ T cells, respectively. $CD4^+$ T (T_H) cells produce cytokines and costimulatory factors that help B cells produce high affinity anti-VACV antibody (functions listed above), as well as promote $CD8^+$ (CTL) T cell activation, expansion, and effector functions. Once the humoral and adaptive immune responses clear VACV from the host, VACV-specific lymphocytes contract to the smaller, memory populations. Adapted from (33).

Figure 5

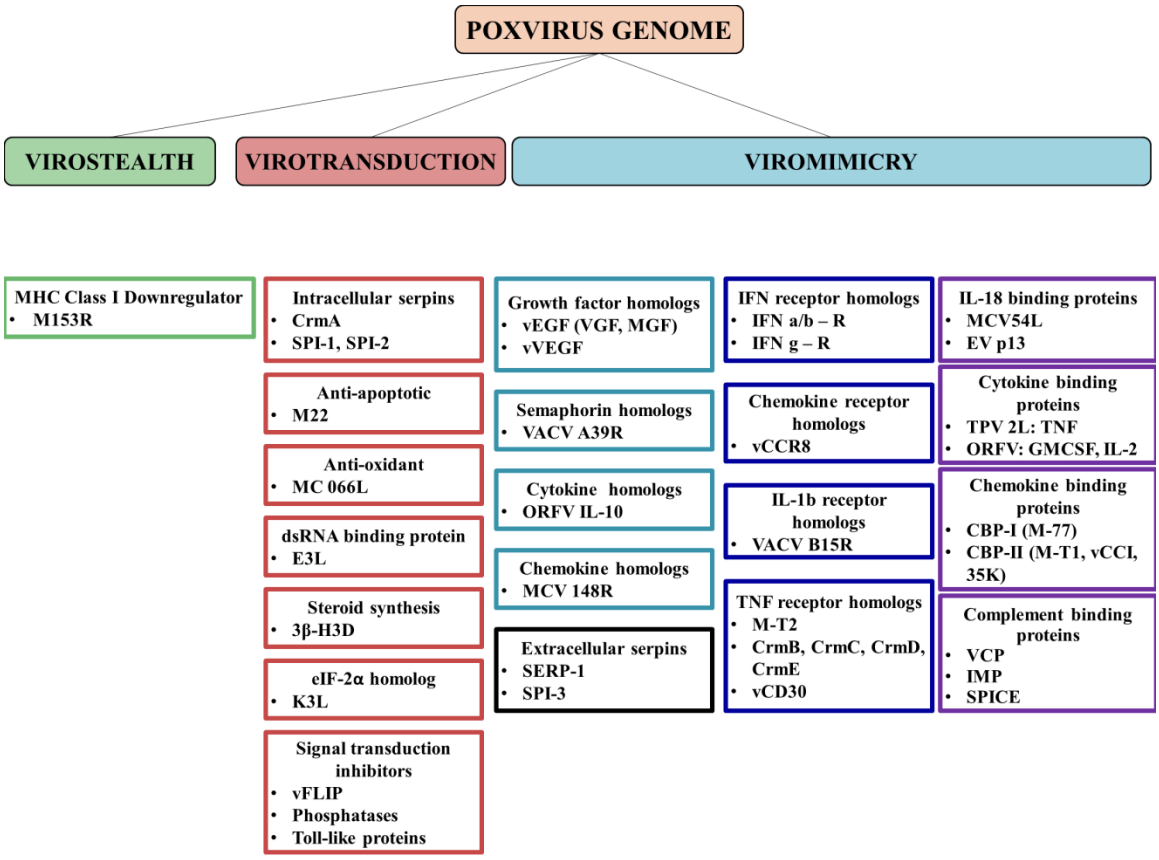


Figure 5. VACV immunoregulatory proteins.
A significant proportion of the VACV genome (~30%) is devoted to immunomodulatory proteins. The functional mechanisms of these proteins are broadly classified as virostealth (proteins that mask infection and reduce cell-mediated immunity, green), virotransducers (proteins that inhibit innate antiviral, cell and host tropism signaling pathways, red), and viromimetics [(proteins that mimic host immunosuppressive proteins (black), cytokines and chemokines (light blue), or their extracellular (purple) and surface receptors (dark blue)]. Adapted from (68, 110).

1.5 Cytopathic effects during virus infection

1.5.1 An introduction to cytopathic effects (CPE)

Cytopathic effects (CPE) are changes in host cell behavior and structure attributed to virus infection. Common CPEs include: cell lysis, apoptosis (programmed cell death), cell rounding, syncytia formation (*i.e.* cell-to-cell fusion), cell-clumping, blebbing, cell detachment, formation of cytoplasmic projections, actin polymerization, cell motility, cessation of contact-inhibition, antigenic changes on the cell-surface, cell division, release of lysosomal contents and formation of inclusion bodies. While some viruses produce obvious CPE, other viruses cause minimal CPE, or in the case of Hepatitis B virus (HBV), CPE that is only apparent in immunocompromised hosts (111). Hereafter follows an introduction to VACV-induced CPE.

1.5.2 Examples of VACV CPE

VACV infection disrupts numerous cellular pathways, resulting in significant changes in cell morphology. For instance, confluent monolayers of adherent cell lines infected with VACV result in the formation of large, circular plaques. Plaque formation is caused by a combination of CPE and migration of infected cells (112). Poxvirus genes that affect plaque formation can be grouped into three categories. First, many poxvirus genes that are essential for virus replication also contribute to plaque formation (reviewed in 85). Second, plaque formation relies upon actin polymerization to spread of infectious EV on actin tails (reviewed in 54). A third group of genes is required for normal plaque morphology and size but are not high contributors to virus output *in vitro* (112). Loss of proteins involved in 1) morphogenesis, 2) intracellular transport of IMV, or 3) actin tail formation dramatically reduce plaque size despite production of high levels of MV.

VACV mutants with reduced plaque size or altered plaque morphology typically alter virulence. Examples in the third category include: C16 and B14 proteins, both of whose functions are required for normal plaque size and for virulence (114, 115). VACV proteins C2 and A55 have been also shown to alter viral plaque morphology, because infections with mutants lacking either protein produced fewer cellular projections and lacked the characteristic Ca^{2+}-independent adhesion of VACV-infected cells (116–118). Identifying and understanding how the third group of plaque phenotype regulatory proteins functions may reveal novel pox virulence mechanisms which contribute to disease. Plaque size is positively correlated with case fatality rates for VARV isolates (119), which has supported the conventional view that changes in plaque morphology will be mirrored by *in vivo* changes to virus replication, spread and/or virulence.

1.5.3 VACV and cell migration

As mentioned above, one virally-induced CPE is cellular migration. It has long been established that VACV infection induces migration of infected cells in culture (120, 121), and this behavior is reminiscent of the epithelial to mesenchymal transition (EMT) of oncogenic cells. VACV-induced cellular migration has been partially attributed to the F11 protein (122, 123), and studies have shown that the F11 mechanism promotes VACV spread, both *in vitro* and *in vivo* (124). However, in regards to other functionally distinct areas of VAVC-induced migration previously described, the mechanism(s) employed by VACV to induce this CPE remains unclear. Interestingly, VACV infection promotes migration of monocytes (125) and DCs (107, 126). Enhanced DC migration is not unique to poxvirus infection (reviewed in 96), as Ebola (128) and HIV-1 (129) infections have also been shown to promote migration of partially matured DCs. In contrast, VACV also encodes chemokine homologues that impair directional

migration of NK cells (130) and leukocytes (69, 73). While cell migratory behaviors are demonstrably affected by VACV infection *in vitro* and *in vivo*, it is still not fully understood how VACV functions within different cell types alter cell migration patterns.

1.6 Vaccinia virus O1L gene in the literature

1.6.1 O1L gene conservation and protein characterization

Expression of the O1L gene (conventionally called O1 when referring to the protein) has been shown to begin early and continue through the infection cycle (8, 89, 131). Incidentally, the majority of VACV immunoregulatory genes are also expressed early, in theory, to rapidly counteract host defenses (18) . One report suggests that the O1 protein is present in the MV (132); however, it was not identified in other studies (133–136). It is unknown with which viral or host proteins O1 may interact, as a large yeast-two-hybrid study reported no interactions between O1 and other VACV proteins (137), and reports have yet to identify interactions with host proteins (reviewed in 111). A recent bioinformatics study proposed a unique mutation pattern within the O1L gene, and suggested that O1 may have been particularly important for promoting virulence as VARV emerged in humans (139). Understanding how VARV adapted to specifically infect humans is an area of ongoing interest, and identification of genetic hotspots that contributed to VARV anthroponotic speciation may be important for predicting future emergence of pox zoonoses.

It has been hypothesized that the poxvirus genome contains immunomodulatory proteins that are highly conserved in mammalian-tropic pox, with relative genetic conservation diminishing as viruses diverge into non-mammalian tropic pox, to the point where there is little to no homology with non-pox proteins. If these proteins are not essential for viral replication and are not related to any known proteins (pox or otherwise), the theoretical implications are that 1)

the proteins do not have an obvious host origin, and 2) poxviruses developed unique functions in a manner divergent from closely related viruses (like herpesviruses); of these pox-specific functions, some may be conserved to combat the mammalian adaptive immune system. Thus far, this hypothesis has led to the identification of several genes, one of which is immunosuppressive, A35 (84, 140–142). The studies discussed hereafter were initiated to determine whether another of these identified genes, O1L, modifies immune responses in a manner unique to poxviruses.

1.6.2 Antigenic characteristics

Several studies have shown that O1 contains prominent epitopes recognized by $CD4^+$ and $CD8^+$ T-lymphocytes (131, 143–145). In a VACV proteome-wide screen, an assay detecting secreted Th1 and Th2 signature cytokines showed that O1 was among the top Th1 targets, as O1 peptides were able to restimulate both IL-2 and IFN-γ production by splenocytes from VACV-WR–infected mice (146). O1 peptides were also identified among a screen of HLA-A and HLA-B binding epitopes as immunogenic, although the immunogenicity of the O1-encoded peptides were either subdominant or cryptic (143). In that report, Assarsson speculated that subdominant T cells may be depleted, tolerized, or impaired in the course of an antiviral immune response to dominant epitopes. Thus, it is possible that immune responses against O1 epitopes may be reduced in favor of a stronger response to more immunostimulatory VACV epitopes.

Reports indicate that O1 may be a prominent target of B lymphocytes as well (147, 148). In a serological screening targeting VACV proteins expressed individually in eukaryotic vectors, O1 was identified among 19 other VACV proteins as an immunodominant antibody target by sera from vaccinated C57BL/6 mice (148). Additionally, O1 was identified among other VACV proteins that elicited a robust antibody response in Dryvax- and ACAM2000-vaccinated individuals (147). Thus, O1-specific antibody could be an important biomarker of VACV

immunity. Taken together, these data suggest that O1 is an important target of the adaptive immune response to VACV. However, the question of whether the immune system can impede the function of O1 is still outstanding.

1.6.3 O1L and VACV CPE

O1L has recently been identified as another VACV gene that influences plaque morphology in a study using the Chorioallantois Vaccinia Ankara (CVA) and Modified Vaccinia virus Ankara (MVA) strains (89). Due to a combination of missing and fragmented genes, MVA fails to replicate on most mammalian cell lines (149, 150). By reinserting fractured or missing genes, it is possible to generate recombinant MVAs with improved replication in some mammalian cells (151). Since the O1L gene is fractured in MVA, Schweneker *et al.* inserted the full-length O1L gene into MVA in an attempt to restore MVA replication (89). While this O1L reinsertion was associated with significant changes in plaque morphology, replication did not significantly improve *in vitro*. Additionally, when O1 was removed from CVA (CVA-ΔO1), plaque sizes were 30 – 50% smaller than parental CVA in human epithelial kidney cells. Differences in plaque phenotype are often indicative of roles in replication, spread, and host range/cell tropism, although new evidence suggests there may be VACV proteins that affect plaque size without affecting any of the former life cycle functions (112). Since the contribution of O1 to VACV replication and spread are still tenuous at best, it may be possible that O1 is among this novel class of proteins for which a functional link to plaque phenotype has yet to be established.

1.6.4 O1L alters MAPK signal transduction

Mitogen-activated protein kinases (MAPK) are an important group of signaling mediators in the cell. The conserved signaling cascade consists of three sequentially activated kinases: a

MAP kinase kinase kinase (MEKK), a MAP kinase kinase (MEK), and a MAP kinase (MAPK). The mitogen activated protein kinase/extracellular signal regulated kinase 1/2 (MAPK/ERK) pathway plays a critical role in transmission of mitogenic and survival signals in response to a variety of extracellular stimuli. Since cell survival is vital for efficient virus multiplication, it is not surprising that many viruses, including VACV, manipulate this pathway.

MAPK signaling cascades are still not completely characterized in the context of OPXV infections. This is unsurprising, given that basal MAPK activation varies with cell type, and OPXV manipulation of signaling cascades varies among species and strains. It is known that VACV infection triggers the MEK/ERK signaling cascade through the epidermal growth factor receptor (EGFR) which promotes infection; the disruption of these pathways results in decreased viral protein expression, DNA replication, and VACV multiplication (85). The C11 protein, also known as Vaccinia growth factor (VGF) has homology to human epithelial growth factor (EGF) and transforming growth factor β (TGFβ). C11 initiates a mitogenic signal that induces the early activation of ERK, but it is not necessary to sustain ERK activation or its downstream effects. Sustained ERK activation enhances cell proliferation in some cell lines, creating a productive environment for VACV replication. While the exact mechanism is still not fully characterized, some reports link proliferative effects of C11-mediated ERK activation to the proto-oncogene c-fos (88). On the other hand, since c-fos transcription also increased with ΔC11 infection, other VACV protein(s) may be implicated in inducing c-fos activity. C11 also activates the EGF/interferon (IFN)-responsive Cip1 protein in cells expressing high levels of EGFR, resulting in cell cycle arrest (152). Interestingly, Cip1 is also thought to promote cell motility (153), but this has not been investigated in context of VACV infection. C11 functionally synergizes with a number of VACV proteins, including F1, which in turn suppresses apoptosis (154, 155). It is

clear that VACV manipulation of ERK signal transduction though C11 activation of EGFR results in a myriad of downstream effects that are not limited to viral replication, since numerous genes cooperate with C11 to promote ERK activation. However, only C11 has been shown to significantly enhance VACV replication through ERK manipulation in cell culture. Overall, C11-activated ERK may result in any number of effects depending upon the cell type, so the functional relevance *in vivo* will need to be carefully dissected in the future.

O1 has recently been implicated in modulating the Raf-MEK-ERK signal transduction pathway (89). Schweneker reported that CVA-ΔO1 infection reduced phosphorylation of c-RAF, MEK, and ERK1/ERK2, with no effects on JAK, FAK, AKT, or PI3K kinases. This indicates that O1 may function upstream of RAF, resulting in ERK activity that could indirectly influence any number of cellular processes, including cell proliferation (156), apoptosis, cytokine production, angiogenesis (157), cell polarity (158), and migration (159). While the reduction in CVA-ΔO1 plaque size described above was partially attributed to the reduction in ERK signaling, the size of plaques treated with an ERK inhibitor were reduced similarly in both CVA and CVA-ΔO1, with CVA-ΔO1 plaque diameter again reduced by 30% compared to CVA. This suggests that the effects on plaque size are not entirely attributable to O1 stimulation of ERK activity.

Importantly, since threshold ERK activity varies widely among cell types, it is likely that the function of O1 in an immortalized cell line does not accurately reflect viral effects *in vivo*. Furthermore, while prior studies have provided valuable insight into how viral manipulation of ERK signal transduction promotes virus infection and survival in the host, the associated mechanisms are vastly different. ERK manipulation may promote viral entry, expand host range,

and/or inhibit antiviral factors depending on the virus, host, and even cell type. Thus, the functional significance of upregulated MAPK signal transduction remains elusive.

1.6.5 O1L contributes significantly to VACV virulence in mice

Multiple studies have demonstrated that O1 and its orthologs are not essential for viral replication (89, 160–163), and reinsertion of O1 into MVA (where the gene is truncated) proved insufficient to restore wild type levels of viral replication in mammalian cells (89). However, O1 was shown to be a significant contributor to VACV virulence in mice (89), observations that have been independently documented elsewhere [Wilkinson, unpublished; and (164)]. Schweneker *et al*. reported that BALB/c mice ($H2^d$ haplotype) infected with CVA-ΔO1 (10^6 – 10^7 PFU/mouse) exhibited significantly reduced morbidity, and mortality rates were 80% lower than CVA-infected mice. A preliminary study by Wilkinson (unpublished) demonstrated similar effects in C57BL/6 mice infected with ΔO1 and the parental VACV-WR strain. In both studies, no differences were reported in viral titers of murine lungs, indicating that O1 is unlikely to influence VACV replication. On the other hand, Schweneker reported that CVA titers were higher than CVA-ΔO1 in distal organs (ovaries), but only significantly differed six days post-infection. Since VACV preferentially infects ovaries (165), high titers six days post-infection are not surprising. Given no differences in viral replication *in vitro* and in murine lungs, differences in ovary titers six days post-infection may indicate either: 1) enhanced tropism of CVA for ovarian tissue; 2) a delay in CVA clearance; or 3) a defect in CVA-ΔO1 dissemination. None of these are mutually exclusive. Since an intact humoral immune response is required to control VACV dissemination (166), a defect in either $CD4^+$ T cell activity or antibody levels (or both) during CVA infection would allow uncontrolled dissemination and prolonged presence within

the ovaries. As it stands, O1 contributes to virulence in mice, but the mechanism *in vivo* remains poorly understood.

