ABSTRACT

THE POXVIRUS A35 PROTEIN PROMOTES VIRULENCE BY REGULATING THE HOST ADAPTIVE IMMUNE RESPONSE

by

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Poxviruses are large double-stranded DNA viruses with a broad host range and worldwide distribution. The most infamous poxvirus is Variola virus, the causative agent of smallpox, which caused over 500 million deaths in the twentieth century before it was declared eradicated in 1980 by the World Health Organization. Nevertheless, more than 25 years after its eradication from nature, smallpox remains a threat, as there is now concern for its use in an act of bioterrorism. Other poxviruses have emerged in recent years. In 2003, there was a monkeypox outbreak in the United States, with nearly 80 cases. This disease is similar to smallpox, but can be spread not only from human to human, but also from *animal* to human, which emphasizes the fact that many poxviruses are a zoonotic threat. The current poxvirus vaccine (Vaccinia virus, VACV), while highly effective, is unsafe and its use is contraindicated for an estimated 25% of

the population due to its virulence. The virulence of VACV is attributed to the production of numerous proteins that function to evade the host immune response, including inhibitors of interferons, regulators of apoptosis, and cytokine receptor homologs. Despite the large amount of work that has been done to characterize poxvirus virulence mechanisms, there are more than 25 highly conserved genes that remain uncharacterized. The poxvirus A35R gene is highly conserved in all sequenced mammalian tropic poxviruses and has little similarity to any non-pox protein, suggesting an important and novel function. The A35 protein was not required for viral replication in eight cell lines from various hosts, but removal of A35 from the viral genome resulted in a 1000-fold decrease in virulence in the mouse model. We therefore hypothesized that the poxvirus A35 gene product promotes virulence by affecting viral replication in certain cell types *in vivo* or by regulating aspects of the host adaptive immune response. We show that A35 is not required for viral replication in various cell types from six different mammalian hosts and also in mouse tissues early after infection. Using a model major histocompatibility complex (MHC) class II-restricted antigen presentation system, we have shown that VACV and A35 inhibit the amount of numerous cytokines that are produced from both the T cell and the antigen presenting cell (APC) as a result of specific antigen presentation, including IL-2 and nitric oxide. Further data implicated the APC as the viral target, as the T cells used in the assays were unaffected by VACV and A35. The A35 protein did not induce apoptosis of the APC or affect antigen-independent NO production. Furthermore, A35 localized to the endosomal compartments of the APC and decreased MHC class II expression and the amount of peptide presented in the cleft of MHC class II molecules. To determine if A35 would decrease the host adaptive immune response, mice were vaccinated with 500 plaque forming units (pfu) of either VACV (Western Reserve strain, WR) or A35Δ. Our data indicate that A35 decreased VACV-

specific immune response including interferon- (IFN)-γ production, cytotoxic T lymphocyte (CTL) killing, serum antibody, and neutralizing antibody. The decreased immune response was shown to be important as it resulted in increased viral replication in target organs. The A35Δ virus was also an effective vaccine, as it protected mice as well as WR from a lethal challenge. We also made a recombinant A35 deletion mutant virus in the Modified Vaccinia Ankara (MVA) replication-deficient vaccine strain, which has been shown to be safe when used in immunocompromised individuals. Results with MVA were similar to studies performed with WR: A35 was not required for virus replication and decreased VACV-specific immune responses. Furthermore, the MVA35Δ vaccine protected mice as well as MVA from a lethal respiratory challenge. Our data expands the knowledge of what is known regarding poxvirus virulence mechanisms and is applicable to the development of safer and highly immunogenic vaccines. Since poxviruses are now being used widely as a platform for a number of vaccines for various diseases, including human immunodeficiency virus (HIV) and cancer, our work with A35 is broadly applicable to vaccine development.

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A35Δ – A35 deletion mutant virus
A35Res – A35 rescue virus
Ag – antigen
AIDS – acquired immune deficiency syndrome
AP – alkaline phosphatase
APC - allophycocyanin
APC – antigen presenting cell
C3b – complement component 3b
C4b – complement component 4b
CD – cluster of differentiation
CD40L – cluster of differentiation 40 ligand
CEF – chick embryo fibroblast
CLIP – class II-associated invariant-chain peptide
Con A – Concanavalin A
CrmA – cytokine response modifier A
CTL – cytotoxic T lymphocyte
DMEM – Dulbecco's Modified Eagle Medium
dpi – days post infection
DNA – deoxyribonucleic acid
dsDNA – double-stranded deoxyribonucleic acid
EEV - extracellular enveloped virus
eIF2α - eukaryotic translation initiation factor 2 alpha
ELISA – enzyme-linked immunosorbent assay
ELISPOT – enzyme-linked immunosorbent spot assay
FACS – fluorescent activated cell sorter
FBS – fetal bovine serum
Fc – fragment, crystallizable
FITC - fluorescein isothiocyanate
GAD – glutamate decarboxylase
G-CSF – granulocyte colony stimulating factor
GM-CSF – granulocyte macrophage colony stimulating factor
GPMBP - guinea pig myelin basic protein
gpt - *E. coli* xanthine guanine phosphoribosyl transferase gene
GRO/KC - growth regulated oncogene alpha/keratinocyte attractant
h – hour(s)
HBSS – Hank's buffered salt solution
HEL – hen egg lysozyme
HIV – human immunodeficiency virus
hpi – hours post infection
HRP – horseradish peroxidase
IEV - intracellular enveloped virus
IFN – interferon
IFNα – interferon alpha
IFNβ – interferon beta

IFNγ – interferon gamma
IFNγR – interferon gamma receptor
Ig – immunoglobulin
IgG – immunoglobulin G
IgG1 – immunoglobulin G 1
IgG2a – immunoglobulin G 2a
IgM – immunoglobulin M
Ii – invariant chain
IL – interleukin
IL-1α – interleukin 1 alpha
IL-1β – interleukin 1 beta
i.m. - intramuscular
IMV - intracellular mature virus
i.n. - intranasal
i.p. - intraperitoneal
IV - immature virion
JAK – Janus-family tyrosine kinases
kb - kilobases
kD - kilodalton
KLH - keyhole limpet hemocyanin
LD_{50} – 50% lethal dose
LDH – lactate dehydrogenase
LPS – lipopolysaccharide
MCP-1 – monocyte chemotactic protein 1
MEM – minimal essential media
MFI – mean fluorescence intensity
MHC – major histocompatibility complex
MIP-1α - macrophage inflammatory protein – 1 alpha
MIP-1β - macrophage inflammatory protein – 1 beta
MOI – multiplicity of infection
mRNA – messenger ribonucleic acid
MTS/PMS - 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)2H-tetrazolium, inner salt/phenazine methosulphate
MVA – Modified Vaccinia Ankara
MVA35Δ - Modified Vaccinia Ankara A35 deletion mutant virus
NK – natural killer
NO – nitric oxide
OD – optical density
ORF – open reading frame
PacBl – Pacific blue
PBS – phosphate buffered saline
PE – phycoerythrin
PE-Cy7 – phycoerythrin cyanine 7
PEC – peritoneal exudate cells
pfu – plaque forming units
PI – propidium iodide

pi – post infection
PKR – protein kinase R
PMA - phorbol 12-myristate 13-acetate
RANTES – Regulated upon Activation, Normal T cell Expressed, and Secreted
RBC – red blood cell
RNA – ribonucleic acid
RPE – R phycoerythrin
SARS – severe acute respiratory syndrome
SD – standard deviation
SEM – standard error of the mean
STAT – Signal Transducers and Activators of Transcription
Th1 – T helper 1
Th2 – T helper 2
TK – thymidine kinase
TNF - tumor necrosis factor
TNFα – tumor necrosis factor alpha
UN - uninfected
VACV – Vaccinia virus
VARV – Variola virus
vCCI – Vaccinia CC chemokine inhibitor
VCP – Vaccinia complement control protein
VIG – Vaccinia immune globulin
vIL-1βR – Vaccinia interleukin-1 beta receptor homolog
vTNFR – Vaccinia tumor necrosis factor receptor homolog
WR – Western Reserve

CHAPTER 1: REVIEW OF THE LITERATURE

Poxviruses

General Characteristics and Taxonomy

The *Poxviridae* are a family of large, enveloped, double-stranded DNA (dsDNA) viruses that range in size from 130-300 kilobases (kb) (141). Unlike all other DNA viruses, poxviruses have the unique ability to replicate in the cytoplasm of their host, rather than in the nucleus. Poxviruses have worldwide distribution and a broad host range, infecting both insects (Subfamily *Entomopoxvirinae*) and vertebrates (Subfamily *Chordopoxvirinae*). Included in the vertebrate-infecting subfamily *Chordopoxvirinae* are the following genera: *Orthopoxvirus, Parapoxvirus, Avipoxvirus, Capripoxvirus, Leporipoxvirus, Suipoxvirus, Molluscipoxvirus, and Yatapoxvirus.* The focus of this literature review will be the orthopoxviruses, as these are the most significant to human health. Virus species that are included in this genus are Variola virus (VARV), Ectromelia virus (mousepox), Monkeypox, Cowpox, and the prototypical poxvirus Vaccinia virus (VACV). These virus species are highly related, and vaccination or infection with one confers immunity to all others. Parapoxviruses, yatapoxviruses, and molluscipoxviruses can also infect humans, and will be mentioned briefly.