Figure 6

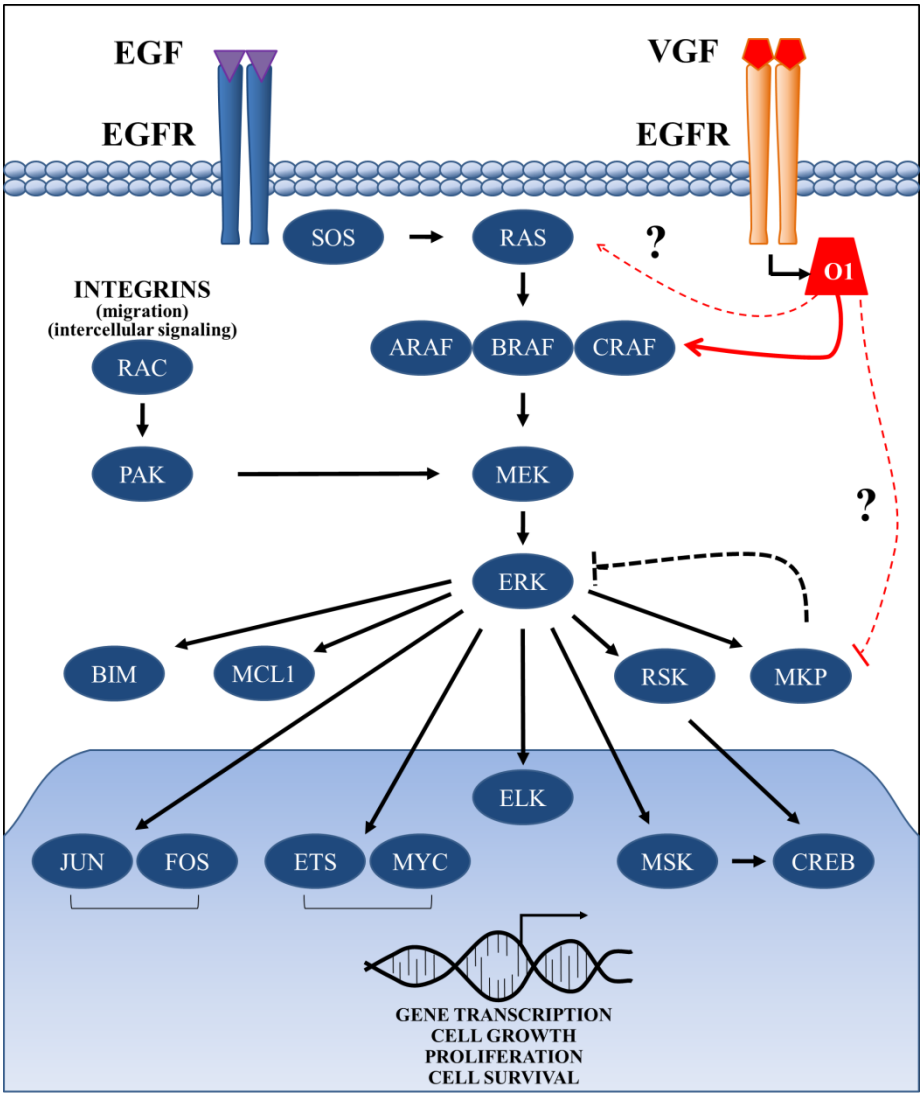


Figure 6. ERK/MAPK signal transduction and putative O1 mechanism

The mitogen activated protein kinase (MAPK) signal cascade is activated by ligation of various cell surface receptors, including the extracellular growth factor (EGF) receptor (EGFR). Activation of the extracellular signal regulated kinase (ERK) MAPK cascade through EGFR results in cellular functions (*e.g.* gene transcription, cell growth and proliferation, and cell survival) that can be further modified by simultaneous signaling through alternate surface molecules (*i.e.* integrins). The VACV C11 protein is a viral growth factor (VGF) homolog of EGF. VGF activates the ERK MAPK cascade, which enhances VACV multiplication by promoting cellular proliferation. The VACV O1 protein purportedly functions downstream of VGF to sustain ERK signaling activated through RAF and MEK (89). Although the exact mechanism of O1 is unknown, sustaining activation of ERK could occur through 1) enhancing upstream stimulators of RAF (*e.g.* RAS, SOS); 2) directly activating RAF; or 3) inhibiting negative regulators of ERK (e.g. MKP). O1 modulation of the RAF/MEK/ERK cascade has insofar not been linked to changes in cellular physiology; thus, the functional significance remains unknown.

1.7 Project Aims and Hypotheses

The overall aim of this project was to understand how the VACV O1 protein promotes virulence in mice, as attaining better insight into O1-specific mechanisms that enhance VACV survival of the immune response may have implications for biotherapeutic applications. Since viral modulation of signal transduction molecules can cause varied effects among hosts and immune cell types, consistency of O1 effects were evaluated across various immune and cultured cell types. Additionally, prior studies of non-essential VACV proteins that contribute to plaque morphology have revealed unique virus-host interactions, some of which influence the host immune response. Therefore, the effects of O1 on VACV-induced CPE was also examined Finally, since structural data on the O1 protein has been limited to sequence analysis predictions, the structural characteristics of the O1 protein were elucidated in order to gain insight into the functional mechanism of this protein.

Thus, experiments were undertaken to **test the hypotheses that O1**:

1) Influences the host immune system (Chapter 3)

2) Functions differently in alternate cell types (Chapter 4)

3) Alters VACV CPE other than plaque phenotype (Chapter 4)

4) Has novel protein characteristics (*e.g.* size, modifications, homology, expression kinetics) (Chapter 4)

If these hypotheses are supported, this would reveal (a) mechanism(s) whereby O1 functions in a novel manner to influence the host immune response to promote virulence. The functional mechanism(s) could be further delineated *in vitro* if alterations in cell-specific behavior are noted in ΔO1 compared to VACV, and if any cell-specific functions are influenced by the unique composition of the O1 protein.

Chapter 2. Materials and Methods

2.1 Virological methods

2.1.1 Cell lines and culture conditions

Mammalian cell lines used included: BS-C-1 green monkey kidney cells, RAW 264.7 murine macrophage cell line, 1153 B lymphocytes and B04 T lymphocytes [both HEL peptide-specific murine cell lines previously obtained from the Janice Blum laboratory (167)], cytotoxic T lymphocyte line (CTLL), Jurkat human T lymphocyte cell line, and HeLa human immortalized ovarian cell line (kind gifts of Dr. Isabelle Lemasson). RAW 264.7 cells were cultured using Dulbecco's modified Eagle's medium (DMEM). BS-C-1 and HeLa cells were cultured in modified Eagle's medium (MEM), and all lymphocyte cell lines were cultured in Roswell Park Memorial Institute (RPMI) media. All media were completed by supplementation with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 μM beta-mercaptoethanol. IL-2 dependent CTLL T cell lines were grown in complete RPMI (cRPMI) supplemented with a 0.5% dilution of IL-2 containing supernatants (produced by Sf9 insect cell culture infected with IL-2 expressing-baculovirus, a kind gift from Dr. Mark Mannie). Bone marrow derived dendritic cells (BMDC) were obtained by harvesting bone marrow and culturing for 6-8 days in cRPMI supplemented with granulocyte-macrophage colony stimulating factor (GM-CSF, 1.5% Sf9 supernatant dilution). All cell lines were cultured at 37˚C in 5% CO_2 atmosphere conditions.

2.1.2 Viruses, propagation, and purification

Vaccinia virus Western Reserve strain (VACV-WR) was used throughout as the wild-type (O1-expressing) virus. O1L deletion mutants (ΔO1) were constructed previously (168) by homologous recombination of the O1L gene with an enhanced green fluorescent protein (eGFP)

marker. O1 deletion mutants (ΔO1-1A1 and ΔO1-3A2) were independently selected and propagated, and both ΔO1 viruses were used throughout experiments to confirm ΔO1 phenotypes. For virus propagation, BS-C-1 cells were grown to desired confluence (80-90%); viruses were diluted in cMEM to desired multiplicity of infection (MOI), and incubated for 2-3 days at 37˚C. VACV-infected cell lysates were collected and stored at -80˚C for subsequent infections. Crude lysates were purified *via* sucrose gradient centrifugation prior to use in all animal infection and immunological assays.

2.2 Animals and reagents

2.2.1 Mouse infections

All animal experiments described herein were approved by the East Carolina University Animal Care and Use Committee and performed in an AALAC accredited facility. For analysis of VACV immunological effects, BALB/c and C57BL/6 mice were purchased from Charles River. To assess the effects of O1 on the murine immune system, BALB/c mice (n = 5) were intranasally administered aliquots (18 μL total, 9 μL/nares) of either VACV-WR, one of the ΔO1 viruses (ΔO1-1A1 or ΔO1-3A2), or were mock-infected with phosphate buffered saline (PBS). Infectious doses ranged from $7x10^3$-$7x10^4$ PFU/mouse, depending on the experiment, and virus titers were determined on the day of challenge to confirm infectious dose. Weight and signs of illness were monitored daily, and mice were euthanized by isoflurane overdose at the end of the study, or if initial body weight decreased by 20%. Spleens and blood were harvested for ELISA and ELISPOT analysis. For antigen presentation assays, spleens were obtained post-mortem from naïve mice [B6, FOXP3-IRES-GFP knock-in (FIG) mice (B6.Cg-*Foxp3*^{tm2Tch}/J) were crossed with MOG_{35-55} specific TCR transgenic 2D2 mice (Tcra2D2,Tcrb2D21Kuch/J) to obtain 2D2-FIG mice (169), a kind gift from Dr. Mark Mannie] used for other purposes.

2.2.2 Antibodies

Polyclonal rabbit sera (Genemed Synthesis) were raised against two synthetic O1 peptide sequences ($AA_{351-371}$ and $AA_{393-414}$), and used as a primary antibody for immunoblot analysis (1:1000 dilution). An unconjugated anti-mouse IgG-IgM-IgA (H+L, Sigma) was used for ELISA. Fluorescently conjugated primary antibodies used for the studies described herein included: PE-CF594 conjugated anti-CD69 (BD Horizon) BV421 conjugated anti-CD69 (BD Horizon), and PE-Cy7 conjugated anti-CD4. Secondary antibodies included: alkaline phosphatase-conjugated anti-rabbit IgG (Sigma), and alkaline phosphatase-conjugated anti-mouse IgG-IgM (H+L, Invitrogen).

2.3 Analysis of viral protein expression and post-translational modification

2.3.1 SDS-PAGE analysis

Proteins from infected cells were harvested in sodium dodecyl sulfate (SDS)-reducing sample buffer (62.5 mM Tris-HCl, 0.25 M glycerol, 2% SDS, 0.01% [wt/vol] bromophenol blue, 12.5% [vol/vol] β-mercaptoethanol) and boiled at 95°C for 5 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using Tris-HEPES gradient gel (4-20%, Thermo) or prepared Tris-glycine gel (resolving gel of 8% acrylamide solution, 0.375 M Tris-HCl, pH 8.8, 0.1% [wt/ vol] SDS, 0.1% ammonium persulfate (APS), and 0.1% N,N,N=,N=-tetramethylethylenediamine [TEMED]; stacking gel of 4% acrylamide solution [37.5:1], 0.375 M Tris-HCl, pH 6.8, 0.1% [wt/vol] SDS, 0.1% APS, and 0.1% TEMED). Coomassie Blue R-250 (Sigma: 0.5 % [wt/vol] prepared in 50% methanol with 10% acetic acid) staining was used for visualization of total protein.

2.3.2 Immunoblot for protein expression and size analysis

Proteins samples were resolved *via* SDS-PAGE (as described above) and transferred (200 mA, 2h) to PVDF membranes using low methanol transfer buffer (12% methanol, 0.1% SDS). Membranes were probed with anti-O1 rabbit sera as primary antibody diluted in PBS- milk (5% [wt/vol] skim milk in PBS with 0.1% Tween 20) for 30 min. The membrane was washed three times in PBS-milk and probed with alkaline phosphatase conjugated secondary antibodies for 30 min. Immunoreactive protein bands were visualized by incubating membranes in alkaline phosphatase substrate (Western Blue, Promega) for 30-45 minutes (25˚C).

2.3.3 Glycosidase assay for analysis of N-linked glycosylation

To determine whether O1 is N-linked glycosylated, HeLa cells were infected (MOI =10) for 18 hours. Whole cell lysates were digested for 3-5 hours using either endoglycosidase H (Endo H, Promega) or peptide N glycosidase F (PNGase F, Promega). SDS-PAGE was performed under denaturing conditions as described above. Immunoblots were developed using anti-O1 sera or polyclonal anti-B5R sera (BEI Resources) to detect a known vaccinia N-linked glycosylated protein (B5R) as a positive control for enzymatic degradation.

2.4 Analysis of antiviral immune responses *in vivo*

2.4.1 ELISA for *in vivo* total anti-VACV immunoglobulin

Enzyme-linked immunosorbant assay 96 well plates (Immulon H2B, Thermo Electron) were coated with ELISA coating buffer (ddH_2O, 1% H_2BO_4, 0.7% NaCl, pH 8.6) containing VACV-WR lysates (10^5 PFU/well) and incubated overnight at 4˚C. Plates were blocked (PBS, 2% FBS, 0.1% NaN_3) at room temperature for 30 min, washed three times (PBS, 0.02% Tween 20, 0.1% NaN_3), and titrations of sera obtained from each infected mouse were added. Plates were incubated 1.5 hours at room temperature and washed again before the addition of the

secondary antibodies (alkaline phosphatase-conjugated goat anti-mouse IgM-IgA-IgG and alkaline phosphatase-conjugated goat anti-mouse IgG [Southern Biotech]). After a 2 hour incubation period, plates were washed three times, developed (alkaline phosphatase substrate kit; Bio-Rad), and the colored product was analyzed by spectrophotometric detection of absorbance at 405 nm.

2.4.2 ELISpot

Anti-mouse IFNγ antibodies (1:500 dilution) were used to coat 96 well plates (1X PBS, pH 7.2) and incubated overnight. Plates were blocked with blocking buffer (2% FBS in PBS, 0.1% NaN_3) and washed with PBS prior to the addition of mouse splenocytes. Splenocytes were then incubated in cRPMI media for 40 hours with VACV-WR infected cell lysates (MOI = 1) to stimulate production of IFNγ. After the removal of splenocytes and virus with wash buffer (PBS with 0.05% Tween 20, 0.1% NaN3), plates were then blocked with biotinylated rat anti-mouse IFNγ (1:250 dilution) and incubated for 2 hours in a humidified chamber. Plates were washed again with wash buffer and blocked with Streptavidin AP. Following a 1 hour incubation and washing, plates were overlayed with BCIP in AMP buffer and agarose to obtain countable blue spots upon development.

2.5 *Ex vivo* immunological assays

2.5.1 Purification and activation of T cells

Splenocytes were isolated from C57BL/6 mice and infected for 3 hours (MOI = 3 and 5) with VACV-WR, ΔO1 or mock (cRPMI), followed by treatment with concanavalin A (ConA, 5 μg/mL) diluted in cRPMI to induce activation. Alternately, $CD4^+$ T cells were purified *via* positive selection using $CD4^+$ T cell magnetic beads (EasySep), infected and activated with

ConA as before. Cells were monitored microscopically for blastogenesis for 24 - 48 hours prior to flow cytometric experiments assessing activation markers.

2.5.2 Purification and activation of B cells

Splenocytes were isolated from C57BL/6 mice and purified *via* negative selection using Pan-B cell magnetic bead kit (EasySep). Cells were infected for 3 hours (MOI = 5) with VACV-WR, ΔO1 or mock (cRPMI) and plated in 96 well plates ($1x10^5$ cells/well). Appropriate groups were treated with lipopolysaccharide (LPS, Sigma: 1-10 μg/mL) diluted in cRPMI. For *ex vivo* antibody production assays, supernatants (100 μL) were collected from plates that had incubated for either seven or 12 days post-infection.

2.5.3 Antigen presentation assays

2D2-FIG mice are TCR transgenic mice with self-reactive TCR that are specific for myelin oligodendrocyte glycoprotein peptide 35-55 (MOG_{35-55}) and cross-reactive with the neurofilament medium ($NEFM_{18-30}$) peptide (169, 170). 2D2-FIG splenocytes contain both APC and T cells specific for these antigens. Upon incubation of splenocytes with the antigenic peptide, splenic APC present antigen to activate T cells, which produce lymphoproliferative cytokines like IL-2. Splenocytes isolated from 2D2-FIG mice were cultured in 96 well plates ($1x10^5$ cells/well) and infected with VACV-WR, ΔO1 (MOI = 1, 3h), or mock-infected (media) as a positive control. Cells were then pulsed with one of the cognate antigenic peptides [either MOG (5 μg/mL) or NEFM (1 μg/mL)] and supernatants were collected 24-96 hours post-infection to determine levels of bioactive IL-2 *via* bioassay

2.5.4 CTLL cell proliferation bioassay

Antigen presentation was quantified using a bioassay measuring proliferation of IL-2 dependent CTLL T cells. CTLL cells were washed with cRPMI twice to remove IL-2 within

culture media, and incubated in 96 well plates ($5x10^4$ cells/well in 150 μL) for 24 hours. Supernatants (50 μL/well) from antigen presentation assays described above were added to CTLL cells, followed by 24 hours of incubation. MTS was added (10 μL/well) and absorbance was measured (492 nm) incrementally over 24 hours.

2.5.5 ELISA for *ex vivo* antibody production

Enzyme-linked immunosorbant assay 96 well plates (Immulon H2B, Thermo Electron) were coated with ELISA coating buffer (ddH_2O, 1% H_2BO_4, 0.7% NaCl, pH 8.6) containing anti-mouse IgM-IgG-IgA (H+L, Invitrogen) and incubated overnight at 4˚C. Plates were blocked (PBS, 2% FBS, 0.1% NaN_3) at room temperature for 30 min, washed three times (PBS, 0.02% Tween 20, 0.1% NaN_3), and dilutions of supernatants obtained from *ex vivo* B cell activation culture were added. Plates were incubated 1.5 hours at room temperature and washed again before the addition of the secondary antibodies (alkaline phosphatase-conjugated goat anti-mouse IgM-IgA-IgG) After a 2 hour incubation period, plates were washed three times, developed (alkaline phosphatase substrate kit; Bio-Rad), and the colored product was analyzed by spectrophotometric detection of absorbance at 405 nm.

2.5.6 Flow cytometric analysis

Splenocytes were analyzed by flow cytometry for $CD4^+$ T cell activation. Splenocytes ($1x10^6$ cells/mL) or purified, activated T cells ($1x10^6$ cells/mL) were fixed in 3% paraformaldehyde, washed, and incubated with an anti-Fc block (BD Pharmingen) to prevent non-specific binding of antibodies to Fc receptors. Fluorescently conjugated anti-mouse primary antibodies were incubated on ice for 1 hour, then washed and resuspended in flow buffer (PBS, 2% FBS, 0.2% sodium azide). Samples were taken (10,000 events/group) on an LSR II flow cytometer (Becton Dickinson) and data were analyzed using FlowJo software.

2.6 Analysis of ΔO1 cytopathic effects (CPE)

2.6.1 Plaque assays

For plaque assays, confluent BS-C-1 monolayers were infected in duplicate using the methods describe above. Viral doses added to wells were calculated to result in 50-100 countable plaques per well. Following five 1:10 serial dilutions, appropriate virus dilutions were added to cell monolayers, and plaques were allowed to form 40 – 48 hours before fixation with crystal violet solution (0.1% in 20% ethanol). Viral titers were defined as plaque forming units per mL (PFU/mL) of initial virus solution, as determined by enumeration of plaques by independent blind observers. Plaques were photographed, and plaque diameters were calculated using Fiji software (10 plaques/virus) for statistical analysis.

2.6.2 Wound-healing assay

For wound-healing assays, confluent BS-C-1 monolayers grown in 6 well plates were serum-starved for 2 hours and then carefully scratched with a yellow 200 µl pipette tip to create a grid pattern. Immediately after scratching, cells were washed in PBS and infected in duplicate as described above. Monolayers were photographed at specified times post-infection, and fixed with crystal violet 24 – 48 hours post-scratch. For statistical analyses, cells migrating into scratched regions (five regions/well) were enumerated by two independent observers.

2.6.3 MTS/PMS cytotoxicity assays

The effects of O1 on the viability of various immune cell types [*e.g.* macrophages (RAW 264.7 cell line), T cells (B04, Jurkat, CTLL lines and splenic primary cells), B cells (1153 line and splenic primary cells), and bone marrow derived dendritic cells (BMDC)] were examined. Cells were infected with VACV-WR or ΔO1 (MOI = 1, 5, and 10 PFU/cell), or mock-infected with media. Twenty-four hours post-infection, 10 µL of 3-(4, 5-dimethylthiazol-2-yl)-5-(3-

carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium/phenazine methosulfate [MTS/PMS (2.0 mg/mL MTS and 0.1 mg/mL PMS)] was added to each well and the resultant colorimetric change was detected at 492 nm over designated intervals.

2.7 Computational analysis

The O1 peptide sequence (WR068, PDB: Q80HX1) was obtained from the NCBI database (https://www.ncbi.nlm.nih.gov/protein) and compared to peptide sequences using UniProt (http://www.uniprot.org). Protein domains were characterized using the InterPro database (http://www.ebi.ac.uk/interpro). Transmembrane regions were examined using the TMPred (embnet.vital-it.ch/software/TMPRED_form.html) and MINNOU servers (minnou.cchmc.org), and Signal P was used to predict the signal peptide sequence (http://cbs.dtu.dk/services/SignalP). O1 protein structural predictions were investigated using various programs on Protein Model Portal (http://www.proteinmodelportal.org), and Phyre2 (www.sbg.bio.ig.ac.uk/phyre2) was used to render 3D models of O1 tertiary structure.

2.8 Statistics

For comparison of more than two conditions at a single point in time, one-way ANOVA was used with Tukey's multiple comparison tests. For multiple comparisons of two or more groups, two-way ANOVA with replicates was employed. For comparison of two conditions, a two-tailed Student *t* test was used. The Data Analysis ToolPak in Microsoft Excel was employed for all statistical analyses.

Chapter 3. Immunogenicity during ΔO1 infection was increased compared to VACV-WR infection

3.1 VACV-specific immunoglobulin levels increased in ΔO1-infected mice

3.1.1 *In vivo* antibody levels were enhanced during ΔO1 infection

Published reports have demonstrated that O1 contributes to VACV pathogenesis in mice (89), and it was hypothesized that O1 mediates virulence by interfering with the mammalian immune response. To investigate the effects of O1 upon the immune response, mice were infected with lower doses of VACV-WR or ΔO1 viruses [7 x 10^3 PFU/mouse] to preclude the effects of systemic illness seen in infections with high doses (171). The specific anti-VACV antibody levels detected in sera collected seven days post-infection from VACV-WR infected mice were similar to levels of sera in mock-infected mice (**Figure 7**). Significantly greater quantities of anti-VACV antibody were detected in sera from ΔO1-infected mice. The diminished levels of total anti-VACV antibody in VACV-WR infection suggest that ΔO1 may be more immunogenic than the parental strain.

Figure 7

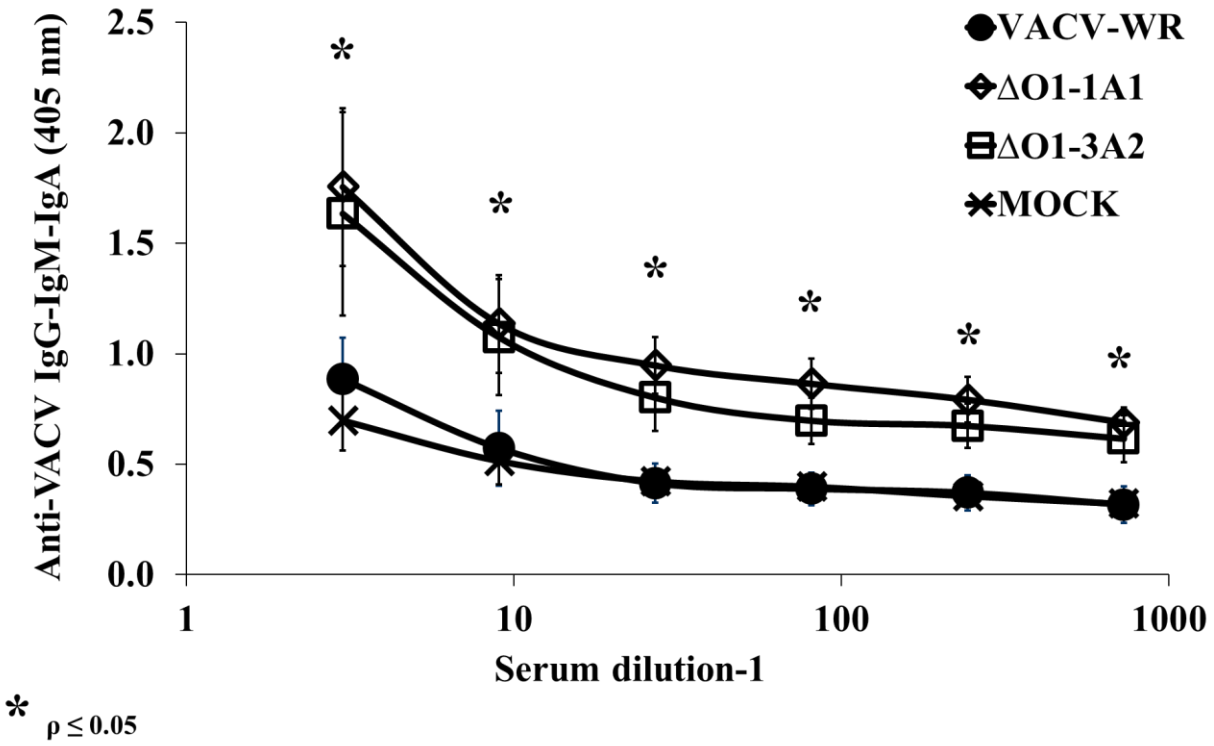


Figure 7. ΔO1 infection induced a stronger humoral response than VACV-WR in mice. BALB/c mice (n = 5) were infected intranasally (2 x 10^3 PFU/mouse) with either VACV-WR (WR), ΔO1 (ΔO1-1A1 or ΔO1-3A2,), or mock-infected (PBS). To quantify the VACV-specific antibody produced, serum isolated from each group seven days post-infection was analyzed by ELISA to detect VACV-specific IgM, IgG, and IgA antibodies. Experiments were performed three times, and the representative data shown are the average absorbance (OD 405 nm) of the serum titration from each group (± SEM). Two-way ANOVA and post-hoc Tukey's assessments were performed to assess the statistical differences between VACV-WR and both ΔO1 mutants. Absorbance of both ΔO1-1A1 and ΔO1-3A2 groups were statistically greater than VACV-WR, with significance at $\rho \leq 0.05$ (compared to VACV-WR).

3.1.2 *Ex vivo* antibody production reduced similarly by VACV and ΔO1 infection

Antibody production was examined *ex vivo* to detect interactions occurring during VACV-WR infection that could result in reduced antibody levels. LPS stimulation of murine splenic B cells moderately increased (20-40%) *ex vivo* antibody levels in all infection groups above unstimulated levels (**Figure 8**)**.** Infection with both viruses significantly reduced antibody levels compared to mock-infected cells, and reductions by VACV-WR and ΔO1 were equivalent. Because splenic-resident T cells and DC are instrumental in helping B cells produce antibody *in vivo*, whole splenocytes were infected as before and stimulated with LPS to induce antibody production (**Figure 9**). Results obtained seven days post-infection (**Figure 9 A**) were similar to those obtained using in purified B cells (*i.e.* LPS induced antibody production that was significantly reduced by infection with either virus). Because the background readings of unstimulated cells seemed high for these assays, splenic cultures were also allowed to incubate longer to ensure sufficient time for antibody production. However, total antibody detected on day 12 (**Figure 9 B**) was diminished compared to seven days post-infection (**Figure 9 A**). Taken together, these data suggest that *ex vivo* assays of LPS-stimulated antibody production are not sufficient to understand how O1 influences antibody production. These data provide suggestive evidence that other mechanisms (*e.g.* cell types or functions) that are operative *in vivo* are likely involved in the processes by which O1 influences antibody production.

Figure 8

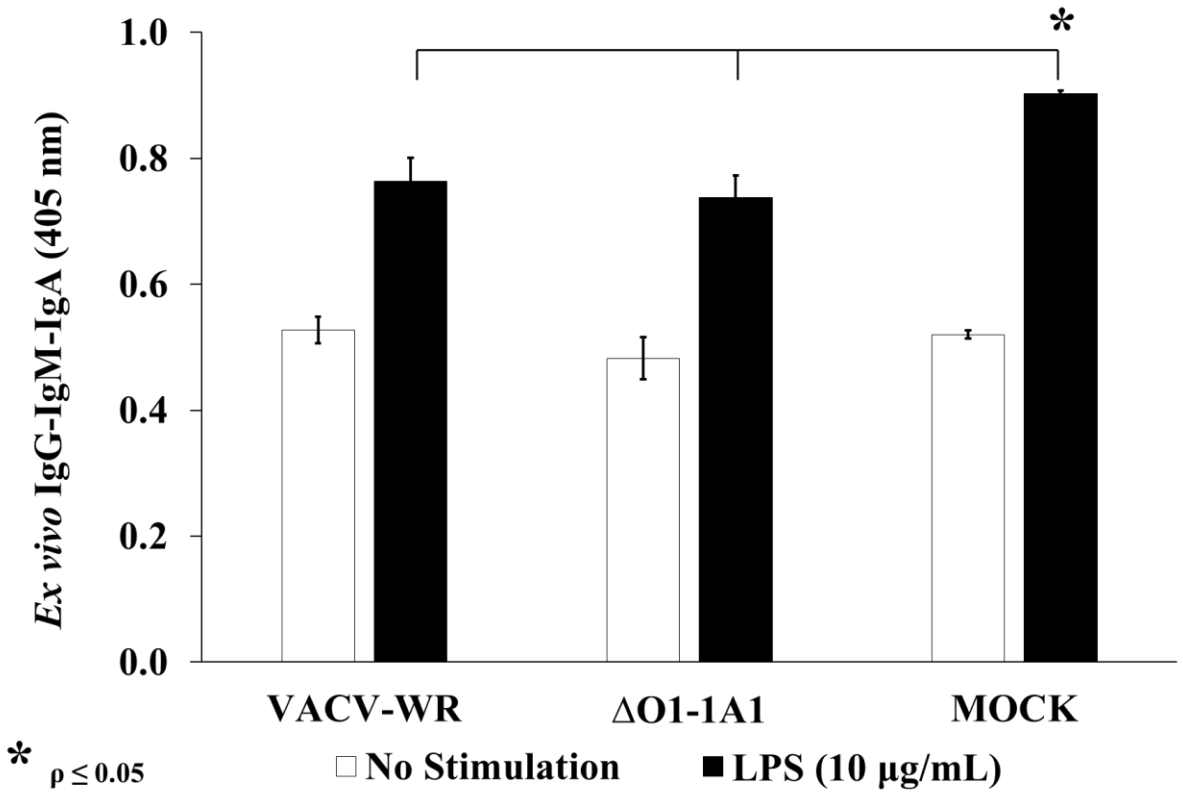


Figure 8: VACV infection reduced LPS-induced immunoglobulin produced by purified mouse splenic B cells.

B cells were isolated (Pan-B negative selection kit, EasySep) from C57BL/6 mouse splenocytes, were infected with either VACV-WR or ΔO1L-1A1, or remained uninfected (MOI = 3, 4 hours). Groups treated with LPS (10 µg/mL) to induce immunoglobulin production are depicted by black bars, and groups of unstimulated B cells are in white. Supernatants were harvested seven days post-infection and assayed *via* ELISA for total mouse antibody (IgG, IgM, IgA) production. OD readings are expressed as the mean (n = 3) ± standard deviation. Experiments were repeated twice, and representative data are depicted. * denotes statistical significant differences between LPS-treated, mock-infected cells and virus groups (black bars, one-way ANOVA, $\rho \leq 0.05$.)

Figure 9

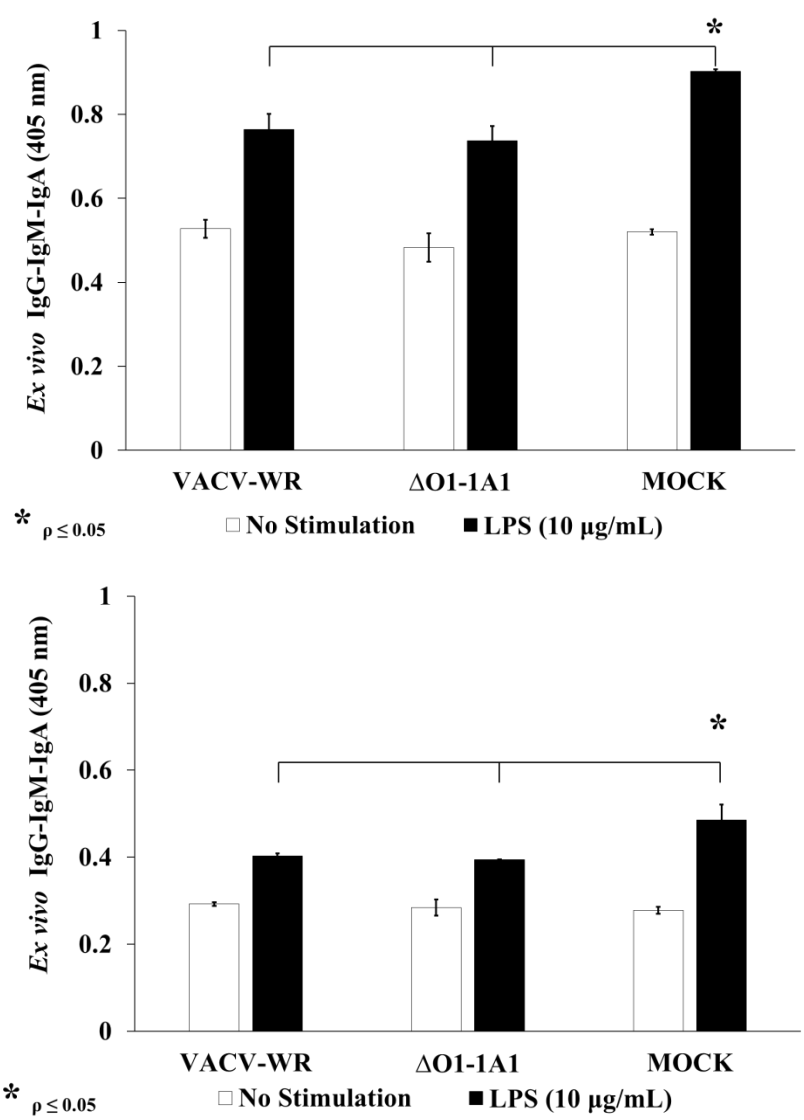


Figure 9: VACV infection reduced *ex vivo* LPS-induced immunoglobulin produced by murine splenocytes.

C57BL/6 mouse splenocytes were isolated and were infected with either VACV-WR or ΔO1-1A1 or were not infected (mock) (MOI = 3, 4 hours). Splenocytes were then treated with LPS (10 µg/mL) to induce immunoglobulin production. Supernatants harvested seven (**Figure 9 A**) and 12 (**Figure 9 B**) days post-infection were assayed *via* ELISA for total mouse antibody (IgG, M, A) production. Each assay was repeated twice, with similar results. OD readings of unstimulated splenocytes (white bars) and LPS-stimulated splenocytes (black bars) are expressed as the mean (n = 3) ± standard deviation * denotes statistical significant differences between LPS-treated, mock-infected cells and virus groups (black bars, one-way ANOVA, $\rho \leq 0.05$.)

3.2 Equal VACV-specific interferon-γ produced by VACV-WR and ΔO1-infected splenocytes

To ascertain whether O1 had an effect on T lymphocytes during VACV infection, ELISpot assays of IFN-γ production were performed. Splenocytes from mice infected with VACV-WR, ΔO1, or mock-infected with saline were isolated, and stimulated with VACV-WR to reactivate VACV-specific T cells *ex vivo*, and IFN-γ was measured as a marker for reactivation. As shown in **Figure 10**, splenocytes from mock-infected mice do not produce IFN-γ, indicating that mock-infected mice do not have recall responses to VACV, as T cells were not introduced to VACV *in vivo*. Splenocytes from mice infected with either VACV-WR or ΔO1 produced equivalent levels of IFN-γ, which suggests that there were no detectable differences in the anti-VACV T cell responses seven days post-infection.