Replication Cycle

Poxviruses have a complicated replication cycle that occurs exclusively in the cytoplasm of the host cell. Thus, upon entry into a host cell, the virion is equipped with the enzymes that are necessary to initiate the transcription of genes required for viral DNA replication (141). Virus entry has been shown to be mediated by the viral envelope protein A27, which attaches to the cellular surface proteins heparin and heparin sulfate (183). The expression of poxvirus genes is temporally regulated, with early, intermediate, and late gene expression (23). Once inside the

host cell, the virion core begins to transcribe early mRNA, which will be translated into proteins that are needed for DNA replication and intermediate transcription. Also synthesized are proteins that function in evading the host immune system, which will be discussed later. Uncoating of the core results in the initiation of DNA replication, followed by intermediate transcription from the newly synthesized DNA. Finally, late transcription occurs, producing early transcription factors and structural proteins. These, along with viral DNA, form the newly assembled virions (141).

Virion assembly occurs in areas of the cytoplasm called virus factories. Assembly begins with the formation of crescent-shaped membrane structures, followed by the packaging of DNA to form immature virions (IV). The IV matures via proteolysis to form the intracellular mature virus (IMV), which then undergoes a membrane wrapping event and acquires two additional membranes from the trans-golgi, forming the intracellular enveloped virus (IEV) (77, 141, 178). The IEV then fuses out from the cell, losing its outermost membrane and forming the extracellular enveloped virus (EEV) (78, 172). Both the IMV and the EEV are infectious forms of the virus. The IMV is released from dying or lysed cells, is extremely stable and thought to be the form of virus responsible for transmission from host-to-host. The IMV can enter the cell by either fusion with the plasma membrane (28) or by endosomal entry (215). The EEV, containing one additional membrane compared to IMV, is also infectious and is believed to be the form that mediates spread of infection in the host (141).

Smallpox

The most infamous orthopoxvirus is Variola virus (VARV), the causative agent of smallpox. VARV has a 30-40% fatality rate and was responsible for nearly 500 million deaths in the 20th century alone before its eradication from nature in the late 1970's (124). The eradication

of smallpox was due to a successful vaccination campaign involving the related poxvirus, Vaccinia virus (VACV). Smallpox is an exclusively human disease and was able to be successfully eradicated due to the fact that no animal reservoir existed, which is not the case with most other poxviruses. Routine poxvirus vaccination in the United States stopped in the early 1970's due to high complication rates. The last natural case of smallpox occurred in Somalia in 1977, and smallpox was declared eradicated by the World Health Organization in 1980 (124). Two official stockpiles of VARV exist in the world, but there are fears that other unknown stockpiles may exist and be used in an act of bioterrorism against the United States. Such release of VARV would be devastating, as the current population is largely unvaccinated and would thus be susceptible to infection.

Emerging Poxviruses

Since the discontinuation of vaccination programs, the incidence of zoonotic orthopoxvirus infections has increased throughout the world. In South America, a wild VACV, known as Cantagalo virus, has infected cattle on over 70 farms and caused disseminated rash in humans (45). The virus is thought to have been transmitted to domestic cattle, where it persisted and mutations occurred, and then transmitted back to susceptible humans (45). There is also concern that Cantagalo is rapidly spreading throughout Brazil (135).

Buffalopox, a close relative of VACV, can infect buffaloes and humans and has been associated with numerous outbreaks in India (100, 227). Buffalopox can cause lesions on the teats and udders of both buffalo and cows, which can then transmit the virus to the hands of humans. A hospital outbreak of buffalopox caused by contaminated first aid supplies was recently reported at a burn center in Pakistan (228). Of particular concern is the evidence that suggests that buffalopox can also spread from human to human (100).

Outbreaks of cowpox have been reported in primate colonies in Europe (129, 132). The outbreaks caused widespread sickness and mortality, raising concerns about the evolution of cowpox into a significant primate pathogen. It was shown that rats captured at the sanctuary were positive for infection and were most likely the source of the outbreak. Human outbreaks of cowpox are mostly associated with contact with infected cats (10), and there is evidence for human to human spread (118).

The most dangerous poxvirus that infects humans is monkeypox virus. This virus causes a smallpox-like illness with a 10% fatality rate (63), is commonly carried by African rodents, and is endemic to equatorial Africa (47). In 2003 there was an outbreak of monkeypox in the United States associated with the importation of African rodents. This outbreak caused more than 80 human infections, but since this was a low virulence strain, no fatalities occurred (34).

Orthopoxvirus is not the only poxvirus genus with members that cause human disease. Tanapox, a member of the genus *Yatapoxvirus*, is endemic to equatorial Africa, infects primates and has been diagnosed in Europe and the United States in recent years (52, 198). Molluscum contagiosum virus, the sole member of the genus *Molluscipoxvirus*, occurs commonly (and exclusively) in humans (39% of a population over 50 years of age tested seropositive in Australia) (101) and accounts for approximately 300,000 doctor visits each year in the United States (140). Molluscum causes groups of wart like lesions, is common in children and acquired immune deficiency syndrome (AIDS) patients, and is now emerging as a sexually transmitted disease. Members of the genus *Parapoxvirus* can cause disease in humans, including orf virus and pseudocowpoxvirus (137, 168), and these infections are mostly transmitted from sheep, goats, or cattle.

Poxvirus Vaccines

Overview and Vaccine-Related Complications

Smallpox was eradicated from the world through vaccination using a related virus, VACV. This is a live virus vaccine that is given in the upper arm using a technique called scarification. A "take" is indicated by the presence of a pustule at the vaccination site (85). The pustule will eventually form a scab and fall off. Vaccine recipients will then have immunity to not only smallpox, but to all other related orthopoxviruses.

Although highly effective, vaccination with VACV was discontinued for the general public due to a high rate of adverse events. Disseminated, or generalized, vaccinia develops as a vesicular rash in otherwise healthy people (114). Eczema vaccinatum can occur in individuals with atopic dermatitis or other inflammatory skin conditions, and thus these individuals or their contacts should not be vaccinated with live VACV. Recently, a 2-year old child developed eczema vaccinatum and became extremely ill after contact with his father who had received the smallpox vaccine because of his military service (111). Progressive vaccinia occurs in those with compromised immune systems, is characterized by a progressive necrosis at the vaccination site, and is often fatal (20). Also associated with vaccination are postvaccination encephalitis and myopericaditis. Indeed, a recent vaccine trial was cancelled due to the increased incidence of heart complications (7, 60, 211). It has been estimated that vaccination with VACV is contraindicated for 25% of the population (96), including those with inflammatory skin conditions, immunodeficiency, or heart disease, and also for any of their close contacts due to the potential for accidental infection of household members. Thus, VACV maintains significant virulence in the human population and mechanisms that contribute to its virulence are of interest.

In addition, in the face of emerging disease and bioterrorism, there is a need for a safer poxvirus vaccine that can be used in the general public.

New Generation Vaccines

Many strategies are being used to reduce the risks associated with vaccination without decreasing either the immunogenicity or efficacy of the vaccine. The first is the sequential passage of virulent wild type VACV in the tissue culture cells of a non-traditional host. During these passages, the properties of the virulent VACV change, resulting in an attenuated virus. Modified Vaccinia Ankara (MVA) is an attenuated virus strain that resulted from over 570 serial passages of wild type VACV Ankara strain in chicken embryo fibroblasts (CEF). During this time, six major deletions of the genome occurred, totaling approximately 25 kb of DNA (136, 138), and the virus lost the ability to replicate in most mammalian cells (17). The avirulence of MVA in mammals is attributed to the deletion of numerous genes, including the K1L host range gene, and genes for the soluble receptors for both type I and type II interferons (IFN) and tumor necrosis factor (TNF) (17). MVA is able to undergo all steps of DNA replication, and all early, intermediate, and late viral proteins are synthesized (98, 204), but morphogenesis stops at the formation of immature virions. This is important, since the production of late viral proteins is crucial in the generation of protective neutralizing antibodies. MVA was used without complication during the smallpox eradication campaign, even in high risk populations (133), although its protective efficacy against human smallpox infection remains to be tested. Recent vaccine trials have shown that MVA can be used without complication in HIV-infected individuals and also in those with atopic dermatitis (90). Animal models using non-human primates indicate that MVA can protect against monkeypox infection (57, 58).