Figure 10

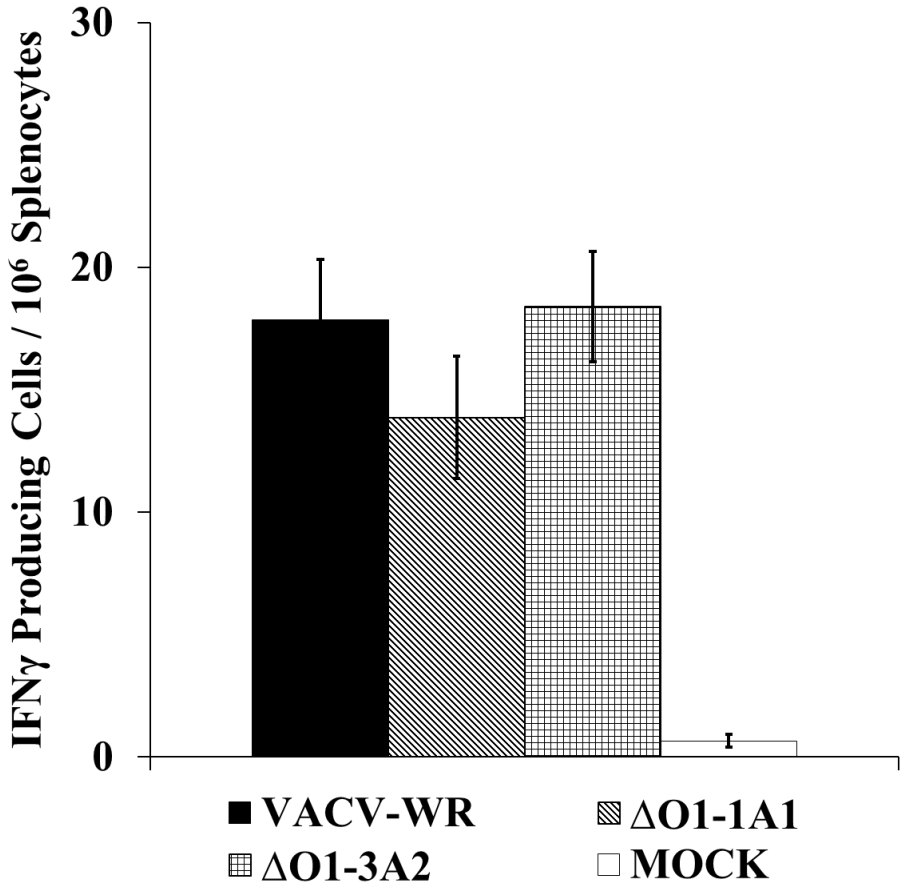


Figure 10: VACV-specific IFN-γ production by VACV-WR and ΔO1 infected murine splenocytes was equivalent.

Effector responses to VACV were quantified by assessing IFN-γ production in response to VACV-WR stimulation *ex vivo*. Splenocytes from VACV-WR, ΔO1 (ΔO1-1A1 and ΔO1-3A2), and mock-infected BALB/c mice (n = 5/group) were isolated seven days post-infection, and stimulated with VACV-WR. IFN-γ producing cells were enumerated via ELISpot assay. Experiments were performed twice, with essentially equivalent results. The mean of each group (± SEM) are depicted, and a one-way ANOVA analysis revealed no statistically significant differences among virus-infected groups ($\rho \leq 0.05$.)

3.3 *Ex vivo* antigen presentation is enhanced during ΔO1 infection

3.3.1 2D2-FIG antigen presentation assay

Because ΔO1 infected mice generated enhanced levels of antibody, it was hypothesized that the O1 function may be immunosuppressive. Since B cells need T cell help for maximal VACV-specific antibody responses, the hypothesis was that lower antibody levels observed *in vivo* could be due to inferior priming of T cells at the level of antigen presentation. Since T cell recall responses were similar (**Figure 10**) during VACV-WR and ΔO1 infection, it was possible that differences *in vivo* may have been too small to distinguish. Alternately, it is likely that O1 may have affected the helper T cell processes necessary for humoral, but not cellular, immunity. Thus, *ex vivo* antigen presentation assays were performed to determine how O1 influences immunological processes.

2D2-FIG mouse splenocytes were isolated and infected with VACV-WR, ΔO1 or mock infected (media), and then stimulated with cognate antigens (MOG_{35-55} or $NEFM_{18-30}$). Supernatants collected at various time points were added to IL-2 dependent CTLL T cell cultures to quantify bioactive lymphoproliferative cytokines produced in response to antigenic stimulation. Supernatants from 2D2-FIG antigen presentation assays promoted CTLL proliferation in peptide-stimulated groups (**Figure 11**). Supernatants from VACV infected groups significantly inhibited CTLL proliferation compared to mock-infected cells. Stimulation with MOG appears to have no effect on VACV-WR infected cells (**Figure 11 A**) whereas supernatants from NEFM treated, VACV-WR infected groups increased CTLL proliferation approximately 10% more than the unstimulated VACV-WR infected group (**Figure 11 B**). However, when cultured with supernatants from antigen stimulated ΔO1-infected groups, CTLL proliferation significantly increased. Although not quite to mock-infected levels, supernatants

from ΔO1 infections enhanced CTLL proliferation by 12 – 20% over VACV-WR groups, and these differences were statistically significant at 24 and 48 HPI. The reduced CTLL proliferation in response to supernatants from the VACV-WR infected, antigen-stimulated group indicate that some step in the antigen presentation process is significantly different during ΔO1 infection. Given the complexity of the antigen presentation process, these data provide a foundational step for the further work needed to determine which processes (*e.g.* antigen processing or presentation, TCR recognition and signaling, IL-2 gene transcription, *etc.*) may be influenced to a greater extent by VACV-WR compared to ΔO1 infection.

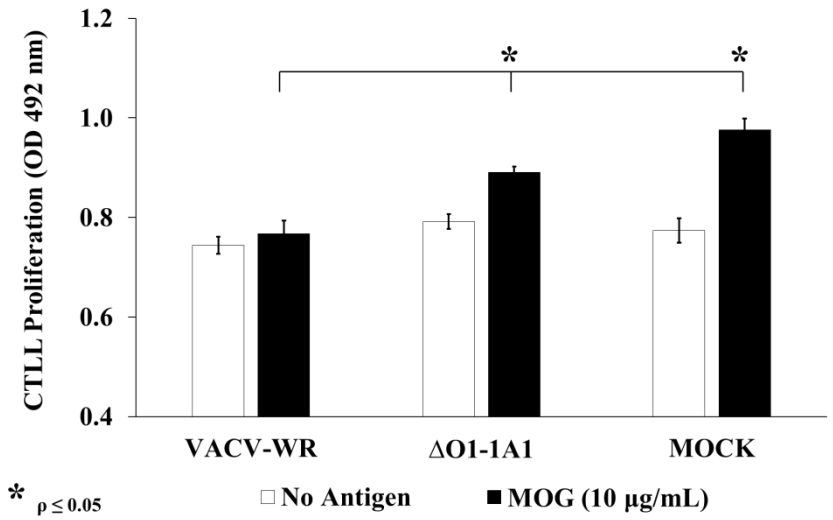
3.3.2 $CD4^+$ T cell activation

Lymphoproliferative cytokines secreted in response to antigenic stimulation may be reduced if T cell activation is altered by VACV-WR infection. Therefore, the possibility was considered that antigen presentation would be reduced in VACV-WR infection if O1 directly affected T cell activation. To investigate this, splenocytes were isolated, infected with VACV-WR or ΔO1, followed by treatment with ConA for 18-36 hours to induce T cell activation. Flow cytometry was used to measure surface expression of CD4 for helper T cells, as well as CD69, a marker of lymphocyte activation. T cells activated with ConA for 18-36 hours were considered the $CD4^+$ $CD69^+$ events present in the upper right quadrant of dot plots (**Figure 12 A**). Treatment with ConA induced a 10 fold increase in $CD4^+CD69^+$ events, with the greatest increase in the ConA-treated mock-infected group. The percentage of $CD4^+CD69^+$ cells trended lower in VACV infected groups (**Figure 12**). VACV-WR and ΔO1 infected groups had an equivalent percentage of $CD4^+CD69^+$ T cells (**Figure 12 B**). These experiments were also repeated using positively selected $CD4^+$ T cells, with similar results (data not shown). Taken together, the equivalent activation of $CD4^+$T cells during VACV-WR and ΔO1 infection suggests that O1 is

unlikely to directly interfere with mitogenic $CD4^+$ T cell activation. Whether or not O1 directly interferes with the antigenic activation of $CD4^+$ T cell activation remains to be seen.

Figure 11

A



B

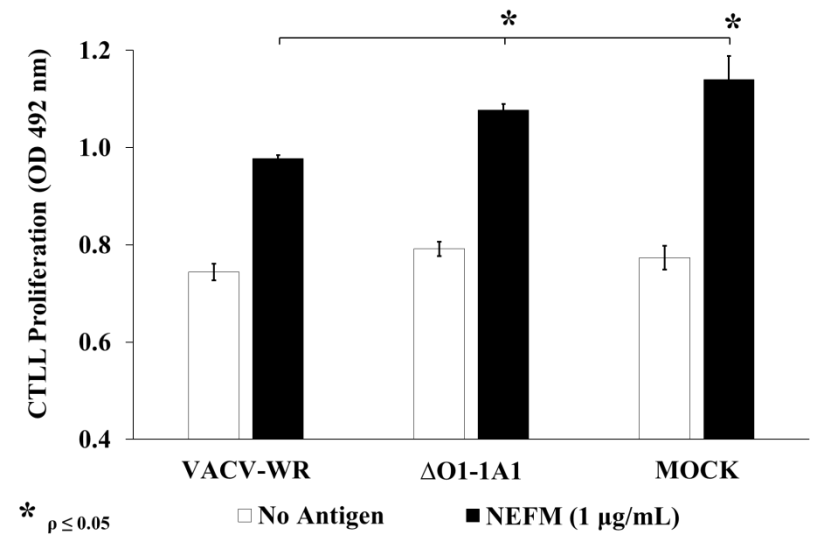


Figure 11. CTLL proliferation was enhanced by antigen-stimulated ΔO1-infected supernatants.

The antigen presentation process, culminating in IL-2 production, was assessed *via* CTLL cell proliferation assay. Splenocytes isolated from 2D2-FIG mice were infected (MOI = 1, 3h) with VACV-WR, ΔO1 (ΔO1-1A1) or mock-infected (mock). Cells were pulsed with cognate antigens (**Figure 11 A**: MOG, 5 µg/mL; **Figure 11 B**: NEFM, 1 µg/mL), and supernatants were collected 24-96 HPI. The IL-2 dependent CTLL T cell line was used to measure IL-2 in collected supernatants. CTLL cells were IL-2 depleted 24 h before addition of collected supernatants (50 - 100 µL). After 24 hr culture, MTS was added and OD read at 492 nm. Experiments were repeated twice, and data from 24 h post-infection is displayed. OD readings are expressed as the mean (n = 3) ± standard deviation. * denotes statistical significant differences between antigen-treated, mock-infected cells and virus groups (black bars, one-way ANOVA, $\rho \leq 0.05$.)

Figure 12

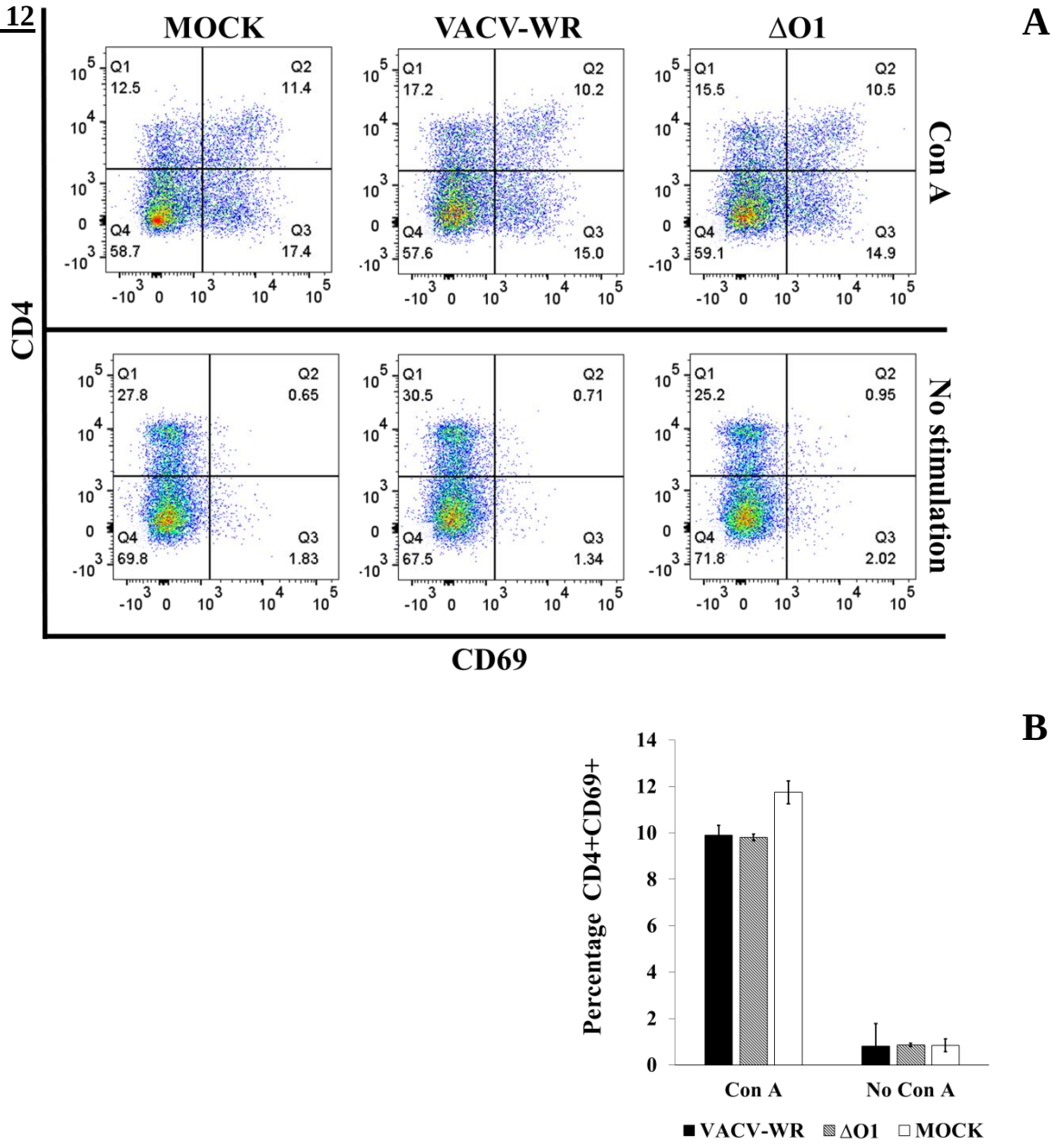


Figure 12: ConA-induced CD4$^+$ T cell activation was equal in VACV-WR and ΔO1 infections.

C57BL/6 mouse splenocytes were isolated, infected (MOI = 3) with VACV-WR or ΔO1 in duplicate, or mock infected (mock) for five hours before treatment with concanavalin A (ConA, 10 µg/mL) to induce T cell activation. After 48 h, flow cytometry was performed to detect activated helper T cells (CD4$^+$CD69$^+$). Cells were gated to exclude debris, and plotted CD4 versus CD69 (**Figure 12 A**) to find the percentage of CD4$^+$CD69$^+$ events (**Figure 12 B**). Data were analyzed via two-way ANOVA, and differences among ConA stimulated groups were not statistically significant. Experiments were performed twice, and representative data is shown.

Chapter 4. O1 protein expression and contribution to VACV CPE

4.1 O1 sequence analysis

The O1L gene (VACV Copenhagen strain designation) encodes the putative O1 protein (666 aa) which is predicted to be approximately 78 kDa. Since there are no reported O1-homologous viral or mammalian proteins with known functions [phylogenetic analysis reviewed elsewhere (139)], comparisons could not be made to predict possible O1 function(s). Thus, software analyses were performed to assess biological composition of O1 to understand how its structure may influence function. The O1L gene is located within the central portion of the VACV genome, which contains a large proportion of genes that are necessary for viral replication and morphogenesis (23). The genes most proximal to O1L include DNA polymerases, DNA binding proteins, and viral core proteins (**Figure 13 A**). However, data from prior studies (89, 168) do not support a role for O1 in viral replication or spread in cell culture or *in vivo*. In **Figure 13 B**, secondary structural domains of the O1 protein include a predicted transmembrane region (TMPred), two nuclear localization sequences (SwissProt), and five N-linked glycosylation motifs (visual inspection of NxS/T sequences). Many peptide sequences in O1 are hydrophobic in nature (**Figure 13 C**), which could suggest membrane association or a tertiary folding pattern such that hydrophobic sequences are sequestered to reduce solvent accessibility. *Ab initio* 3D-rendering of the O1 peptide sequence (**Figure 13 D**, Phyre2) generated a protein with numerous alpha-helices, and several low structure regions. The structural homologs reported by Phyre2 (Table 1) exhibited low-levels of structural identity (7 – 50%, with confidence ranging from 5.2 – 54.3 %). Several enzyme families were overly represented among the proteins identified (more than five hits in six different groups **Table 1 A**), and at least two structural domains were identified more than five different times (**Table 1 B**).

While this may be indicative of potential O1 functional orthologs, the low sequence identity suggests that the function of O1 may be completely novel. Future biochemical studies will be necessary to confirm whether the described peptide regions and structural motifs contribute to O1 location and/or function.