A second strategy that has been used to increase the safety of vaccination is the identification and selective removal of genes that contribute to virulence. The NYVAC vaccine is missing 18 open reading frames (ORFs), resulting in an attenuated virus. NYVAC is avirulent in animal models (39, 72), but recent studies suggest that its immunogenicity is lacking compared to the traditional vaccines (139). Single gene deletions have also been tested for their ability to produce an effective immune response and to protect against disease. Deletion of the viral dsDNA binding protein, gene E3L, from wild-type VACV results in a 1000-fold decrease in pathogenicity in the mouse model (216), increased IFNγ-secretion from splenocytes of vaccinated mice (87), and protection against lethal challenge (87, 216). An A41L gene (viral chemokine binding protein) deletion results in an increased T cell response and protection from lethal challenge, although this deletion was slightly more virulent than the wild-type virus (36). An MVA-based vaccine missing the soluble viral interleukin 1-beta (IL-1β) receptor (gene B15R) was avirulent in mice, induced a better T cell response, and provided better protection against challenge compared to wild-type virus (196). Thus, identification and deletion of virulence genes appears to be a useful strategy in the production of safer poxvirus vaccines.

Poxviruses as Platform Vaccines

Poxviruses are being used as platform vaccines for other infectious diseases and cancer, due to their ability to induce a robust immune response and to accommodate large pieces of foreign DNA. Since wild type VACV has safety issues associated with it, poxvirus-based vaccine vectors are mostly made from the attenuated strains, such as MVA and NYVAC, or from host-restricted poxviruses, such as avipoxviruses, since these do not replicate in mammalian tissues. Poxvirus-based recombinant vaccines have been made to express antigens from severe acute respiratory syndrome (SARS) (16, 35), influenza (24, 106), *Leishmania major* (203), HIV

(50), malaria (56), and rabies (154), among many others. These vaccines produce long term memory and protection in animal models. A promising new study reported that a canarypox-based HIV vaccine was effective at preventing HIV infection in a community in Thailand (165). Thus, attenuated poxviruses are not only important as potential new generation vaccines for poxviruses, they are also attractive vectors for vaccines for other antigens.

Poxviruses and the Immune Response

Immune Responses to Poxvirus Infections

Poxviruses are known for their ability to induce a robust immune response. Studies with humans and animal models have shown that a strong anti-virus antibody response follows infection with various poxviruses (57, 67, 218, 224), and that this response plays a role in protection. In humans, antibodies are produced that can neutralize both IMV and EEV particles (48, 49) and virus-specific antibody is detectable for decades after immunization (43, 75, 156). Human Vaccinia Immune Globulin (VIG) is used in the treatment of many of the vaccine-related complications, highlighting the importance of antibody in viral neutralization and clearance.

In mice, virus neutralizing IgM can be detected in the sera as soon as four days after infection (143). Virus-specific isotypes of IgG can be detected by day 7 post infection (193) and remain elevated as far out as six weeks after infection in mice (221). The production of virus-specific IgG antibody is dependent on CD4+ T cells (224). Mice that lack CD4+ T cells or major histocompatibility complex (MHC) class II fail to produce sufficient amounts of VACV-specific IgG and are deficient in their ability to clear virus after primary infection with VACV (224). The generation of antibody during an immunization has also been correlated with protection in mice after challenge with VACV (14). In mice infected with ectromelia virus (mousepox), a humoral response was required to survive infection (32). Mice lacking B cells

succumbed to infection later than those deficient in the CD8+ T cell effector response, suggesting a role for antibody in the later stages of a viral infection in this model. The adoptive transfer of B cells or immune-serum from vaccinated mice to infected B cell deficient mice resulted in complete recovery (32). Antibody, but not CD8+ T cell function, was shown to be necessary in the protection of primed mice from a challenge using virulent mousepox (151). In this model, the antibody response was dependent on CD4+ T cell help. This was shown by infecting mice that lacked either CD40 or MHC II, and were therefore unable to activate B cells through the CD40-CD40L interaction or lacked CD4+ T cells completely, respectively. Similar to the mice deficient in B cell function, these mice succumbed to infection (151).

In non-human primate models, IMV and EEV neutralizing antibody can be detected after about one week post infection (57), and antibody is necessary for protection from a lethal monkeypox challenge, as primates depleted of antibody-producing B cells prior to challenge did not survive (61). Together, there is an overwhelming amount of data to indicate that antibody is important both for the clearance of a primary infection and in mediating protection after vaccination.

Poxviruses also induce a strong anti-virus T cell response, resulting in the clonal expansion of both CD4+ T cells and CD8+ T cells. In humans, VACV-specific CD4+ and CD8+ T cells can be detected approximately one week after vaccination (131), and peak around two weeks (6). The virus-specific CD8+ T cell response was greater than the CD4+ T cell response at these early times after vaccination (6) and both populations secreted mostly Th1 cytokines, including IFNγ (6). Decades following vaccination, virus-specific T cells can be detected, but memory CD4+ T cells are longer lived than the memory CD8+ T cell population (6, 75). In humans, cellular immunity appears to be crucial in the control of viral replication, as individuals

with deficiencies in cellular immunity can develop severe adverse reactions upon vaccination with VACV.

In mice, anti-viral IFNγ-secreting CD4+ and CD8+ T cells can be detected approximately 6-7 days following infection (76, 224) and both play an important role in the anti-viral defense against VACV or other poxviruses. Mice depleted of CD4+ T cells were impaired in their ability to clear virus from target organs following infection with VACV, while those depleted of CD8+ T cells were similar to wild-type mice in their ability to control replication (224). CD4+ T cells are important in the production of anti-viral CD8+ T cells (9, 92). Mice depleted of CD8+ T cells do not survive primary infection with ectromelia virus (32), although they were not required for protection from a lethal secondary infection following vaccination (151). CD8+ T cells can also provide protection against a VACV infection, as the adoptive transfer of memory CD8+ T cells to naïve mice resulted in decreased viral replication (224). In another study, it was shown that mice that were vaccinated and then depleted of CD8+ T cells were not protected from sickness (191). Thus, both CD4+ and CD8+ T cells play roles in immunity to poxviruses in mice.

Poxvirus Immune Evasion Strategies

While poxviruses can induce a robust immune response, they also encode numerous proteins that function to evade the host immune response. These immunomodulators contribute to the virulence of poxviruses. Many of these proteins were identified by their sequence similarity to known cellular proteins, indicating that they were likely captured from host genomes, while some evolved independently in the virus. Described below are some of the proteins that poxviruses use to subvert the host immune response.

Vaccinia complement control protein. The complement system involves an immunological cascade of proteins that function to opsonize pathogens. Complement proteins can bind directly to the surface of a pathogen (alternative pathway) or can bind to either IgM or IgG that is bound to antigen (classical pathway). The end result of either pathway is the formation and activation of the membrane attack complex and lysis of the cell. VACV encodes a soluble 35 kilodalton (kD) polypeptide that has sequence similarity to host complement regulatory proteins (103). This is now known as the Vaccinia virus complement control protein (VCP) and is encoded by VACV gene C21L (103). Rabbits inoculated intradermally with a mutant virus lacking the C21L gene developed smaller lesions on the skin compared to rabbits inoculated with wild type VACV, indicating that the protein is an important virulence factor (83). C21L was shown to inhibit the hemolysis of IgM-sensitized sheep red blood cells (RBC) in a hemolysis assay using serum as a source of complement (103). When medium from wild type infected cells was added to the assay, lysis of the RBCs was prevented, but when medium from cells infected with a mutant virus lacking the 35 kD protein was added, the sheep RBCs were not protected from lysis (103). VCP has subsequently been shown to bind to and inactivate two critical proteins of the complement cascade, C3b and C4b, and can inhibit both the classical and alternative pathways (15, 102, 134, 145).

IL-18 binding protein. IL-18 is an important inducer of IFNγ and the Th1 immune response, both critical components of the anti-viral defense. This pro-inflammatory cytokine can be secreted by many cell types and is stored intracellularly as pro-IL-18 (69). Before its release from the cell, pro-IL-18 is cleaved to its active form by the IL-1β-converting enzyme. IL-18 then works together with IL-12 to activate pathways that lead to the production of IFNγ, activation of natural killer (NK) cells, and induction of the Th1 response. Many

poxviruses encode a soluble protein, encoded by the VACV gene C12L, with sequence similarity to cellular IL-18 binding proteins that can bind human and mouse IL-18 (18, 222), and can disrupt the activity of IL-18 *in vitro* (18, 190, 205, 222). The protein also plays a role in virulence, as mice that were infected with a mutant virus lacking the IL-18 binding protein showed less weight loss and signs of illness compared to those that were infected with wild type virus (159, 205). Further, the protein plays a direct role in modulating IL-18-driven immune responses. Mice that were infected with the mutant virus had higher levels of virus specific NK (18, 159) and cytotoxic T lymphocyte (CTL) cytotoxicity (159) and had greater levels of IFNγ in samples collected from the lungs (159) than mice infected with wild type virus.

Soluble tumor necrosis factor receptor homologs. Tumor necrosis factor (TNF) is a potent pro-inflammatory cytokine that is secreted by numerous cell types. TNF contributes to the antiviral immune response by binding its cellular receptor, and inducing apoptosis of virally infected cells and inhibiting viral replication. TNF also works together with IFNγ to promote a Th1 immune response. Thus, TNF is a key player in the host defense against viruses. Many poxviruses, including myxoma virus, tanapox, VACV, VARV, ectromelia virus, and cowpox encode and secrete a TNF receptor homolog (vTNFR) (71, 121, 158, 176, 181, 226). The vTNFR from each of these virus species can bind host TNF and block the biological activity of TNF *in vitro* (121, 158, 176, 181). It has also been shown that deletion of the vTNFR in VACV results in decreased virulence in the mouse model (158)

Soluble IL-1β receptor homolog. IL-1β is a pro-inflammatory cytokine secreted mostly by monocytes/macrophages. It is also known as endogenous pyrogen since its major function is the induction of fever during an infection. IL-1β is similar to IL-18 in that both are stored intracellularly as either pro-IL-1β or pro-IL-18 and then cleaved to their active forms by caspase-

1 before being released from the cell (69). Once released from the cell, IL-1β can bind its cellular receptor, leading to the production of pro-inflammatory cytokines, recruitment of lymphocytes to areas of infection and the induction of fever. Not surprisingly, many poxviruses, including VACV (2), cowpox (192), and ectromelia virus (188) encode a secreted IL-1β decoy receptor (VACV gene B15R). These virus encoded IL-1β receptors (vIL-1βR) can bind IL-1β (2, 192) and block its activity *in vitro* (192). Interestingly, a vIL-1βR deletion mutant virus delivered intranasally to mice induced fever and was *more* virulent than wild-type virus (2). Vaccine strains known to naturally lack this decoy receptor have also been associated with greater virulence and induction of fever, and expression of the vIL-1βR in these strains reduced virulence and prevented fever (1). These data led to the finding that IL-1β was the major fever-inducing cytokine during a poxvirus infection (1). An MVA virus lacking the vIL-1βR maintained its avirulent phenotype and induced greater T cell responses and better protection against lethal challenge compared to wild-type MVA (196).

Chemokine regulators. Chemokines are specialized cytokines that aid in the migration of immune cells to sites of infection. These chemoattractants can be classified as either CC, CXC, or C chemokines, based on the order of conserved cysteine residues. Different chemokines can be secreted by many cell types and bind specific receptors found on their target cells, thus orchestrating the influx of cells and the resulting immune response to the infection. Poxviruses have been shown to secrete a 35 kD protein (called CC chemokine inhibitor, vCCI) that preferentially binds CC chemokines (including RANTES, MCP-1, MIP-1α, and MIP-1β) *in vitro* (4, 25, 73, 187), thus blocking the binding of the chemokine to its cellular receptor. vCCI does not have sequence similarity to any other known protein (4, 187). This 35 kD protein can also inhibit the migration of cells in an *in vitro* monocyte chemotaxis assay (4, 187), and rabbits

infected with a mutant virus lacking the expression of the vCCI had significantly more lymphocytes in the pock lesions on the dermis (73). Expression of vCCI in virus strains lacking the protein resulted in attenuation of disease and decreased numbers of lymphocytes in the lungs in the mouse model (160). The protein has also been shown to be a promising therapeutic agent in diseases involving lung inflammation, such as asthma (44). Poxviruses can also regulate chemokine activity by encoding chemokine homologs (46, 122).

Interferon binding proteins. Interferons (IFNs) are a group of potent antiviral cytokines. IFNs bind their cellular receptor, activating the JAK/STAT pathway leading to the synthesis of many anti-viral proteins. IFNs can be divided into two groups: type I IFNs (IFNα and IFNβ) and type II IFNs (IFNγ), each group having a distinct receptor. IFNγ is secreted by T cells and NK cells, and induces the Th1 immune response, suppresses the Th2 response, activates macrophages, upregulates the expression of MHC molecules, and enhances CTL activity. The production of Th1 cytokines is crucial to the clearance of poxvirus infections, while a Th2 response can increase virulence. Indeed, mice that are given IFNγ during a poxvirus infection recover quicker than mice that do not receive treatment (93). The importance of IFNγ in combating poxvirus infections is underscored by the numerous mechanisms that this group of viruses has evolved to counteract this response. The VACV gene B8R encodes a soluble protein with sequence similarity to the extracellular domain of the human and murine IFNγ receptor (IFNγR). The IFNγR homologs in VACV, cowpox, and camelpox have been shown to bind and inhibit the activity of IFNγ from human, cow, and rat, but not mouse (3, 142, 207). The myxoma virus IFNγR can only bind IFNγ from rabbits, its natural host (212). The protein is an important virulence factor, depending on the model used. Disease is attenuated in rabbits, but not in mice, after infection with VACV lacking the IFNγR gene (142, 194, 207). In contrast, the ectromelia

virus homolog is an important virulence factor in mice and inhibits the generation of an optimal immune response in this model (174).

IFNα/β are also important anti-viral cytokines. Type I IFNs can be secreted by immune cells and fibroblasts, and they upregulate the expression of MHC class I molecules, leading to enhanced recognition of virally infected cells by the host. The VACV B18R gene encodes a secreted protein that can bind extracellular type I IFNs from numerous species and inhibit their anti-viral effects (37, 206, 214, 219), and also contributes to virulence in the mouse model (206). Interestingly, the soluble viral type I IFN binding protein can also bind to the surface of both uninfected and infected cells, where it blocks the action of the anti-viral cytokines, resulting in continued viral replication and spread (5). The mousepox ortholog of VACV B18R binds mouse type I IFNs (189, 225) and is an important virulence factor (225). Importantly, mice inoculated with a recombinant viral type I IFN binding protein were protected from a lethal challenge (225), suggesting that this protein may be used as a target for future vaccination.

Intracellular inhibition of interferon activity. Since IFNs are such potent anti-viral cytokines, it is not surprising that poxviruses have evolved multiple strategies to thwart their effects. In addition to the extracellular IFN binding proteins described above, poxviruses also encode proteins that inhibit the intracellular activity of IFN-responsive genes, most notably protein kinase R (PKR). Once synthesized, PKR binds to dsRNA, a common product in the cell during a viral infection, and then, along with the eukaryotic translation initiation factor 2α (eIF2α), becomes phosphorylated. eIF2α is then no longer able to initiate translation, and viral replication stops. Another IFN-responsive gene that is expressed during a viral infection is 2'-5' oligoadenylate synthetase that also binds dsRNA, ultimately resulting in the degradation of RNA and the inhibition of viral replication. Poxviruses have two proteins that regulate IFN activity

intracellularly. The E3L protein binds dsRNA, thus preventing it from binding to PKR and 2'-5' oligoadenylate synthetase (31, 109, 128, 147). The K3L gene product is similar to eIF2α and functions as a "pseudosubstrate" for PKR, preventing the phosphorylation of the cellular eIF2α (182). The function of both proteins is to allow for the continued translation of viral proteins.

Apoptosis inhibitors. Apoptosis, or programmed cell death, is a highly regulated cellular process that can occur in response to viral infection. The goal of the host is to eliminate the virally infected cells before progeny virions can be produced and infect other cells. CD8+ cytotoxic T lymphocytes can recognize viral antigen presented in the context of MHC class I molecules and then selectively kill these cells by releasing cytolytic granules into the cell or by inducing apoptosis through the interaction of Fas with Fas Ligand. Poxviruses encode genes that counteract both of these pathways. Cowpox encodes a protein, CrmA, that inhibits the activity of caspase-8 (54), which is cellular protease involved in the transduction of apoptotic signals initiated by TNF or Fas receptors (209). VACV encodes a gene similar to CrmA, designated B13R (53). Both proteins can protect cells from Fas or TNF-induced cytolysis (53, 209). A mutant VACV missing the B13R gene induced apoptosis in *in vitro* infected cells (99) and deletion of the cowpox ortholog resulted in attenuation of disease in the mouse model (120). Molluscum contagiosum also expresses a protein that interferes with apoptotic signaling via the Fas/TNF receptors (146, 184, 210). Other poxviruses known to encode anti-apoptotic genes include ectromelia virus (21) and myxoma virus (64).