Figure 13

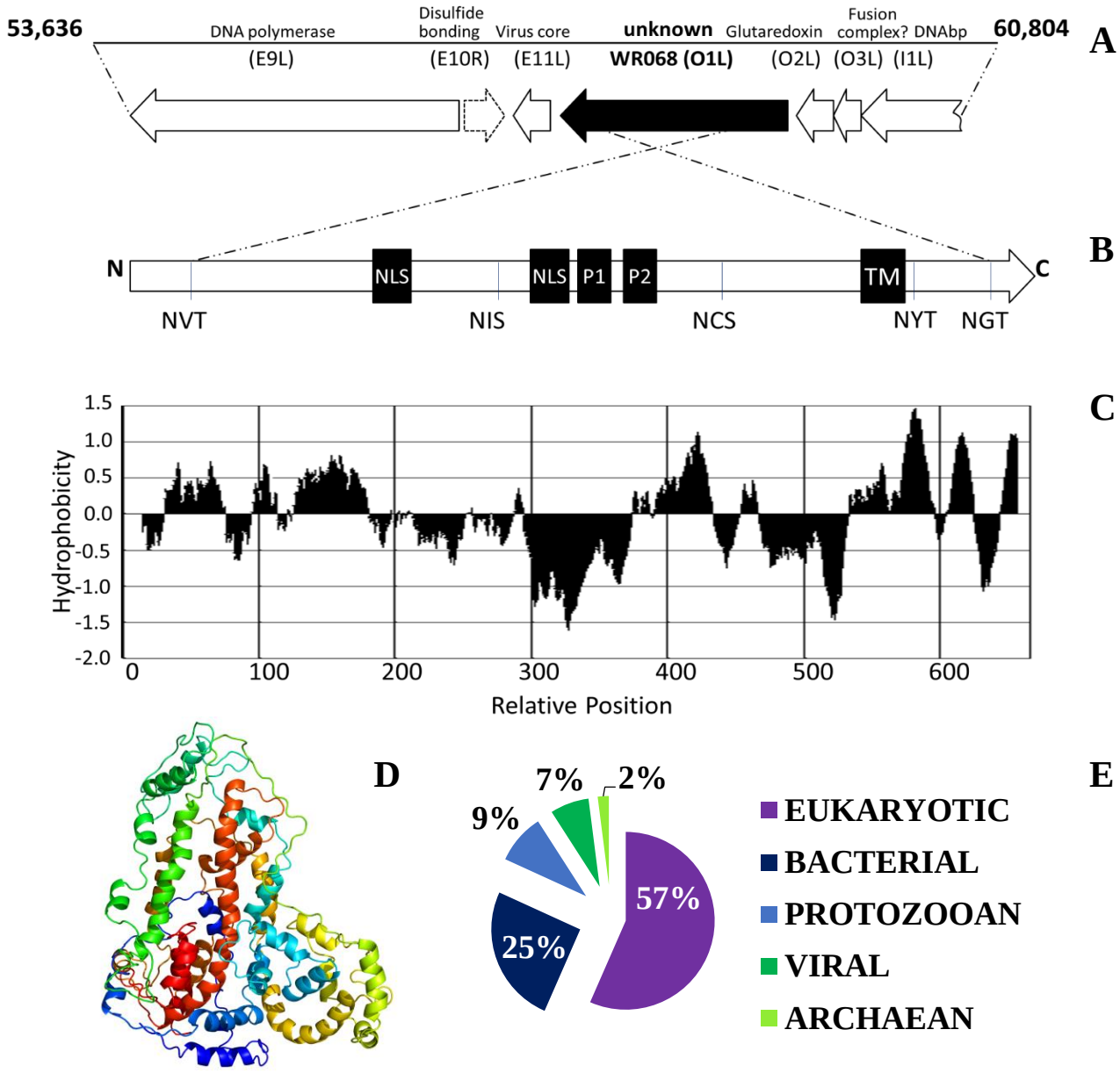


Figure 13. O1 sequence analysis

Figure 13 A: The O1L gene sequence is depicted in context of proximal VACV genes. **Figure 13 B**: Secondary sequence searches revealed predicted N-linked glycosylation sites (designated NxS/T motifs as marked below arrow), transmembrane region (TMPred software, denoted TM), and nuclear localization signals (NLS, SwissProt). Peptide regions selected for anti-rabbit sera generation are denoted P1 and P2. **Figure 13 C:** Plot of O1 sequence hydrophobicity depicts peak regions of hydrophobicity, including one transmembrane region also predicted by TMPred. **Figure 13 D**: A model of the tertiary structure of the O1 protein, as predicted using Phyre2 software. **Figure 13 E** depicts frequency of non-pox proteins reported (Phyre2) to share any degree of sequence identity with O1 within the listed taxonomic domains.

Table 1

A

Protein Family	# hits	% Identity	Confidence
ribosome	8	17.0-27.0	6.8-30.3
ligase	8	7.0-20.0	10.2-28.4
lyase/metal binding	6	12.0-33.0	7.5-22.1
aldolase	6	12.0-18.0	7.5-9.9
hydrolase	5	7.0-16.0	6.7-10.2
oxidoreductase	4	14.0-21.0	6.6-9.7
transcription	4	11.0-56.0	6.2-6.6
CytB endotoxin	3	16.0	33.2-54.3
transferase	3	12.0-15.0	7.5-8.5
viral	2	21.0-30.0	6.2-6.6
phosphorylase	1	17.0	13.6
p53	1	29.0	8.3
TrsP immunogenic peptide	1	50.0	6.1
chaperone	1	18.0	6.0

B

Domain	# hits	% Identity	Confidence
DNA binding	7	9.0-35.0	5.2-9.9
SAM/Pnt	5	20.0-22.0	6.0-13.7
LEM/SAP (HeH motif)	4	23.0-27.0	7.2-10.1
TIM beta/alpha barrel	3	10.0-18.0	8.0-17.4
thioredoxin-like fold	2	7.0-15.0	11.9-17.6
GRIP	2	20.0-23.0	10.4-17.0
ATC-like	2	12.0-13.0	7.0-8.5
MOP-like	2	13.0	6.2-8.2
CARD (7, 11)	2	11.0-19.0	7.5-9.5
ankyrin-like	1	19.0	11.9
C2H2 zinc finger	1	33.0	8.2
Ig variable (V)	1	0.0	7.2
TM helix	1	36.0	7.0
serum albumin-like	1	23.0	6.7
ferrodoxin	1	24.0	6.6
SH3-like	1	16.0	6.5
SM-like fold (TrmB)	1	50.0	6.6
globin-like	1	14.0	5.9

Table 1. O1 shared sequence identity with protein families and domains

The O1 peptide sequence (PDB designation: Q80HX1) was analyzed with Phyre2 to determine structural homology with proteins of known structure. **Table 1 A** summarizes the protein families reported to be among the top 100 proteins to have any degree of sequence homology. **Table 1 B** summarizes the types of protein domains and structural motifs reported. The number of separate structures per domain/family type (# hits), the level of sequence similarity (% identity), and the probability that O1 is a structural homolog of these proteins [% confidence, (100% maximal)] is listed.

4.2 Characterization of O1 protein expression during VACV infection

4.2.1 O1 protein was determined to be approximately 78 kDa

Polyclonal rabbit sera were raised against two synthetic O1 peptide sequences [($AA_{351-371}$ and $AA_{393-414}$), Genemed Synthesis]. The two sequences selected consist of relatively hydrophilic amino acids (**Figure 13 B and C**), in order to increase the likelihood of antibody epitope availability in both a linear and conformation state. To detect expression of O1, VACV-WR and ΔO1 infected cell lysates were separated *via* SDS-PAGE and immunoblots were probed with each of the four anti-O1 rabbit sera generated. Initially, none of the sera were able to differentiate between bands present within VACV-WR and ΔO1 cell lysates (data not shown), so rabbits were boosted with O1 peptides to enhance levels of anti-O1 antibody in sera. Subsequent immunoblots revealed a single ~81 kDa band that was present in VACV-WR lysates but not in ΔO1 lysates (**Figure 14 A**) or mock-infected controls (data not shown), suggesting the band detected within VACV-WR lysates is the O1 protein. Further electrophoresis was performed to determine molecular weight more precisely (**Figure 14 B**), and the O1 band was calculated to be ~78 kDa.

4.2.2 Expression of O1 was detected from 3 to 24 h post-infection

The O1L gene sequence is predicted to contain an early promoter (8), and mRNA has been detected as early as three hours and throughout infection (89). Protein expression was examined *via* immunoblot at various times (3, 6, 8, and 24 HPI). A single O1 band (~78 kDa) was detected at each time point tested (**Figure 15**).

Figure 14

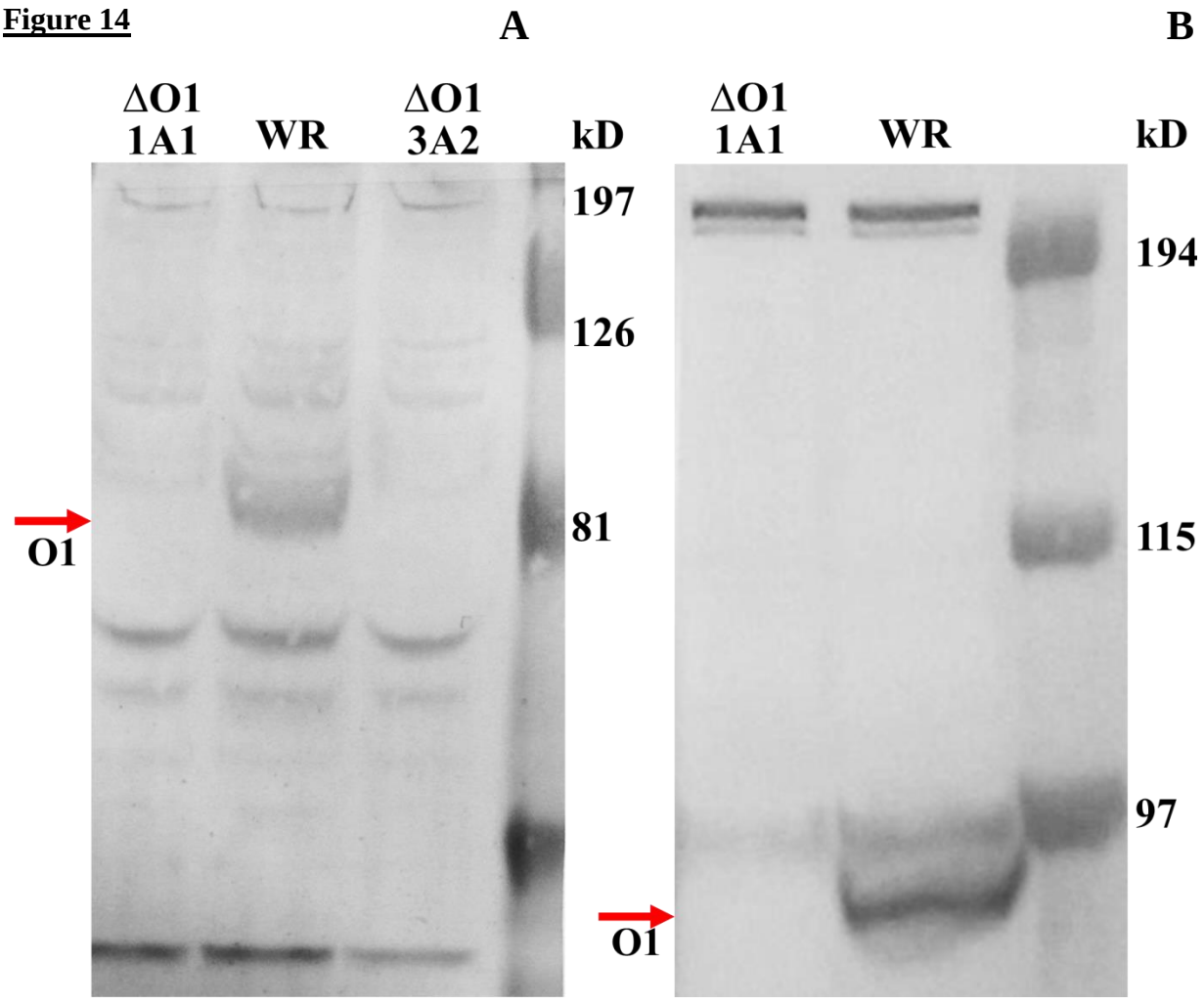


Figure 14: O1 protein was approximately 78 kDa.
Polyclonal rabbit antisera were raised against two O1 peptides (**351-LEDILAHIDNARKNSKVSIED-C and 393-LSDIDIKTKIMVLKIVKDWKSC**). To detect the O1 protein, BS-C-1 cells were equally infected with either VACV-WR (WR) or an ΔO1 mutant (ΔO1-1A1 and ΔO1-3A2). Whole cell lysates were obtained and separated on 4-20% SDS-Tris-HEPES gels. All immunoblots performed as described were repeated at least 3 times, and representative blots are displayed.

Figure 15

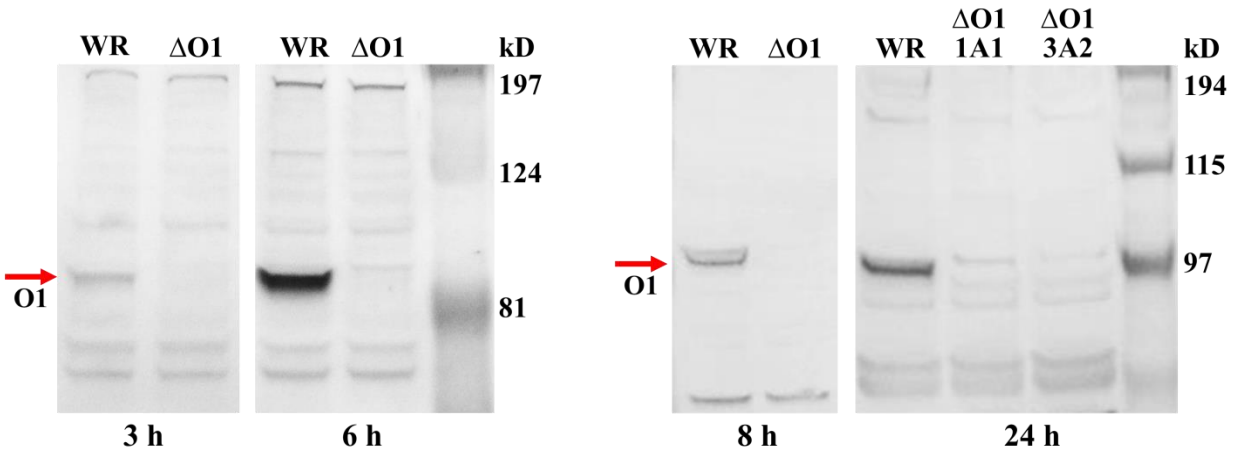


Figure 15. O1 protein expression was detected from 3 to 24 hours post-infection.
HeLa cells were infected (MOI = 10 for 3 and 6 h; MOI = 5 for 8 and 24 h) with either VACV-WR (WR) or an ΔO1 mutant (ΔO1-1A1 and ΔO1-3A2). Nuclei were isolated, separated via SDS-PAGE, and immunoblots were performed using rabbit polyclonal anti-O1 sera. Electrophoresis was performed as described at least twice, and representative immunoblots are displayed. Arrows indicate putative O1 band.

4.3 N-linked glycosylation (NXS/T) is predicted by O1 sequence, but was not detected *via* immunoblot

The O1 peptide sequence contains numerous predicted glycosylation sites, including five N-linked glycosylation motifs (NxS/T, **Figure 13**). To determine whether O1 is post-translationally glycosylated, VACV-WR and ΔO1 infected cell lysates were incubated with either endoglycosidase H (EndoH), which cleaves the chitobiose core of high mannose and some hybrid oligosaccharides, or peptide:N glycosidase F (PNGase), an amidase which cleaves the bond between the asparagine residue and the innermost N-acetylglucosamine moiety of high mannose, hybrid and complex oligosaccharides. Immunoblot analysis of treated lysates demonstrated that B5R, a known N-linked glycosylated VACV protein, was degraded in the presence of both EndoH and PNGase F (**Figure 16**), resulting in several faster migrating peptides in the EndoH and PNGase F treated lanes. However, in both EndoH and PNGase treated groups, the O1 band resolved as a single band at the same molecular weight (~78 kDa) as in untreated lysates. Therefore, no evidence to support N-linked glycosylation of the O1 protein was detected in this assay.

Figure 16

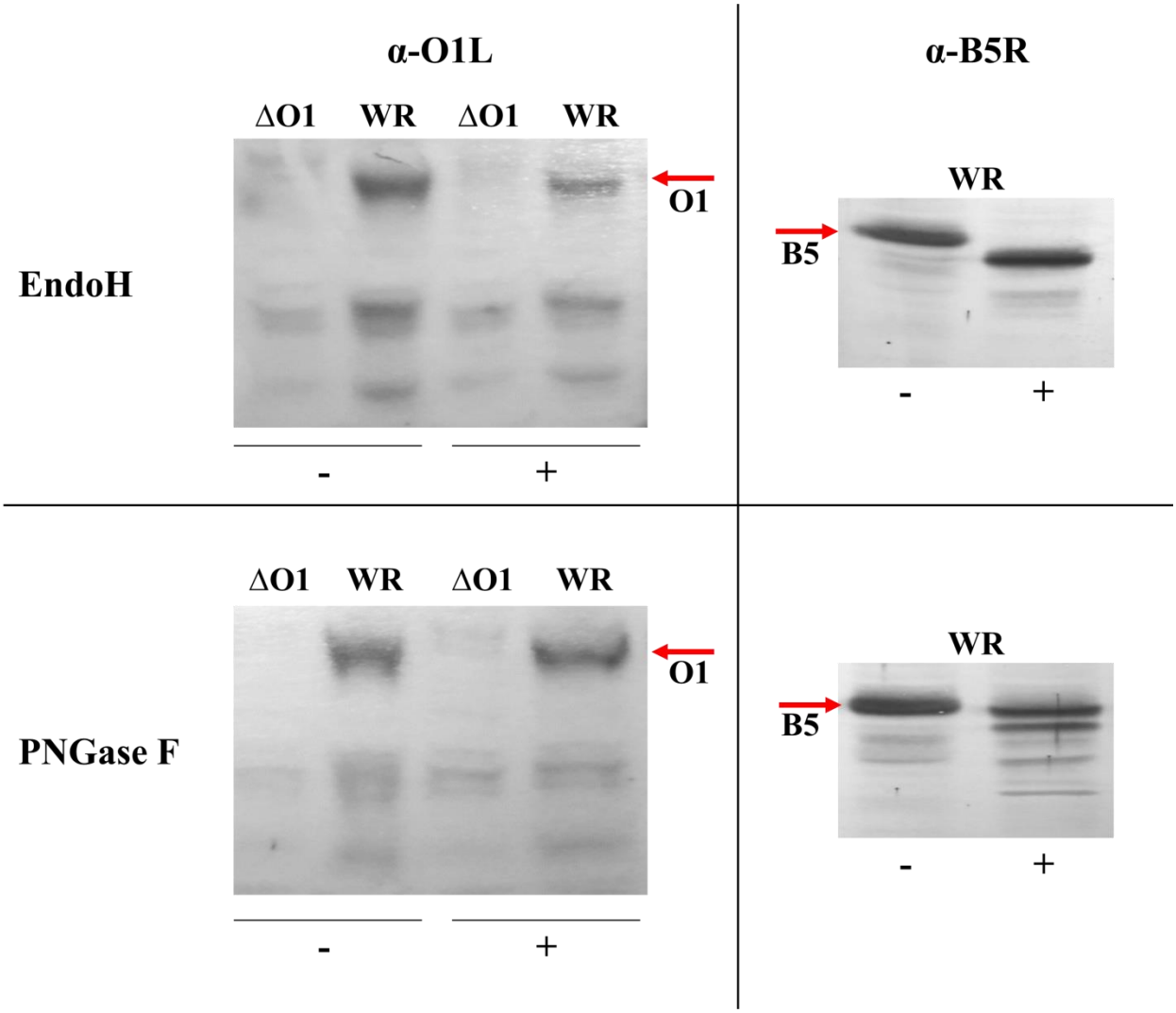


Figure 16. N-linked glycosylation of O1 was not detected.
HeLa cells were infected (MOI = 10 PFU/cell, 12 h) with either VACV-WR or an ΔO1 mutant. Whole cell lysates were treated with or without endoglycosidase H (Endo H, **top**) or peptide:N-glycosidase F (PNGase F, **bottom**). Samples were separated via SDS-PAGE and immunoblots developed using polyclonal rabbit anti-O1 sera. As a positive control for each sample glycosidase digestion, polyclonal anti-B5R sera was used to detect the Vaccinia protein B5R, a protein known to be N-linked glycosylated and susceptible to deglycosylation by both EndoH (**top right**) and PNGase F (**bottom right**). Immunoblots were performed at least twice and representative data are displayed.