Regulation of MHC class II antigen presentation. MHC class II heterodimers present extracellular antigen to cognate CD4+ T cells. Viral peptides, usually derived from the cytosol, can also be presented via MHC class II molecules by autophagy within the cell or when infected

cells are engulfed by professional antigen presenting cells (APC). MHC class II has been shown to be important in poxvirus infections, as mice either depleted of CD4+ T cells or genetically void of MHC II molecules were deficient in their ability to clear a VACV infection, where those lacking CD8+ T cells cleared infection comparable to wild type mice (224). *In vitro* MHC class II-restricted antigen presentation has been shown to be regulated by VACV using several different model systems. Li et al (116) showed that VACV inhibited the presentation of numerous antigens (endogenous and exogenous) from several different professional APCs and fibroblasts. MHC class II surface expression was unchanged as a result of infection and the underlying mechanism was shown to involve a decrease in the amount of peptide that was loaded into MHC class II molecules (116). Another laboratory reported that *in vitro* VACV infection of primary Langerhans cells decreased their ability to become activated and present a model antigen, keyhole limpet hemocyanin (KLH), to a cognate CD4+ T cell line, HDK-1 (51). Primary rodent bone marrow derived macrophages infected *in vitro* with VACV were deficient in presenting lysozyme peptide to T cells (126). It has also been shown that splenic dendritic cells from VACV-infected mice had reduced MHC class II surface expression and were impaired in their ability to present antigen to CD4+ T cells (227). Thus, there is accumulating data to indicate that VACV can disrupt the processing and presentation of antigen via the MHC class II pathway, although none of the above mentioned manuscripts have identified the gene or genes responsible.

Vaccinia Virus A35R Protein

While much work has been done to characterize poxvirus genes, more is needed. In the subfamily *Chordopoxvirinae*, there are 90 genes that are completely conserved, and over 25 of these have yet to be characterized (213). It has previously been shown that the VACV A35R

protein is highly conserved in all sequenced mammalian tropic poxviruses, but has little similarity to any other known protein, suggesting an important and novel function (170). The hydrophobicity and location of the gene within the genome suggested that it was a membrane protein, but in a Triton X-114 partitioning assay, A35 partitioned to the aqueous phase, indicating that it is a cytoplasmic protein (170). A35 is expressed early during infection, is non-glycosylated, and is resolved at about 23 kD when probed with anti-A35 antibodies on a Western blot (170). To determine the function of A35, a mutant virus was constructed in which A35 was replaced by a selectable marker, the *E. coli* xanthine guanine phosphoribosyl transferase (*gpt*) gene, and is designated as A35Δ (170). A35Δ replicated similarly to the wild type Western Reserve (WR) strain of VACV, forming normal size plaques and normal quantities of intracellular and extracellular virus particles in BS-C-1 African green monkey kidney cells (170). Futhermore, A35 was not required for replication in numerous cell types from various hosts, indicating that A35 is likely not a host range gene (170). To determine the role of A35 in virulence, mice were intranasally (i.n.) infected with 10^4 plaque forming units (pfu) of either WR, A35Δ, or an A35 rescue virus (A35Res) (or mock-infected with phosphate buffered saline, PBS, as a control), and their weights were measured daily as an indicator of sickness (170). Mice that were infected with either of the viruses containing A35 (WR and A35Res) rapidly began to lose weight at about 6 days post infection (dpi) (2 out of 6 WR died), while those infected with the A35Δ virus grew similar to PBS control. Mice were also infected with 10^5 and 10^6 pfu, and it was determined that the A35 protein increases virulence by 100- to 1000-fold.

Previous data with A35 indicated that the protein was not involved in replication in eight different cell lines from various hosts, but that it was required for full virulence in the mouse model. We therefore hypothesized that the protein may promote virulence by affecting viral

replication in certain cell types or tissues *in vivo* or by regulating the host immune response. To test for the role of A35 in replication, we have compared the replication of WR to A35Δ using various cell lines, primary cells, and *in vivo* infected mouse tissues. To test for the role of A35 in regulation of the immune response, we have used two approaches. The first involves an *in vitro* MHC class II restricted antigen presentation system using primary rat macrophages as APC. We found this to be an appropriate system to look at since rats are natural reservoirs for poxviruses and peritoneal exudate cells (PEC) are a rich source of macrophages. For most of the *in vitro* assays, both mouse and rat cells were used. We were also interested in looking at MHC class II antigen presentation because during a VACV infection, mice deficient in MHC class II or CD4+ T cells are suppressed in their ability to control viral replication (224) and have lower amounts of virus-specific antibody and CTL. This is in contrast to mice deficient in MHC class I or CD8+ T cells that are able to clear an infection similar to wild type mice (224). The second approach to determining the role of A35 on the host immune response involves infection of mice with either A35Δ or WR, and then performing *ex vivo* assays to determine virus-specific antibody production, CTL killing, and IFNγ production. We also constructed an A35 mutant virus in the attenuated vaccine strain MVA (MVA35Δ) and similar experiments were performed comparing the replication and immunogenicity of MVA to the MVA35Δ virus. We have also tested the protective efficacy of the A35Δ and the MVA35Δ viruses compared to wild type WR or MVA, respectively. The data presented provide strong evidence that A35 is an important poxvirus immunoregulator, and that A35 deletion mutant viruses can provide protection against a lethal respiratory challenge similar to wild type viruses.

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CHAPTER 2: VACCINIA VIRUS DECREASES MHC CLASS II ANTIGEN PRESENTATION, T CELL PRIMING, AND PEPTIDE ASSOCIATION WITH MHC CLASS II

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Abstract

Vaccinia virus (VACV) is the current live virus vaccine used to protect humans against smallpox and monkeypox, but its use is contraindicated in several populations because of its virulence. It is therefore important to elucidate the immune evasion mechanisms of VACV. We found that VACV infection of antigen-presenting cells (APCs) significantly decreased major histocompatibility complex (MHC) II antigen presentation and decreased synthesis of 13 chemokines and cytokines, suggesting a potent viral mechanism for immune evasion. In these model systems, responding T cells were not directly affected by virus, indicating that VACV directly affects the APC. VACV significantly decreased nitric oxide production by peritoneal exudate cells and the RAW macrophage cell line in response to lipopolysaccharide (LPS) and interferon (IFN)-γ, decreased class II MHC expression on APCs, and induced apoptosis in macrophages and dendritic cells. However, VACV decreased antigen presentation by 1153 B cells without apparent apoptosis induction, indicating that VACV differentially affects B lymphocytes and other APCs. We show that the key mechanism of VACV inhibition of antigen presentation may be its reduction of antigenic peptide loaded into the cleft of MHC II molecules.

These data indicate that VACV evades the host immune response by impairing critical functions of the APC.

Introduction

Poxviruses are large, double stranded DNA viruses (~200 kb) that replicate in the cytoplasm of host cells (141). Poxviruses have worldwide distribution and are highly successful pathogens, infecting a tremendous variety of animals including insects, reptiles, birds and mammals. The most infamous member of Family Poxviridae is smallpox, which is estimated to have caused 500 million human deaths in the 1900's before its eradication from nature (124). The eradication of smallpox was due to the most successful vaccination campaign in history using a related virus, Vaccinia virus (VACV). Today there remain multiple poxvirus threats to humans, including the use of smallpox as a bio-terrorism weapon in a now largely unvaccinated population. New poxviruses are identified each year in animal populations, and several zoonotic poxviruses appear to be emerging worldwide: Cantagalo in South America (45), Tanapox in Africa, Europe and the USA (52, 198), and buffalopox in India (100). Molluscum contagiosum virus causes wart-like lesions, occurs commonly in humans (101), is now emerging as a sexually transmitted disease, and accounts for approximately 300,000 doctor visits each year in the U.S. (140). The most dangerous poxvirus extant today is monkeypox virus, which causes a smallpox-like illness in humans, is endemic to Africa, and caused an outbreak in humans in the USA in 2003 (34). Studying the immune evasion tactics of this virus family will shed light on how these viruses evolved into such successful pathogens.

While VACV is used as a live virus vaccine, it can also be pathogenic. In a report on almost 39,000 volunteers vaccinated as first responders, it was found that 1 of every 450 vaccinees had to be hospitalized due to adverse vaccine reactions and that 1 death occurred per

13,000 vaccinations (29). Vaccination is contraindicated for individuals with any history of eczema or immunodeficiency disorders because fatalities may occur. Otherwise-healthy individuals can acquire a disseminated poxvirus rash, myopericarditis, and rarely fatal encephalitis from vaccination (7, 29, 60, 211). Understanding viral evasion mechanisms will aid in the development of safer vaccines.