4.4 O1 contribution to VACV CPE

4.4.1 VACV-WR plaques were significantly larger than ΔO1 plaques

Distinct alterations in ΔO1 plaque morphology (89) appear to mimic characteristics of several VACV proteins (reviewed in 141) that promote cytopathic effects but not viral replication or morphogenesis. As described previously, this also seems to be the case when O1 is removed from the VACV CVA strain. However, this has not been established for O1 deletion from the VACV-WR strain. Accordingly, plaque assays were performed to quantify ΔO1 alterations in plaque morphology. Titration of VACV-WR on BS-C-1 cell monolayers produced plaques with approximately 30% larger diameter (**Figure 17**) than plaques formed in ΔO1-infected wells. Additionally, clearance from the center of ΔO1 foci was significantly reduced. These data support prior studies of O1 mutants in the VACV CVA strain, and indicate that O1 contributes to VACV CPE in cell culture.

4.4.2 ΔO1-induced metabolic perturbations significantly differed from those induced by VACV-WR only in RAW 264.7 macrophages

MTS/PMS cell viability assays were performed on several cell lines and isolated primary immune cells to address the possibility that cytotoxicity is altered with ΔO1 infection. There are two lines of evidence that suggest O1 may affect cytotoxicity: 1) reduced cellular debris observed within WR-VACV plaque foci may result from more dead cells being released from the matrix; and 2) purported O1 effects on ERK signaling may differentially influence various immune cells processes, including cell viability/metabolism. **Figure 18** shows that VACV infection had minimal effects on the lymphocyte cells tested [*i.e.* T cells (**Figure 18 A and B**), and B cells (**Figure 18 B and C**)]. However, in the innate APCs, VACV infection significantly reduced metabolism (**Figure 19**). In immature BMDC and BMDC induced to mature with LPS,

the metabolism of VACV-WR and ΔO1 infected cells was similar (**Figure 19 A**). In RAW 264.7 macrophages (**Figure 19 B**), the decline in metabolism was proportional to viral dose in both VACV-WR and ΔO1 infections. However, metabolism was further reduced by ΔO1, and this was significantly different at the highest viral dose tested. Because the differences noted between macrophage metabolism during VACV-WR and ΔO1 infections were statistically significant at higher doses, these data may indicate an immune cell type in which O1 is functionally relevant. However, since the magnitude of effect was modest, the biological significance remains uncertain.

4.4.3 O1 contributed to VACV induced cell motility

The migratory potential of infected cells is another component of CPE that influences plaque size. VACV-WR promotes migration of infected cells (120, 122), which may enhance viral dissemination or immune evasion *in vivo*. Contributions of the O1 protein to this phenotype were assayed using BS-C-1 cells in a wound assay. As shown in **Figure 20**, uninfected cells remained relatively stationary, whereas VACV-WR infected cells migrated into a wound created by 'scratching' a monolayer of BS-C-1 cells with a pipette tip. VACV-WR infected cells migrated maximally between 16 and 24 HPI (**Figure 20 B**). In contrast, ΔO1 infected cell migration was significantly lower ($\rho < 0.01$) from 16 through 44 HPI. The greatest differences between migration effects were observed prior to 24 hours. This reflects prior reports that maximal migration of VACV-WR infected cells occurs from 9 to 18 hours post-infection (120). Thus, data indicates that the parent VACV-WR infected cells are induced to migrate to a significantly greater extent than ΔO1 infected cells. This may suggest that the O1 function enhances cell migration during VACV-WR infection.

Figure 17

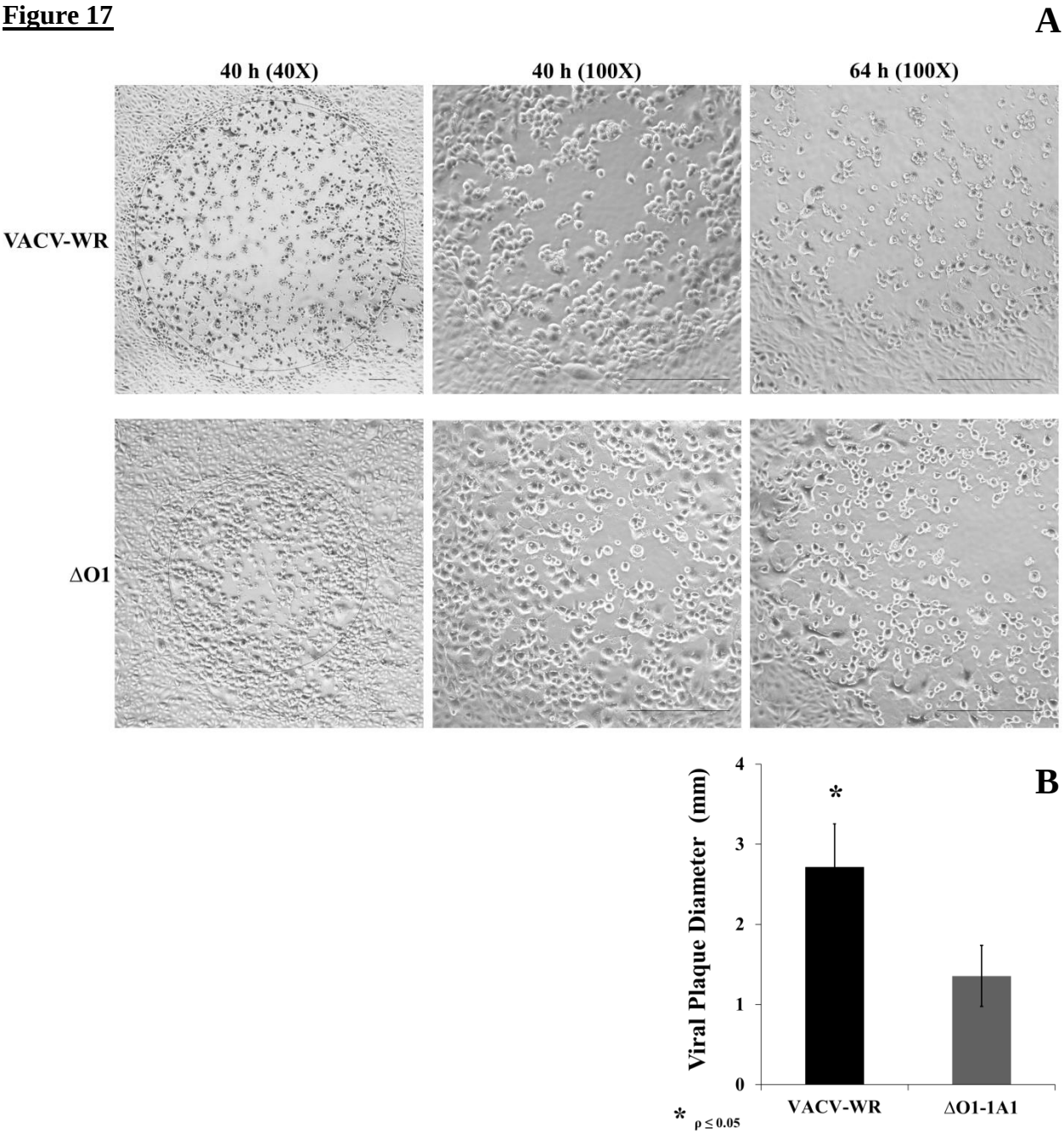


Figure 17: Plaque size and foci clearance were reduced with ΔO1 infection.
Plaque morphology was evaluated by the titrating viruses (VACV-WR and ΔO1) on BS-C-1 monolayers and staining with crystal violet (0.1% in 20% ethanol) 40 HPI. **Figure 17 A)** Plaques formed by 40 HPI were photographed, and the average diameter of plaques formed by each virus was determined. **Figure 17 B)** Plaque sizes are expressed as the mean diameter (mm, n = 10) ± standard deviation. * denotes statistical significance, (Student's t test, $\rho \leq 0.05$.)

Figure 18

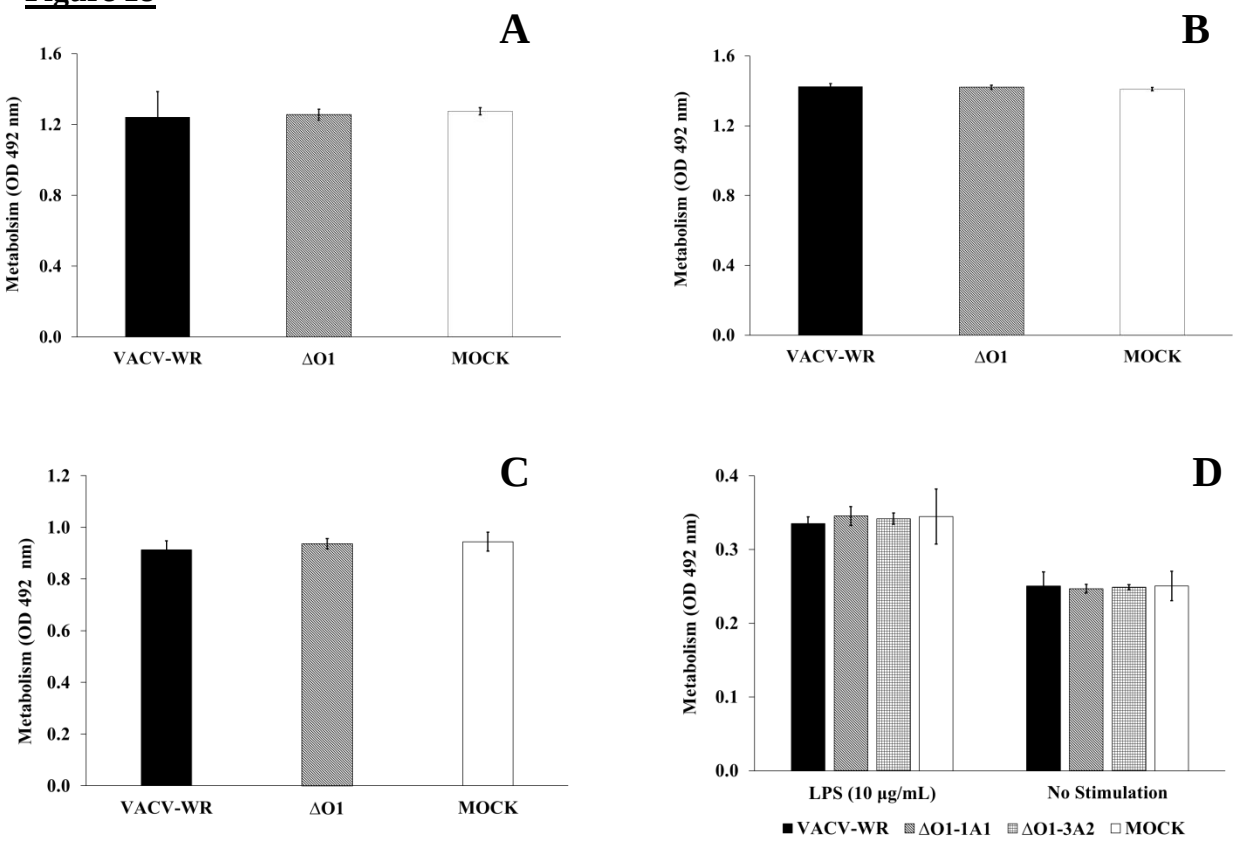


Figure 18. No significant differences in lymphocyte viability were detected via MTS assay. MTS assays were used as to measure metabolism as an indicator of cell viability. To test whether O1 reduces T lymphocyte viability, Jurkat (**Figure 18 A**) and B04 (**Figure 18 B**) T lymphocyte cell lines were infected in triplicate with VACV-WR or ΔO1 or mock-infected. To test whether O1 reduces B cell viability, the 1153 B lymphocyte cell line (**Figure 18 C**) was infected in triplicate with VACV-WR or ΔO1 or mock-infected (media). Purified mouse splenic B cells were isolated (EasySep Pan-B negative selection kit), infected as above, and treated with LPS (5 µg/mL) to induce B cell activation (**Figure 18 D)**. MTS/PMS was added 24 hours post-infection, and cellular metabolism was measured (492 nm) as an indicator of cell viability. These experiments were repeated at least twice with similar results, and representative data (MOI = 5, 24 h) are displayed for each experiment. One-way AVOVA revealed no significant differences between mock and virus infected cells (**Figure 18 A-C**), and no statistically significant differences among LPS-stimulated cells (**Figure 18 D**).

Figure 19

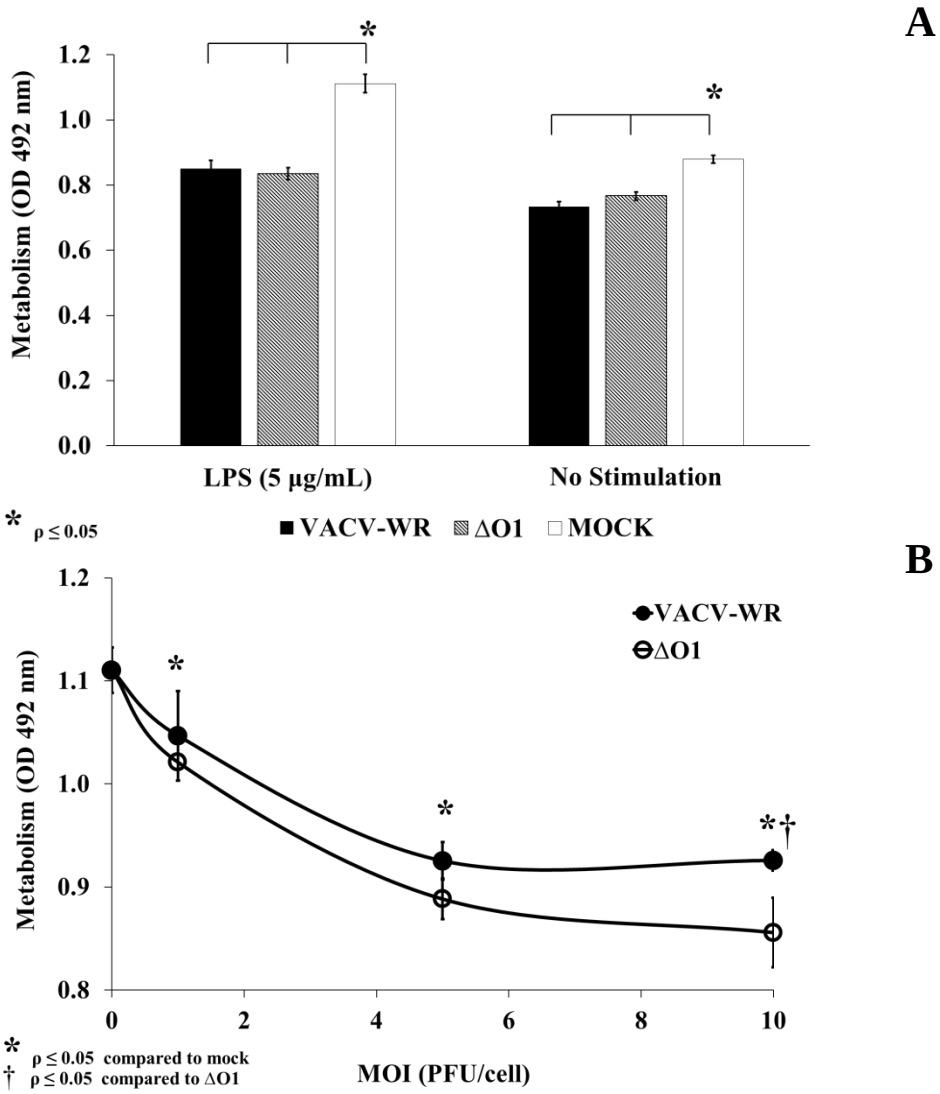


Figure 19. VACV infection significantly reduced innate APC metabolism, but ΔO1 infection significantly differed only in RAW 264.7 macrophages.

(Figure 19 A) C57BL/6 mouse BMDCs cultured for seven days (cRPMI + GMCSF 1.5%) were infected in triplicate with either VACV-WR or ΔO1-1A1 (MOI = 5) or mock-infected (media) for 3 hours prior to LPS treatment (5 µg/mL). **(Figure 19 B)** Macrophages (RAW 264.7 cell line) were infected in triplicate with either VACV-WR or ΔO1 (MOI = 1, 5, and 10) or mock-infected (mock, MOI = 0). MTS/PMS was added to culture 24 HPI, and metabolism was measured (OD 492 nm) as an indicator of cell viability. Experiments were performed at least twice, with similar results. * denotes significant difference (ANOVA, $\rho \leq 0.05$) between infection groups and mock infection (MOI = 0). † denotes significant difference (ANOVA, $\rho \leq 0.05$) between VACV-WR and ΔO1.