For healthy individuals, an effective immune response limits viral replication of the less pathogenic poxviruses. However, poxviruses encode a plethora of immuno-modulatory proteins, including proteins that block apoptosis, chemokine and cytokine binding and synthesis, and cell signaling (74, 141, 181). In addition, poxviruses directly infect human and rodent immune cells both *in vivo* and *in vitro*, including lymphocytes, natural killer (NK) cells, and monocytes/macrophages (149, 175) and have been shown to decrease antigen presentation in several types of APC (116, 227). We wanted to further explore the effects of VACV on antigen presentation interactions between APC and the responding T cells and were especially interested to study *primary* APC responding to an inflammatory stimulus, as this has not been previously studied. We chose to use rat peritoneal macrophages elicited by injection of killed *Corynebacterium parvum* to study how virus interacts with primary APC that would be recruited to a site of infection/inflammation (38, 108). Also, we were interested to explore how VACV interacts with the rat immune system, as rats are a natural reservoir for orthopoxviruses and can transmit them to primates (34, 129). While the natural host of VACV is unknown, several orthopoxviruses spread naturally in rodents, including rats (34, 129). Here we describe the effects of VACV on a MHC class II restricted antigen presentation system in which rat macrophages present a peptide of myelin basic protein to cognate antigen specific CD4+ T lymphocytes (127, 155). CD4+ T cells are crucial for viral clearance in poxvirus infection (92,

151). In addition, we explore viral effects on T and B lymphocytes, dendritic cells and macrophage lines. Our data indicate that VACV-infection rapidly disables various APC by affecting relevant cell surface proteins, and in some cases inducing apoptosis of APC, thus blocking downstream T cell activation events and cytokine production. Further, our data indicate that the mechanism of immune interference is not necessarily apoptosis-dependent as has been previously suggested (95), but rather VACV blocks presentation of antigenic peptides in MHC class II molecules.

Materials and Methods

Cells and Virus

VACV Western Reserve (WR) stocks were propagated using BS-C-1 cells as previously described (170). For titrations of VACV, BS-C-1 monolayers were fixed and stained with 0.1% crystal violet in 20% ethanol. All cells were grown in media containing 10% fetal bovine serum (FBS). CD4+ RsL-11 T cell clones were derived and maintained as previously described (127). CTLL-2 (ATCC # TIB-214) were maintained in RPMI supplemented with 0.4% IL-2 (127). RAW 264.7 (ATCC # TIB-71), a mouse macrophage cell line, was maintained in DMEM. The mouse B cell line 1153 and the T cell hybridoma B04 (HEL_{74-88} specific) (kind gifts from Janice Blum) were maintained in RPMI (116).

Peritoneal Macrophage Isolation and Antigen Presentation Assays

Lewis rats (bred and maintained at the AAALAC-certified Brody School of Medicine animal care facility at East Carolina University) were injected intraperitoneally (i.p.) with 200 μg of inactivated *C. parvum* in 5 ml of HBSS. Two to three days later, the rats were sacrificed and peritoneal exudate cells (PEC) were harvested by washing the peritoneum in cold HBSS. PEC were washed, infected for 5 h, and placed into 96-well plates. PEC were incubated in

quadruplicate with 50-500 nM guinea pig myelin basic protein (GPMBP) for 30 minutes, followed by addition of 25,000 Lewis rat CD4+ RsL.11 clones specific for GPMBP. For experiments involving 1153 B cells, 25,000 B cells (I-A^b) were infected for 6 h. Cells were then plated in a 96-well format and incubated with varying concentrations of the antigen HEL_{74-88} (gift from Janice Blum) for 1 h. 25,000 B04 T cells specific for the HEL_{74-88} peptide in the context of I-A^b were added to each well. The antigen presentation plates were incubated for 24-48 h at 37ºC, 5.0% CO_2. After incubation, 50 µl of supernatants were transferred into an empty 96-well plate and frozen for the following assays. All animal experiments were in compliance with East Carolina University Animal Care and Use committee (K139) and NIH guidelines.

Bone Marrow-Derived Dendritic Cells

Rat bone marrow was harvested from femur and tibia. The cells were placed in RPMI supplemented with 10% FBS, 1% L-glutamine, 1% penicillin-streptomycin, and 0.1% granulocyte macrophage-colony stimulating factor- (GM-CSF-) containing baculovirus supernatant. After 3 days of culture, the culture medium was removed and replaced with fresh culture medium. On day 7, the dendritic cells were collected and used for the antigen presentation assay and flow cytometry analysis.

CTLL IL-2 Bioassay

IL-2 was measured using previously described methods (127). Briefly, 10,000 CTLL clones (quadruplicate) were washed, resuspended in RPMI, and added to the collected supernatants. The plates were incubated for 48 h at 37ºC, 5.0% CO_2, followed by the addition of 10 µl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)2H-tetrazolium, inner salt/phenazine methosulphate (MTS/PMS) (2.0 mg/mL MTS, Promega; and 0.1 mg/mL PMS, Sigma) (40). The absorbance was read at 24, 48, and 72 h post MTS/PMS at

492 nm filtered, and 690 nm reference. Media only was used to define the background control level and known IL-2 containing supernatants were used as positive control.

Nitric Oxide (NO) Measurement

Griess Reagent (50 μl) (1% sulfanilamide–0.1% N-[1-naphthy] ethylenediamine in 2.5% phosphoric acid) was added to 50 μl of the quadruplicate harvested supernatants. The absorbance was read at 540 nm after a 5-minute incubation at room temperature (27).

Measurement of Cytokines

50 μl of the harvested supernatants (triplicate) were analyzed using the LincoPlex 24 Rat Cytokine/Chemokine Luminex Bead Immunoassay Kit according to the manufacturer's instructions (Linco Research). The supernatants were incubated with a panel of anti-cytokine antibodies immobilized on Luminex beads (Bio-Rad Laboratories). The following cytokines were analyzed: IL-1α, IL-1β, IL-2, IL-6, IL-17, IL-18, MIP-1α, GM-CSF, IFN-α, growth regulated oncogene alpha/keratinocyte attractant (GRO/KC), RANTES, TNF-α, MCP-1, eotaxin, G-CSF, IL-4, IL-9, IL-13, IL-5, and IL-10. Samples were run according to the manufacturer's instructions (Bio-Rad) and analyzed on the BioPlex protein array reader (Bio-Rad) in the Duke University Human Vaccine Institute Immune Reconstitution Core Facility (Durham, NC).

RsL.11 Stimulation Assay

CD4+ RsL.11 T lymphocytes were washed and resuspended in RPMI. The cells were infected for 3-4 h, plated in tripliciate in a 96-well format, and stimulated with PEC/50 nM GPMBP, 25 μg/ml Con A (Sigma), or 100 nM phorbol 12-myristate 13-acetate (PMA)/2 μM ionomycin (Sigma). The plates were incubated at 37^{o}, 5% CO_2. 50 μl of the harvested supernatants were collected at 20- and 40-h and assayed for IL-2 using the CTLL bioassay already described.

Metabolism Assays

PEC or RAW 264.7 macrophages were infected with VACV multiplicity of infection=2 (MOI=2) and 1153 B cells at an MOI of 5. Metabolism was measured by the addition of 10 μl MTS/PMS 4-24 hpi, and analyzed similarly to the method described above for the CTLL IL-2 Bioassay. Absorbance was read at 492 nm at various times post infection.

Flow Cytometry

Cells were infected for 4 hours and then washed in FACS buffer (PBS containing 0.1% heat-inactivated FBS and 0.1% sodium azide). 3 x 10^5 cells were stained with an anti-MHC II concentrated supernatant (Y3P, AF6120, and MKS4) or OX1 anti CD45 for 1 h on ice, washed once, and then incubated with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Southern Biotech). MHC II expression was measured using a FACScan (Becton Dickinson) equipped with the Cell Quest software. For apoptosis measurement, cells were infected and then stained with annexinV-FITC/propidium iodide (PI) (BD Pharmingen) per the manufacturer's instructions.

One-Step Growth Curve

A one-step growth curve was obtained to measure viral replication in permissive cells and PEC as previously described (170).

Peptide-MHC Association

The Y-Ae antibody specifically detects a complex of peptide 52-68 from I-E^d MHC class II bound in the cleft of MHC class II I-A^b (84, 173). This complex is formed in mice that express both alleles. Spleens were harvested from B10.A-H2^i5 H2-T18^a/(5R)SgSnJ mice (Jackson Labs); C57BL/6 mice were used as a negative control. Unfractionated splenocytes were infected with purified virus for 3 h (MOI=10) and then incubated with biotin-conjugated Y-

Ae (eBioscience). Cells were washed and incubated with streptavidin-R phycoerythrin (RPE) (Southern Biotech). Samples were analyzed using a FACScan equipped with Cell Quest software.

Statistical Analysis

Experiments were repeated 3 times and representative data with standard deviation bars are shown. A two-tailed Student's *t* test was used to compare uninfected with VACV-infected groups. p values < 0.05 were considered significant.

Results

VACV Decreases IL-2 Production

As antigen presentation is the seminal event triggering an adaptive immune response, we wanted to assess the effects of VACV infection on this pivotal point of the immune response using primary cells recruited to a site of inflammation. Peritoneal exudate cells (PEC) were harvested from rats, infected with VACV, and pulsed with a model antigen (GPMBP). Various numbers of infected cells were then incubated with cognate CD4+ T cells. In order to measure antigen presentation responses, supernatant IL-2 was measured. VACV infection significantly (Student's t test $p<0.05$) reduced IL-2 production by T cells 15-48 hpi (Fig 1a). VACV also significantly decreased IL-2 production using rat splenocytes (Fig 1b), dendritic cells (Fig 1c), and PEC that had been rested overnight prior to infection (not shown) as APC. In the absence of antigen or either cell type, no IL-2 was produced. Together, these data indicated that VACV significantly decreased the amount of IL-2 that is produced by CD4+ T lymphocytes.