Figure 20

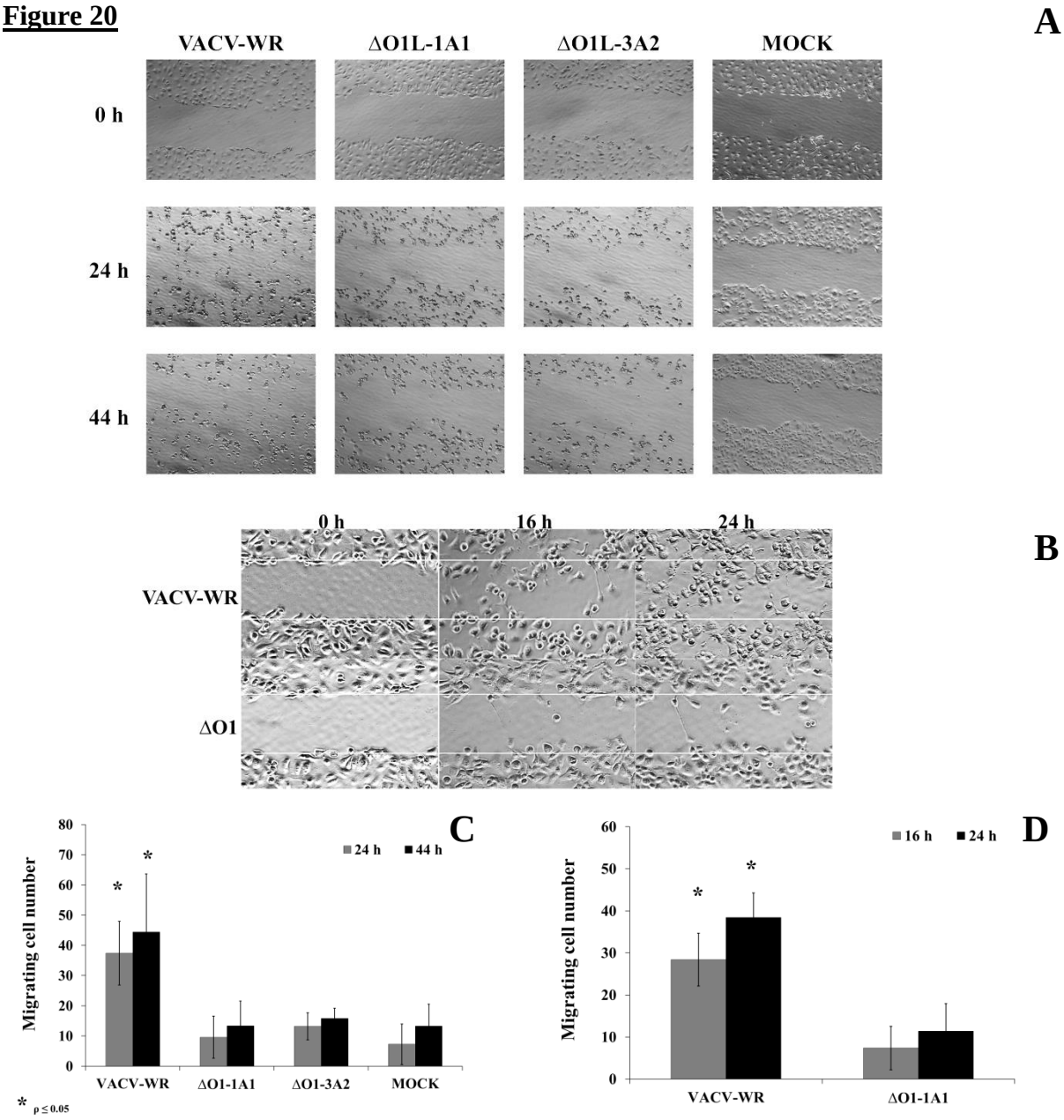


Figure 20. VACV-induced motility was ablated with ΔO1 infection.

Confluent monolayers of BS-C-1 cells were serum starved for 2 h, then infected with VACV-WR (WR) or ΔO1 (MOI = 1). After virus adhesion (one HPI), wounds were scratched into the cell monolayer with pipette tip. Cells were washed three times, and incubated in serum-free media. Wounds were photographed (100X magnification) 0 – 44 HPI. Experiments were performed at least 3 times, and representative data are presented. The number of cells that migrated into scratch regions (n = 5) were enumerated, and the mean ± standard deviation of **Figures 20 A and B** are indicated in **Figure 20 C and D**, respectively. One-way ANOVA was performed separately for each time point and * denotes significance ($\rho \leq 0.05$).

Chapter 5. Discussion

That the O1 protein regulates the host immune system to promote virulence during VACV infection was the major hypothesis tested by this study. This hypothesis was initially based on evidence from sequence and literature analysis that the O1L gene is a large gene most highly conserved within poxviruses that specifically infect mammals. It is unlikely that poxviruses would retain a large gene unless it conferred some survival advantage, either through viral multiplication or as a defense against a host. Since O1L is a gene not essential for viral replication, it was surmised that this gene, among a number of other possibilities, was retained to allow poxviruses to combat the antagonistic environment of a host immune response. Furthermore, the levels of O1L gene homology are highest among mammalian-tropic poxviruses, compared to poxviruses that infect other host species. Since mammals have a more complex adaptive immune system that is lacking in lesser vertebrates, it was hypothesized that the O1L gene may more specifically interact with the host adaptive immune response.

5.1 ΔO1 and VACV-WR immunogenicity

Investigations into the function of O1 in context of the adaptive immune system originated with a survey of the ability of a murine host to initiate a humoral immune response to VACV infection in the presence (VACV-WR) or absence (ΔO1) of the O1 protein. The humoral immune response during ΔO1 infection was enhanced, as evidenced by the substantial increase in the level of anti-VACV antibody detected in sera from ΔO1 infected mice compared to VACV-WR-infected mouse sera (**Figure 7**). Although studies have identified O1 as one of 21 VACV proteins that compose the human antibody response to vaccination with different strains of VACV (147), and O1 is one of 19 VACV proteins preferentially targeted by mouse sera (148), how O1-specific antibody influences a primary VACV-WR infection in mice has not been

directly tested. Future experiments should be performed to clarify whether the reduced antibody levels detected in sera from VACV-WR infected mice are due to a mechanistic suppression of the immune response, or if the reduced signal in the ELISA assays (**Figure 7**) was due to significant levels of O1-neutralizing antibody *in vivo*. If VACV-specific antibody in the serum of VACV-WR infected mice was bound to the O1 protein (not present in ΔO1 infection), this may have sequestered antibody available for *ex vivo* detection *via* ELISA. Since these observations were initially deemed sufficient indication that O1 may interact with the host immune response, experiments to define a putative immunoregulatory mechanism were pursued.

ELISpot assays were performed to elucidate whether T cell responses were affected in the same time frame as observed alterations in the humoral response. Cellular responses to VACV-WR and ΔO1, as detected by IFN-γ ELISpot, appeared to be similar in magnitude. This was surprising, given the number of reported CD4- and CD8-specific epitopes within the O1 protein (131, 143, 144, 173, 174). These recall responses to VACV antigens were measured seven days post-infection, so it is possible that splenocytes at this time had not sufficiently expanded *in vivo* to allow for a detectable difference in VACV-specific recall response to VACV-WR and ΔO1 infection *ex vivo*. Future studies investigating a difference at a later time point post-infection (>12 days post-infection) may therefore provide more information of O1 effects on VACV-specific T cell development *in vivo*.

5.2 ΔO1 and *ex vivo* antibody production

Given the observed alteration in humoral response, and lack of detectable differences in T cell response *in vivo*, it was hypothesized that O1 may directly influence the ability of B cells to produce antibody. Splenocytes from naïve mice that were infected and activated *ex vivo* with LPS produced a reduced quantity of antibody compared to mock-infected cells; however,

reductions induced by both VACV-WR and ΔO1 were similar (**Figures 8 and 9**). Although LPS stimulation was sufficient to induce levels of antibody production significantly greater than unstimulated cells, there is the potential that the experimental approach did not accurately reflect the mechanisms needed to induce the biologically relevant levels of antibody observed *in vivo*. Since LPS activation of B cells is not the primary mechanism for inducing antiviral antibody production during VACV infection, it is likely that ΔO1-infected B cells may still be better equipped to mount a humoral response. Optimal production of antibody by B cells *in vivo* relies on a combination of signals, including BCR activation, and T cell dependent stimulation through ligation of co-stimulatory receptors and production of cytokines like IL-4. LPS is known to stimulate ERK signal transduction (175), so if O1 constitutively sustains ERK activation, it is possible for the O1 expressed in VACV-WR infection to functionally synergize with the LPS signal, effectively minimizing detectable differences in antibody production between VACV-WR and ΔO1. For future studies to confirm that the O1 protein does not directly inhibit B cell production of antibody, it would be beneficial to employ an antibody induction assay where B cell activation reflects *in vivo* conditions more closely.

5.3 Ablated antigen presentation with equivalent T cell activation

Taking a step back, it was hypothesized that if O1 did not directly affect B cells, O1 may modify the global process that precedes humoral immunity – antigen presentation. If appropriate interactions between APC and T cells are ablated, any number of subsequent alterations in the nature of T cell physiology (*i.e.* activation, differentiation, or proliferation) or function (*i.e.* cytokine production, etc.) would theoretically result in the reduced antibody production observed previously. To preclude interference of T cell specific epitopes present in the O1 protein, the well-established 2D2 antigen presentation model was employed (169). Compared to VACV-WR

infection, ΔO1-infected splenocytes were better activated upon antigenic stimulation, resulting in higher levels proliferation by IL-2 dependent CTLL cells. Despite the significant difference between the parental infection and the mutant, the magnitude of difference between the two viruses was smaller than expected. This small but significant difference was observed 24 HPI (**Figure 11**) through 48 HPI (data not shown), but diminished at later time points. There are several explanations that may provide insight for future studies. First, it is possible that, over time, excess bioavailable IL-2 was either degraded or consumed by proliferating T cells in splenocyte culture, resulting in the inability to detect potentially greater differences in the subsequent bioassay. Secondly, although IL-2 containing media was used as a positive control, since anti-IL-2 antibodies were not used to confirm that IL-2 was actually contributing to CTLL proliferation; it is possible that differences in CTLL proliferation with ΔO1 were due to production of another lymphoproliferative cytokine that also promotes CTLL proliferation, but is produced in smaller quantities (*e.g.* IL-4). Additionally, multiple OPXVs encode IL-2 binding proteins and decoy IL-2 homologs, including VACV (176). If these VACV IL-2 modulatory proteins function in VACV as they reportedly do in other OPXV, this would alter detectable IL-2 in both virus groups. Since it is unknown how O1 influences the function of other VACV proteins, including putative IL-2 regulatory virulence proteins, it is not possible to draw a direct association between O1 and IL-2 production with these data.

Furthermore, Yao *et al*. reported that lower doses of VACV activate DC better than higher doses, and that viral doses $>10^{-3}$ PFU/cell resulted in inhibition of DC maturation (98). Since the MOI used (1 PFU/cell) may have been too high, the lower magnitude of difference between VACV-WR and ΔO1 would make sense considering DC function may have been reduced overall. Reports have also shown that splenic DC (SPDC) are more resistant to infection

in vitro (98) compared to BMDC, so the splenocyte culture used for antigen presentation assays may have consisted of cells that were more refractory to infection (*e.g.* SPDC), thus reducing the magnitude of antigen presentation observed. Finally, it is conceivable that if O1 function is restricted to a specific cell type, the quantity of these putative cells may have been insufficient within antigen-activated splenocyte culture to induce a greater measurable difference in antigen presentation.

Since there were multiple observations that antigen presentation was increased in ΔO1-infected, antigen-activated splenocytes, it was of interest to dissect this system to identify putative intercellular and/or cell-specific interactions that may have contributed to these differences. Splenocytes that were infected and activated with ConA were assessed via flow cytometry to detect expression of the lymphocyte activation marker CD69 on the surface of $CD4^+$ T cells. Data in **Figure 12** demonstrate that ConA was sufficient to induce significant levels of $CD4^+CD69^+$ cells; however, CD69 expression was equivalent between VACV-WR and ΔO1 infections. The results obtained were similar when ConA was used to activate positively selected $CD4^+$ splenic T cells that were infected with either virus (data not shown). Since the data indicated no difference between VACV-WR and ΔO1 infection in the ability of $CD4^+$ T cells to express the activation marker CD69, it is more likely that if O1 is indeed affecting some aspect of T cell biology, it is a process subsequent to T cell activation.

5.4 Cell survival and metabolism during VACV infection

Another method was employed to determine whether ΔO1 functioned in splenocyte resident cell types differently than VACV-WR. MTS assays were performed, and focused on cell types found in spleen – B cells, T cells, macrophages, and DCs. Since VACV-WR infection is lethal in certain cell types, it was expected that if cells were permissive to infection, they should

be killed with sufficient quantities of VACV-WR. In the T cell lines assayed, doses up to 10 PFU/cell did not significantly reduce the OD readings in either VACV-WR or ΔO1 (**Figure 18 A**). This is not surprising, given that resting T cells are refractory to VACV infection (101, 177), and it has been previously reported that high doses of VACV-WR have minimal effects on T cell metabolism (99). However, Byrd *et al*. recently reported that when activated, T cells derived from primary human leukocytes become permissive to a complete cycle of VACV binding, infection, and replication (177). The lack of VACV membrane receptors on the surface of these cultured rodent T cells, as well as an intracellular environment lacking key replication factors, are likely explanations for the absence of metabolic effects during T cell infection with either virus.

Similarly, VACV infection had no effects on the metabolism of a murine B cell line or purified primary murine splenic B cells, and there were no differences between VACV-WR and ΔO1 infections. When B cells were treated with LPS (**Figure 18 D**), the MTS OD significantly increased in both virus and mock infected groups. This suggests that LPS treatment increased the overall number of B cells in culture, consistent with literature reports that LPS induces B cell activation and proliferation (178–181). According to one report, B cells are permissive to VACV binding, but not infection (177), so the observation that VACV infection had little effect on B cell metabolism/viability suggests that B cells may not have been infected. The question that remains is how VACV could reduce antibody production *ex vivo*, if B cells were not infected. Taken together, the *ex vivo* antibody production and B cell viability assays suggest that VACV directly reduces antibody levels, without reducing B cell viability. Future studies should be performed to identify whether these B cells are permissive to infection, and if VACV binding to

B cells is sufficient to block antibody production, or if antibody produced *ex vivo* is neutralizing VACV in culture, which would explain reduced levels detected *via* ELISA.

The ability of VACV-WR to reduce cell viability was observed in the BMDC (**Figure 19 A**) and RAW 264.7 macrophage cell infections (**Figure 19 B**), as doses between 3-5 PFU/cell were sufficient for both VACV-WR and ΔO1 to reduce OD. In BMDCs, the addition of LPS also enhanced metabolism, although the influence of VACV-WR and ΔO1 infections on metabolism was essentially the same. These data suggest that O1 does not influence BMDC metabolism, and by proxy, cell viability.

ΔO1-infected macrophages were less metabolically active than VACV-WR infected cells, and the magnitude of difference increased with viral dose ($\rho > 0.05$ at MOI =10, **Figure 19 B**). Since MTS assays are limited to the ability to correlate the metabolic state of living cells present in culture, the differences in OD observed could indicate several possibilities, including: 1) ΔO1 infection reduces the metabolic state of macrophages compared to the parent virus; 2) that ΔO1 infected macrophages proliferated to a lesser extent, effectively reducing the quantity of metabolically active cells, or 3) macrophages remain viable for longer during VACV-WR infection compared to ΔO1. The latter two possibilities are not mutually exclusive, however, as increased proliferation and sustained viability could occur simultaneously.

Given any of the circumstances discussed above, a model of the O1 mechanism in the context of the macrophage environment might include: enhanced proliferation, metabolism, or anti-apoptotic functions. As one example of how this might work: VGF is a homolog of the human EGF, and has long been established as a virulence factor that induces cell proliferation during VACV infection. Cells in a prolonged proliferative state are thought to provide a productive environment for optimal viral replication. Since it is has been reported that O1

functions downstream of VGF (89), it is not unlikely that O1 synergizes with VGF signaling to enhance cellular proliferation in certain cells. Increased cellular proliferation in VACV-WR infected RAW 264.7 cell line would be a simple explanation for why metabolism was greater than in ΔO1 infection.

An interesting proposition is one where O1 concurrently influences: 1) cellular tropism, by enhancing infectivity of EGFR-expressing cells, 2) viral dissemination, as motility of VACV-infected cells enhances viral spread, and infiltration of macrophages into uninfected tissues is thought to be one of the primary mechanisms for VARV spread; and 3) immune evasion. If O1 influences the preference of VACV for infecting anti-inflammatory macrophages (102), this would be a stealth mechanism for avoiding early immune detection while the virus waits for safer environments to establish infection (*e.g.* in ovaries). The betaherpesvirus human cytomegalovirus (HCMV) has been shown to simultaneously affect the first two possibilities, with speculation that the third is also possible (182). Like VACV, activation of EGFR by HCMV promotes infection. Chan *et al.* demonstrated that inhibiting EGFR abrogated HCMV infectivity of monocytes, and led them to conclude that EGFR acts as an entry receptor for HCMV. Furthermore, EGFR activation promoted monocyte motility and trans-endothelial migration during HCMV infection. The demonstration by Chen *et al.* that these effects were PI3K-independent was important for establishing that the aforementioned effects occurred subsequent to HCMV glycoprotein B (gB) activation of EGFR. HCMV gB induction of EGFR activity is functionally orthologous to VACV C11, including anti-apoptotic functions. It would be interesting to determine whether the HCMV manipulates ERK downstream of EGFR to influence entry and motility. Given that herpesviruses and poxviruses are closely related, it is

possible that there is some betaherpesvirus protein that is functionally orthologous to O1, which bears further study.

Upon demonstrating that ERK activation and plaque sizes were equivalent in CVA and CVA-ΔO1 infected, ERK inhibitor-treated chick embryo fibroblast cell line, but significantly different in a number of other cell lines tested, Schweneker *et al*. surmised that O1-sustained activation of ERK clearly depends upon cell type (89). Whether O1 functions similarly in macrophages (*i.e*. by sustaining ERK activation) remains to be established. If so, it is conceivable that this would provide an experimental model in which to study the O1 functional phenotypes observed to date in a single, biologically relevant, environment. Finally, since the ERK/MAPK signal cascades are also known to regulate metabolism, apoptosis, and macrophage differentiation, whether O1 has any functional significance in these contexts will be of great interest.

5.5 ΔO1 plaque and cell migration phenotypes

Another aspect of this study was to evaluate O1 behavior in cell culture. Investigations were initiated on two levels: 1) determine a mechanism whereby O1 influences CPE; and 2) characterize the O1 protein to understand how the structural biology of this pox virulence protein lends itself to novel functions.

Prior reports (89) showed that CVA-ΔO1 formed plaques that were up to 50% smaller in some cell types. It was of interest to quantify similar effects with the VACV-WR strain and ΔO1. Although predecessors in the laboratory where these studies were performed reported little to no difference between VACV-WR and ΔO1 plaque sizes (168), the data reported herein demonstrate a 30% reduction in the diameter of ΔO1 plaques formed on BS-C-1 cell monolayers. Additionally, clearance of cellular debris from plaque foci was also reduced in ΔO1 infection, in

agreement with Schweneker *et al.* Furthermore, data collected by predecessors to this study demonstrated equivalent levels of VACV-WR and ΔO1 replication and spread in single and multi-step growth analyses. Taken together, these results suggest that O1 may be classified among an emerging group of plaque phenotype-modulating proteins that affect cell morphology but not viral life cycle (113).