VACV Decreases NO Response

During antigen presentation, both the T lymphocyte and the APC become activated, and macrophages are stimulated to produce nitric oxide (NO) (123), an important antiviral defense.

Using the same antigen presentation system described above, supernatants were assayed for the production of NO. Uninfected PEC produced increasing amounts of NO with increasing numbers of APC (Fig 1d), while VACV-infected PEC were markedly impaired in their ability to secrete NO. NO was not produced in the absence of antigen or either cell type (data not shown), indicating that this is a specific antigen presentation response. These results indicated that both T lymphocyte and macrophage responses were suppressed by VACV.

RsL.11 T Cells are Refractory to VACV

We next sought to determine which cell type was being directly affected by VACV in this antigen presentation system. While we intentionally incubated VACV with APCs (macrophage, splenocytes and dendritic cells) prior to addition of T cells, the RsL.11 T cells could have become infected during co-incubation with the infected APC. To assess the effects of virus infection on IL-2 production by the RsL.11, RsL.11 T cells were incubated in triplicate with VACV for 3 h, washed, and then incubated with a titration of *uninfected* PECs pulsed with antigen, similar to the above-described experiments. As shown in Fig 2a, VACV did not significantly decrease IL-2 production when pre-incubated with T cells. We also assessed the effect of VACV infection on RsL.11 responses to mitogenic and chemical stimulation. RsL.11 T cells were incubated with VACV for 4 h, and then stimulated for 24 to 48 h with Con A, PMA and ionomycin, or uninfected antigen (Ag)-pulsed PECs. VACV did not significantly decrease IL-2 production by RsL.11 T cells in response to any of these stimuli (Fig 2b). Control groups included the stimulants in media with no RsL.11 T cells and did not give readings above background in the IL-2 bioassay. These data suggest that RsL.11 IL-2 synthesis and secretion is not affected by VACV.

To determine whether RsL.11 T cells could be affected by VACV, we also measured cellular proliferation after incubation with virus. RsL.11 T cells were incubated with VACV for 10 h, and then MTS/PMS was added to measure metabolism. VACV did not significantly affect the RsL.11 T cell metabolism up to 64 h post infection (hpi) (Fig 2c). These data suggest that RsL.11 T cells are completely refractory to VACV. We therefore incubated RsL.11 T cells with infectious virus and compared progeny virus production to a known permissive cell line, BS-C-1. After 72 h, a 100-fold increase in virus was measurable in infected BS-C-1 cells, but no increase in virus could be detected in cells or supernatants of RsL.11 T cells, indicating that they are non-permissive for VACV infection (data not shown). Thus, there is no evidence that these cells even become infected under these conditions.

CTLLs and VACV Infection

As CTLL cells were used to measure IL-2 in supernatants, it was also possible that VACV might be affecting the ability of the CTLL to proliferate in response to IL-2. To assess virus effects on CTLL**,** we incubated the CTLL with VACV for 5 h and then measured proliferation in response to IL-2-containing supernatants (155). The addition of IL-2 to the media increased the metabolism/proliferation of the uninfected CTLLs over the proliferation of CTLLs incubated with no IL-2 (Fig 2d), and VACV did not significantly reduce this response. As the amount of virus used in this experiment was four times higher than levels contained in supernatants from antigen presentation assays, it is unlikely that VACV affected CTLL responses to IL-2 in the antigen presentation assay. Together these data suggest that VACV acts directly on the APCs, not the T cells used in these assays.

Antigen Concentration

As the T lymphocytes in these assays were minimally affected by VACV infection, we theorized that the APCs were being affected by VACV and that increasing antigen concentrations might overcome the VACV-induced decrease in antigen presentation capacity. In infected PECs presenting antigen to RsL.11 T cells (similar to experiments depicted in Fig 1), a titration of GPMBP antigen (50-250 nM) showed that higher antigen concentrations augmented the weak antigen-presenting capacity of infected APCs (Fig 3). In contrast, increasing antigen in this concentration range did not significantly enhance the efficiency of uninfected APCs to stimulate IL-2 production.

B Cell Antigen Presentation

We next determined if VACV affects antigen presentation by a murine B cell line. The mouse 1153 B cell line presents the HEL_{74-88} peptide to the CD4+ B04 T cell line (116). 1153 B cells were infected for 6 h, incubated with varying concentrations of the HEL_{74-88} peptide, and then incubated with the cognate B04 T cell line. The presence of VACV significantly suppressed B cell antigen presentation (IL-2 production, Fig 4a) (Student's t test $p<0.01$). To assess viral effects on the B04 T cells, T cells were incubated with virus for 24 h, at which time MTS/PMS was added in order to measure metabolism. VACV did not affect the proliferation/metabolism of the B04 T cells up to 70 hpi (Fig 4b), suggesting that the T cells were also not directly affected by VACV in this antigen presentation system. These data indicate that VACV blocks antigen presentation of multiple antigens presented by various APCs from both rats and mice.

VACV Inhibits NO Production from PEC and RAW Macrophages

In order to study VACV regulation of isolated APCs, we used LPS and IFNγ to stimulate PECs and the RAW 264.7 murine macrophage cell line (157). PECs and RAW cells were infected for 4 h, stimulated with LPS, IFNγ, or both, and supernatants were assayed for NO. All stimuli induced NO production in uninfected PECs and RAW cells; however, this induction was significantly (Student's t test $p<0.05$), and in some cases completely, blocked by VACV-infection (Fig 5). These data suggest that macrophages are highly sensitive to VACV infection.

VACV Infection Globally Alters Cytokine Responses in Antigen Presentation

As the result of antigen presentation, both the APC and the responding T cell produce numerous cytokines initiating the specific immune response. As VACV inhibited the amount of both IL-2 and NO produced in this rat model of antigen presentation, we assessed the effect of VACV on synthesis of other cytokines and chemokines. Using a multiplex bead-based assay, we determined that VACV reproducibly and significantly ($p<0.05$) inhibited the production of MIP-1α, IL-1β, IL-1α, IL-2, IFNα, GRO/KC, RANTES, TNFα, and MCP-1 (Fig. 6) in response to PEC antigen presentation to RsL.11 T cells. These data confirm the IL-2 bioassay results in Fig 1. In addition, VACV decreased the secretion of IL-6 and GM-CSF; however the reduction was not always significant. Interestingly, VACV infection did not inhibit IL-18 production.

VACV Decreases MHC Class II Expression

Because VACV infection decreased MHC class II-mediated antigen presentation, we assessed the effects of VACV on the expression of MHC II molecules on the surface of PECs. PECs were infected for 4 h, and MHC II expression was measured by flow cytometry. In three experiments, VACV mildly decreased MHC class II surface expression. For example, compared to uninfected PECs, VACV decreased the percentage of high-expressing MHC II-positive cells

from 47% to 37% (Fig 7; mean fluorescence intensity, MFI, 26 +/- 0.3 versus 19.7 +/- 1.8). We also assessed the ability of VACV to inhibit MHC II expression in 1153 B cells. 1153 B cells were infected and MHC II expression was measured as above. In uninfected 1153 B cells, 38% expressed high levels of MHC II, while only 15% of VACV-infected B cells were class II bright (Fig 7; MFI 144 +/- 13.4 versus 76 +/- 2.8). These data indicated that VACV decreases MHC II expression on APCs soon after infection. In the same experiments, VACV infection did not decrease CD45RO expression, indicating that the reduction observed was specific and not just a consequence of general membrane protein perturbation caused by viral infection.

VACV Induces Apoptosis in PEC and RAW Macrophages

Because VACV infection blocked PEC responses, we assessed the effects of VACV on the viability of the PECs. PECs were infected with VACV in triplicate, and metabolism was measured through the reduction of MTS/PMS. Differences in metabolism between the groups became apparent 24 hours post infection (hpi) (Fig 8a), suggesting that the virus is killing cells. However, VACV had only a minor effect on 1153 B cells (Fig 8b), even at 100 hpi. We therefore measured the ability of VACV to induce apoptosis in these cells using PI and annexin V. It was previously reported that VACV induces apoptosis of the murine macrophage J774 cell line (80). VACV infection increased early apoptotic cell percentages in PECs from 16% to 42% and in the RAW 264.7 macrophage line from 14% to 53% (Fig 9) at 4 hpi. VACV induced apoptosis similarly in rat bone marrow-derived dendritic cells and mouse splenic dendritic cells (data not shown). However, using similar assays we could not detect VACV induction of apoptosis in three experiments in the 1153 B cell line (Fig 9) from 6 to 24 hpi, even though VACV also suppressed antigen presentation by this line.