One of the proteins in the aforementioned group, F11, has been shown to similarly enhance VACV plaque size, and subsequent studies have demonstrated that F11 promotes the motility of VACV-infected cells (122, 124, 183). Thus, it was hypothesized that O1 may influence VACV-WR induced cellular migration. Indeed, VACV-WR infected cells were significantly enhanced in their migratory capacity compared to ΔO1 infected cells. The greatest magnitude of VACV-WR induced cellular migration into a monolayer wound occurred between 0 and 16 HPI, which closely reflects prior kinetic studies demonstrating that VACV-induced migration occurs maximally 10-14 HPI (120). Furthermore, Sanderson *et al.* also reported that early VACV gene expression was necessary and sufficient to induce cell migration, but not formation of cellular projections (120). This supports a role for O1 in the temporal regulation of VACV-WR induced migration, since O1 is expressed early and the increased cell migration of VACV-WR infected BS-C-1 cells was within established kinetics. Interestingly, in recent observations of RAW 264.7 macrophages, there appeared to be distinct cellular projections in VACV-WR infected cells that were not present with ΔO1 infection at 16 HPI (data not shown). Byrd *et al* also recently published similar observations that VACV alters macrophage CPE at 16 HPI (102). Although these preliminary data are informative, to further explore the role of O1 in cell migration, and whether VACV-WR infection induces migration of macrophages and potentially contributed to virulence *in vivo,* additional studies should be conducted. The current

data provide strong evidence to suggest that O1 promotes migration of VACV-WR infected cells, which may contribute to the expanded plaque size during VACV-WR infection.

5.6 Structural characteristics of the O1 protein

Finally, knowledge of the structural biology of O1 has been limited to analysis of DNA and peptide sequences to uncover protein homology and predict functional motifs (89, 139, 160), or PCR analysis of mRNA expression (8, 89). Given that the majority of gene functions depend upon protein structure, the O1 protein was characterized to determine 1) if the O1 protein was expressed; 2) whether the protein size was as the amino acid sequence predicted or if cell-dependent modifications (*e.g.* predicted N-linked glycosylation motifs) were functional; and 3) relative expression kinetics. Rabbit sera raised against two separate O1 peptides sequences used in immunoblot analyses detected a protein band of ~78 kDa that was present in VACV-WR infected cell lysates but not in ΔO1 lanes (**Figures 14-16).** These data are the first reported studies detecting the O1 protein expression during VACV-WR infection.

Although the O1 protein band detected was equivalent to the size predicted based on the peptide sequence, it is possible that this same size range would also be observed if the O1 protein undergoes cleavage and post-translational modification following expression. With at least five putative N-linked glycosylation motifs in the O1 sequence, it was of interest to determine whether O1 was, in fact, N-linked glycosylated. Two enzymes that cleave glycosylation moieties at different positions were used, but the O1 band did not migrate differently upon enzymatic treatment, suggesting that the O1 protein is not N-linked glycosylated. This is not surprising, given the early expression and activity kinetics of O1. If glycosylation did occur, the O1 functional kinetics would rely upon cellular processes, and the time required to traffic through the cell would delay potential functions. Since O1 mRNA is detected as early as 30 minutes post-

infection (8, 89), and RAF/MEK/ERK phosphorylation is reduced with ΔO1 infection within 30 minutes (89), it is questionable whether any post-translational modifications occur prior to O1 function. This is not to say that later modifications do not occur; however, data obtained from cellular lysates 8-10 HPI (**Figures 15 and 16**) indicate that N-linked glycosylation is not among them. Furthermore, the O1 band migrates to the same ~78 kDa range from 3 through 24 HPI (**Figure 15**), suggesting that any protein modifications that may occur would be minimal. Since many VACV proteins that are highly glycosylated are expressed later and are membrane associated, the absence of N-linked glycosylation argues against possible integral membrane localization for O1. Additionally, since ERK is mainly cytoplasmic until activated, and O1has been speculated to function upstream of RAF, it could be inferred that O1 is a soluble cytoplasmic protein. Taken together, these findings are the first to show that O1 is expressed as a 78 kDa protein that lacks N-linked glycosylation modifications.

Finally, Phyre2 software was used to generate a 3D model of the O1 protein based on the amino acid sequence. The homology of the protein structure generated *ab initio* was compared to previously modeled proteins reported in PDB (**Table 1**). *Ab initio* modeling is notoriously imprecise, so it is unsurprising that reported structural homology to the O1 protein (of which little is known) was given low confidence scores. However, proteins from different species can share low sequence similarity, yet be functional orthologs; therefore, these findings were given due consideration. Interestingly, low levels of sequence identity with O1 (5 – 30%) were shared among a number of protein families with functions ranging from bacterial toxins to mammalian enzymes involved in DNA replication. Since O1 contains a predicted DNA binding domain, it was unsurprising that there were a number of proteins associated with DNA replication. However, with numerous demonstrations that O1 is not necessary for VACV replication, it is

unlikely that O1 functions within this realm. On the other hand, the putative O1 nuclear localization signal (NLS) is interesting in the context of ERK signal cascade, since ERK is known to translocate to the nucleus upon activation. Whether the predicted O1 NLS is functional, and whether this has any bearing on ERK activity during VACV infection would be useful to know, as nuclear translocation by a virulence protein would be a completely novel signal cascade manipulation for poxviruses.

The sequence identity to various lyase domains, although limited, is also of significant interest, since it has been shown that the HMG CoA lyase indirectly regulates B-RAF mediated cell proliferation (184). Additionally, ATP citrate lyase, which functions downstream of HMG CoA lyase, has also been implicated in EGF-initiated MAPK-mediated anti-apoptotic signaling (185). It could therefore be possible that O1 functions as a lyase to enhance ERK activation. O1 also had a putative thioredoxin-like fold. Thioredoxins negatively regulate signaling of apoptosis signal-regulating kinase (ASK) through direct binding (186). Thioredoxin overexpression elevates ERK phosphorylation (187), and thioredoxin knockdown inactivates EGF-induced ERK activation (188). Since all known VACV apoptosis regulators are inhibitory (189), it would be interesting to determine whether O1 moderates pro-apoptotic stress responses in order to maintain the cell viability needed for VACV infection.

O1 also exhibited low levels of predicted structural homology to the ligase family of proteins, with multiple E3 ubiquitin ligase homologs represented. Ubiquitin ligases target proteins for degradation, and it has been shown that an E3 ubiquitin ligase targets the ERK phosphorylase MKP-1 (190). Since MKP-1 downregulates ERK, inhibition of this negative feedback regulator would be one method that O1 could employ to sustain VGF-mediated RAF/MEK/ERK signaling (**Figure 6**). Since VACV encodes an MKP homolog [H1 (191)], it is

likely that O1 activity influences the function of H1, so it will be important to determine if O1 functions in tandem with this protein.

Since O1 is such a large protein, it is possible that the shared sequence identity with smaller proteins is not as low as predicted. Given that enzyme active sites don't encompass the entirety of a protein, and proteins can have a number of active sites with different functions, it is possible that there may be more than one functional ortholog among the protein families discussed. Although many of the protein domains discussed exhibited lower levels of structural homology to the O1 protein, there are some poxvirus proteins that have been demonstrated to function similarly to homologous proteins with as little as 25% sequence similarity (138, 192), so there is some relevance to these considerations. Overall, these predictions may provide mechanistic insight into the O1 functions observed, and provide targets when designing future studies.

5.7 Implications for biotherapeutics

In less than a century, the poxvirus field has progressed beyond desperation to eradicate the scourge of smallpox to the ability to actually use sister OPXV viruses to treat many other human menaces, from cancer to Ebola virus. In the context of these studies to understand the role of O1, the alacrity in which novel foundational research into O1 has been translated into a functional treatment for cancer is especially noteworthy. When Schweneker and colleagues first published data on O1 in 2012, there was minimal interest in continuing research on a protein that behaved contrary to expectation, and the project was discarded by that group (personal communication, 2014). As the research in this report was ongoing, Nakamura *et al.* (164) were able to utilize the expanded catalogue of poxvirus information provided by Schweneker, and a novel VACV oncolytic vector lacking both VGF and O1 was developed in Japan in 2014. The

concept was that enhanced cell proliferation promotes efficient VACV infection, so removing VGF and/or O1 would only allow efficient VACV replication in cells where basal RAF/MEK/ERK is elevated. In this case, tumor cells become the only proliferative environment for VACV, so the tumor cells that are targeted for VACV multiplication are subsequently lysed upon resolution of viral life cycle. Thus, VGF-/O1- virus use to preferentially target tumor cells for oncolysis was experimentally tested, and received patents worldwide in 2016. Given the laudable accomplishments that have employed established phenotypes in the *absence* of O1, it will be of great importance in the future to differentiate this from what happens when O1 is *present*. With certain classes of poxvirus virulence proteins (cytokine inhibitors/mimics, complement inhibitors, protease inhibitors) having been tested individually in the absence of virus for their potential as novel biotherapeutics (67), it is conceivable that the O1 protein may have a positive future after all.

Chapter 6. Key Findings and Conclusions

Because the O1 protein is most highly conserved by poxviruses that infect mammals, but is not essential for the viral life cycle, it was hypothesized that the O1 protein may be retained by mammalian-tropic poxviruses in order to combat the host immune response. Mice were infected with the VACV Western Reserve strain (VACV-WR) or with VACV-WR virus lacking the O1 protein (ΔO1), and higher levels of VACV specific antibody were detected in sera from ΔO1-infected mice. However, VACV-specific T cells from mice infected with either virus were reactivated equally *ex vivo*. To determine if VACV-WR directly ablated antibody production, murine splenocytes and primary B cells infected with VACV-WR and ΔO1 infection were examined *ex vivo* for their ability to produce antibody. Infection with both viruses reduced antibody levels equally, suggesting that alterations in antibody levels during ΔO1 infection may occur through an indirect mechanism.

VACV inhibition of antigen presentation is a mechanism that indirectly reduces antibody production *in vivo*, therefore the influence of VACV-WR and ΔO1 infection on the ability of antigen-stimulated mouse splenocytes to present antigen and subsequently induce T cells to produce interleukin-2 (IL-2) was tested *ex vivo*. Compared to VACV-WR, ΔO1-infected splenocytes induced significantly higher levels of IL-2. However, the number of activated helper T cells ($CD4^+CD69^+$) did not increase with ΔO1 infection, suggesting that the improved antigen presentation noted with ΔO1 infection may not occur at the level of T cell activation. Taken together, it appears that ΔO1 infection increases immunogenicity but may not directly enhance B or T cell activity.

O1 purportedly enhances MAPK signal transduction, the functional relevance of which is speculated to be cell type dependent. Thus, it was hypothesized that O1 function differs among

immune cell types. Of the adaptive (*e.g.* T and B lymphocytes) and innate [*e.g.* DCs and macrophages] immune cells examined for differences in cell viability, the macrophage cell line was the only one found to be differentially affected by VACV-WR or ΔO1 infection, signifying that macrophages may be a biologically relevant immune cell in which O1 functions.

Plaque assays were performed to investigate the contribution of O1 to VACV CPE in cell culture. VACV-WR plaques were significantly larger than ΔO1, supporting prior reports of reduced CPE with another VACV strain lacking O1. VACV-induced cell migration contributes to CPE, so it was hypothesized that O1 may enhance migration during infection. Cellular migration was significantly delayed with ΔO1 infection, whereas VACV-WR infected cells migrated optimally as previously reported. O1 effects on migration may be an important mechanism for virulence *in vivo*, especially if O1 functions similarly in an immunologically-relevant cell type.

Expression of O1 has been detected between three and 24 hours post-infection. Despite numerous NxS/T motifs, N-linked glycosylation of the O1 protein was not detected. Analysis of the O1 secondary structure revealed a number of proteins with low levels (5 – 30%) of sequence identity. Although several pox proteins reportedly have low sequence identity with their functional orthologs, the low levels of structural homology detected suggest that the O1 protein structure is unique to poxviruses, and may have a completely novel functional mechanism.

In summary, this work has revealed novel properties of O1 biology, and provided data suggesting that the immunogenicity of the VACV-WR strain can be increased by the removal of O1. Data support prior reports that O1 function may be cell type dependent, as results showed that RAW 264.7 macrophage cell line was the only one tested where metabolic activity, and implied cellular viability/proliferation, significantly differed between VACV-WR and ΔO1. This

data may provide a biologically relevant immune cell type in which O1 functions optimally to promote virulence *in vivo*. The influence of the VACV O1 protein on enhancing virally-induced cellular migration provides insight into how VACV-WR causes CPE *in vitro*, which may provide a foundation for future studies that seek to clarify potential associations between plaque phenotype with poxvirus virulence mechanisms *in vivo.* Finally, the function of the VACV virulence protein O1 has been of significant interest in emerging oncolytic vectors, so any mechanistic insight provided by these studies may be applicable to future biotherapeutic development.

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Appendix A

Master's Pre-Thesis Research Approval Form

Before beginning master's thesis research, and at least one semester before defending a thesis, this check list must be completed by the master's candidate in conjunction with the thesis director. Please NOTE: Student is required to provide a copy to all committee members; all thesis research must be approved by the thesis director and the Unit Graduate Program Director. All students whose thesis projects involve human subjects must have their proposed research approved by the University and Medical Center Institutional Review Board (UMCIRB) before beginning the studies involving those subjects. Likewise, all students whose projects involve animals must have their proposed research approved by the Institutional Animal Care and Use Committee (IACUC) before beginning those studies. A copy of the appropriate approval must be included in the Appendix of the completed thesis. The Graduate Program Director completes and submits this form to Marquerite Latham (bassm@ecu.edu)

Date: 07 June 2017	**Student name, phone number, and email address:**
Degree Program/Dept: Masters, Biomedical Sciences	Anastasia C. Weeks
Banner ID: B00600274	(601) 214-6386 (cell) weeksa12@students.ecu.edu

Working Title of Thesis Research:

Structural and functional analysis of the Vaccinia virus O1 virulence protein

Mentor/Director of Master's or doctoral work:

1. **Mark Mannie** **X** Graduate or Associate Graduate Faculty

   (Type or print name and Banner ID here)

All Graduate Student Advisory Committees must have at least three ECU Graduate or associate Graduate Faculty members (some ECU programs may require more, please check with your Graduate Program Director), which includes the mentor/director. Requests for External members need to be submitted by the Graduate Program Director, as defined in the Faculty Manual (Faculty Manual, Part II, Section IV, subsection F)

Tentative Graduate Student Advisory Committee members:

If so, please list:

2. **Isabelle Lemasson** **X** Graduate or Associate Graduate Faculty
   (Type or print name and Banner ID here)

3. **Marty Roop** **X** Graduate or Associate Graduate Faculty
   (Type or print name and Banner ID here)

4. **Jamie DeWitt** **X** Graduate or Associate Graduate Faculty
   (Type or print name and Banner ID here)

X Has your proposed research been reviewed and approved by your director?

___ Does your research involve human subjects?

___Has it been approved by the UMCIRB?

If not, when will it be reviewed for approval? _____

X Does your research involve animals?

X Has it been approved by the IACUC?

If not, when will it be reviewed for approval? _____

X Does your research involve potential biohazards such as recombinant DNA, viral vectors, infectious agents, human blood products etc.?

X Has it been approved by the by the Biosafety Committee?

If not, when will it be reviewed for approval? _____

Approvals:

Thesis Director Signature Date

Unit Graduate Program Director Signature Date

Acknowledgement of Receipt by Graduate School:

Dean of the Graduate School or designee Date

East Carolina University

Animal Care and Use Committee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834
252-744-2436 office
252-744-2355 fax

April 12 2011

Rachel Roper, Ph.D.
Department of Micro/Immuno
Brody 5E-106
East Carolina University

Dear Dr. Roper:

Your Animal Use Protocol entitled, "Analysis of Poxvirus Virulence and Improved Vaccines Using Murine Vaccination and Challenge Models" (AUP #K157) was reviewed by this institution's Animal Care and Use Committee on 4/12/11. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies.

Sincerely yours,

Scott E. Gordon, Ph.D.
Chairman, Animal Care and Use Committee

SEG/jd

enclosure

East Carolina University

Animal Care and Use Commitee
212 Ed Warren Life Sciences Building
East Carolina University
Greenville, NC 27834
252-744-2436 office
252-744-2355 fax

April 11, 2014

Rachel Roper, Ph.D.
Department of Micro/Immuno
Brody 5E-106
ECU Brody School of Medicine

Dear Dr. Roper:

Your Animal Use Protocol entitled, "Analysis of Poxvirus Virulence and Improved Vaccines Using Murine Vaccination and Challenge Models" (AUP #K157a) was reviewed by this institution's Animal Care and Use Committee on 4/11/14. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to hazard use

A copy is enclosed for your laboratory files. Please be reminded that all animal procedures must be conducted as described in the approved Animal Use Protocol. Modifications of these procedures cannot be performed without prior approval of the ACUC. The Animal Welfare Act and Public Health Service Guidelines require the ACUC to suspend activities not in accordance with approved procedures and report such activities to the responsible University Official (Vice Chancellor for Health Sciences or Vice Chancellor for Academic Affairs) and appropriate federal Agencies. **Please ensure that all personnel associated with this protocol have access to this approved copy of the AUP and are familiar with its contents.**

Sincerely yours,

Susan McRae, Ph.D.
Chair, Animal Care and Use Committee

SM/jd

Enclosure

The Brody School of Medicine
Office of Prospective Health
East Carolina University
188 Warren Life Sciences Building • Greenville, NC 27834
252-744-2070 office • 252-744-2417 fax

EAST CAROLINA UNIVERSITY

Occupational Medicine
Employee Health

Radiation Safety

Infection Control

Biological Safety

TO: Dr. Rachel Roper
Microbiology

FROM: Eddie Johnson
Chad Spruill CLS
Biological Safety Officers

RE: Registration Provisional Approval

Date: March 27, 2014

Your Biological Safety Registration, RoperR, 01-B; 14-01, **BSL1 and BSL2 poxviruses including vaccinia virus strains, mousepox, fowlpox, rabbitpox, myxoma, cowpox; human blood, serum, human primary cells, human cell lines; mutant viruses and plasmids (recombinant)** was given **provisional approval** by the IBC on March 20, 2014, to be conducted at Biosafety Level 2 and Animal Biosafety Level 2. Final approval will be granted pending the following:

1. Page 1, B, check Ingestion
2. Page 2, A2, please provide more information on cell lines
3. Page 3, A4, will animals be exposed by ingestion? If so, please check ingestion box
4. Page 6, provide training date for PI, update positions for Metcalf and Rocket
5. Page 6, BBP training for all
6. Page 17, update AUP #

Please do not hesitate to contact Biological Safety at 744-2070 if you have any questions, concerns, or need any additional information. Best wishes on your research.

cc: Dr. Jeff Smith, Chair, Biosafety Committee
Janine Davenport, IACUC
Dr. Susan McRae, IACUC
Dale Aycock, Comparative Medicine

Appendix B

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Expected completion date	Jun 2017
Estimated size (number of pages)	120

Anastasia C. Weeks
[redacted]

12 June 2017

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1. Figure 5 (page 2114)
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