Viral Replication

Induction of apoptosis in virally infected cells is one host defense against virus replication, and VACV has been reported to induce apoptosis in macrophages and dendritic cells (22, 80). We therefore determined the ability of VACV to replicate in PECs compared to BS-C-1 cells, which are permissive for viral replication. Figure 10 shows that VACV-WR was able to increase infectious virus particles 2-logs by 30 hpi in BS-C-1 cells, but no replication was apparent in PECs at 10, 20, or 30 hpi. Thus VACV may induce apoptosis in professional APCs and block their function, but the virus also sacrifices the ability to replicate in the cells.

Peptide Association with MHC Class II

One level of immune regulation, both in homeostasis and infection, lies in controlling the association of peptides with the MHC molecules that present them (82). As VACV inhibits antigen presentation, but only modestly affects class II MHC expression, we tested the effect of VACV on peptide loading in the MHC class II employing the Y-Ae antibody. The Y-Ae antibody specifically detects a complex of peptide 52-68 from I-E^d MHC class II bound in the cleft of MHC class II I-A^b (84, 173). MHC class II molecules frequently present self peptides from surface proteins that recirculate through endosomes and are degraded, similarly to exogenous antigens that are internalized. The Y-Ae antibody recognizes a major determinant (approximately 12% of MHC molecules) in mice expressing both of these class II molecules (173, 200). We measured the amount of peptide bound in the cleft of MHC class II molecules on the surface of uninfected and VACV-infected spleen cells. As Fig 11 shows, 63% of uninfected cells expressed MHC class II molecules complexed with this I-E peptide, while only 30% of VACV-infected cells were positive 3 hpi (MFI 78.2 +/- 9.6 for uninfected cells compared with 38 +/- 2.8 for VACV-infected cells). In contrast, surface MHC class II I-A^b levels were only

modestly reduced (Fig 11). These data indicate that VACV interferes intracellularly with the expression of surface proteins required for antigen presentation and specifically blocks expression of peptide-loaded MHC class II.

Discussion

We have shown here that VACV blocks class II MHC antigen presentation by primary rodent cells, including unfractionated splenocytes, PECs, dendritic cells, and by a B cell line. This likely represents an evolved immune evasion mechanism that is advantageous to the virus even though the virus sacrifices the ability to replicate progeny virions in APCs. Responses to antigen presentation of both CD4+ T lymphocytes and the APCs are inhibited (Fig 1) and this has the overall effect of limiting downstream cytokine and chemokine production (Fig 6) and presumably multiple aspects of the anti viral immune response. Notably, many of the cytokines that are regulated by VACV are chemotactic factors (MIP-1α, IL-1, TNF-α, GRO/KC, RANTES, and MCP-1), which aid in the migration of immune response cells to sites of inflammation and infection.

We have found the T lymphocytes used here to be refractory to virus effects, with VACV directly affecting the APCs. It has been reported in humans that VACV preferentially infects subsets of CD14+ cells, B cells to a lesser extent, and only *activated* primary T cells (30). Our results are similar to work recently published showing that VACV decreases MHC class II antigen presentation in B cell, fibroblast, and macrophage cell lines, as well as primary immature dendritic cells (116). Antigen presenting capacity could be partly restored in our studies by increasing the amount of antigen, suggesting that some process of antigen uptake or processing or presentation is blocked. Data from the Blum laboratory indicate that intracellular antigen is not the limiting factor (116). We found that VACV alters class II MHC expression in APCs

within a few hours after infection. This may simply be a consequence of apoptosis induction as we and others have shown that VACV induces apoptosis in human and rodent macrophages and dendritic cells (22, 80). Other groups have shown a decrease in CD86 and MHC class II on human dendritic cells correlating with apoptosis induction and reduced ability to present antigen (62, 95). VACV modulation of class II MHC has been controversial. Other groups have shown VACV-induced *increases* in MHC class II, but with inhibition of maturation-induced MHC class II, class I, CD86, and CD80 (86). Some differences may be attributable to the type of preparation of virus used to infect APCs, since the 'crude' virus preparation normally used for infections is a cell lysate, which can act as an inflammatory stimulus. Another group recently reported that VACV decreased MHC II-restricted antigen presentation by specifically inhibiting the expression of surface MHC class II on splenic dendritic cells isolated from VACV-infected mice (227). We did not measure the amount or stability of MHC class II proteins in our studies because other laboratories have shown that the amount of MHC protein is not directly related to its functional surface expression. For example, peptide-MHC complexes can be inhibited with no decrease in the amount of MHC class II proteins in cells (116), and MHC class II mRNA levels can be decreased with no decrease in MHC class II surface expression (227). In summary, there is growing evidence to indicate that VACV modulates MHC class II-restricted antigen presentation by specifically affecting functions of the APC.

Interestingly, the inhibition of antigen presentation appears distinct for professional APCs and the 1153 B cell line. Class II MHC expression was reduced in this B cell line, but, unlike other APCs, apoptosis was not detected. This suggests that VACV may decrease class II even in the absence of apoptosis induction. Importantly, we have shown that VACV strongly reduced the amount of peptide in the cleft of MHC class II on infected primary APCs by flow cytometry.

These data are supported by a study in a human B cell line where VACV infection decreased peptide association with MHC class II measured by western blot (116). As this effect is demonstrable with different antigens in different cells, it is likely that this is a key mechanism used by VACV to suppress immune responses. How this occurs is currently under investigation.

We do not believe that VACV simply reduces antigen presentation because it kills infected cells. First, we show in Fig 3 that increasing antigen concentrations restore antigen-presenting capacity in VACV-infected cells, suggesting that the cells are not dead, but rather disabled in terms of antigen presentation. In Fig 8, we show that differences in the metabolism of APCs are not seen until almost 24 hpi, but that VACV reduces the surface expression of MHC class II and the amount of peptide in its cleft by 5 hpi, and the antigen presentation-induced T-cell responses have been diminished by 15 hpi of APC. In addition, VACV does not induce apoptosis in 1153 B cells, but still blocks their antigen-presenting capacity and MHC class II expression (Figs 4 and 7). Together, these data suggest a specific VACV-induced inhibition of antigen presentation.

We studied antigen presentation in rodent cells. As most extant poxviruses have evolved a broad host range, the immunomodulatory mechanisms appear to be conserved and functional in a number of host species. For example, rats are a natural host for cowpox virus and can transmit it to primates, as demonstrated by a recent outbreak in a primate colony in Europe with widespread fatalities (129, 132). Most VACV APC work has been undertaken in the mouse model; our results show that VACV blocks antigen presentation and T cell priming in a rodent known to transmit orthopoxviruses to primates.

VACV caused an apparent broad decrease in post-antigen presentation cytokine production. The notable exception to VACV inhibition was Il-18. IL-18 is stored intracellularly as pro-IL-18 and rapidly released upon stimulation (69). As IL-18 is an important factor in the induction of anti-viral IFNγ production, its storage for rapid release may be an evolved host mechanism to contravene immunomodulation by viral gene products. The importance of this cytokine in antiviral defense is underscored by the possession of an IL-18 binding protein encoded by poxviruses (159). VACV produces several soluble cytokine binding proteins that may interfere with detection of these cytokines if, and only if, they block binding of the detection antibody. However, because there is a general decrease in cytokine production it seems unlikely that the decrease would be caused by VACV-specific binding proteins.

VACV rapidly decreases antigen presenting capacity, alters expression of class II MHC and induces apoptosis in multiple APC types, including peritoneal macrophages responding to inflammatory stimuli. These data expand our knowledge of viral interference mechanisms and suppression of immunity that contribute to poxvirus pathogenesis, however, the viral gene products involved in this inhibition and the intracellular mechanisms remain to be elucidated. An increased understanding of poxviral pathogenesis will aid in the development of antiviral drugs and novel tailored vaccine strains, which exclude immunosuppressive poxviral genes, but which retain and efficiently express the protective antigenic target epitopes that stimulate the immune system.

Figures

Figure 1: VACV infection of primary APC inhibits IL-2 and NO production. Freshly isolated PEC (a), splenocytes (b), or dendritic cells (c) were isolated from a rat, infected with VACV WR (MOI=2) for 5 h, pulsed for 30 minutes with 50 nM GPMBP, and then incubated with the cognate CD4+ RsL.11 T cell line. Uninfected PECs served as a control. At 15 to 48 hpi, supernatants (50 μl) were collected and assayed for either IL-2 using the IL-2 dependent cell line CTLL (a-c) or for NO using Greiss reagent (d). For the CTLL bioassay, media only was used to define the background control level and known IL-2 containing supernatants were used as a positive control. Proliferation was read as absorbance at 492 nm. IL-2 production was determined as the mean optical density (OD) value of the experimental group minus the background control level. * $p<0.05$

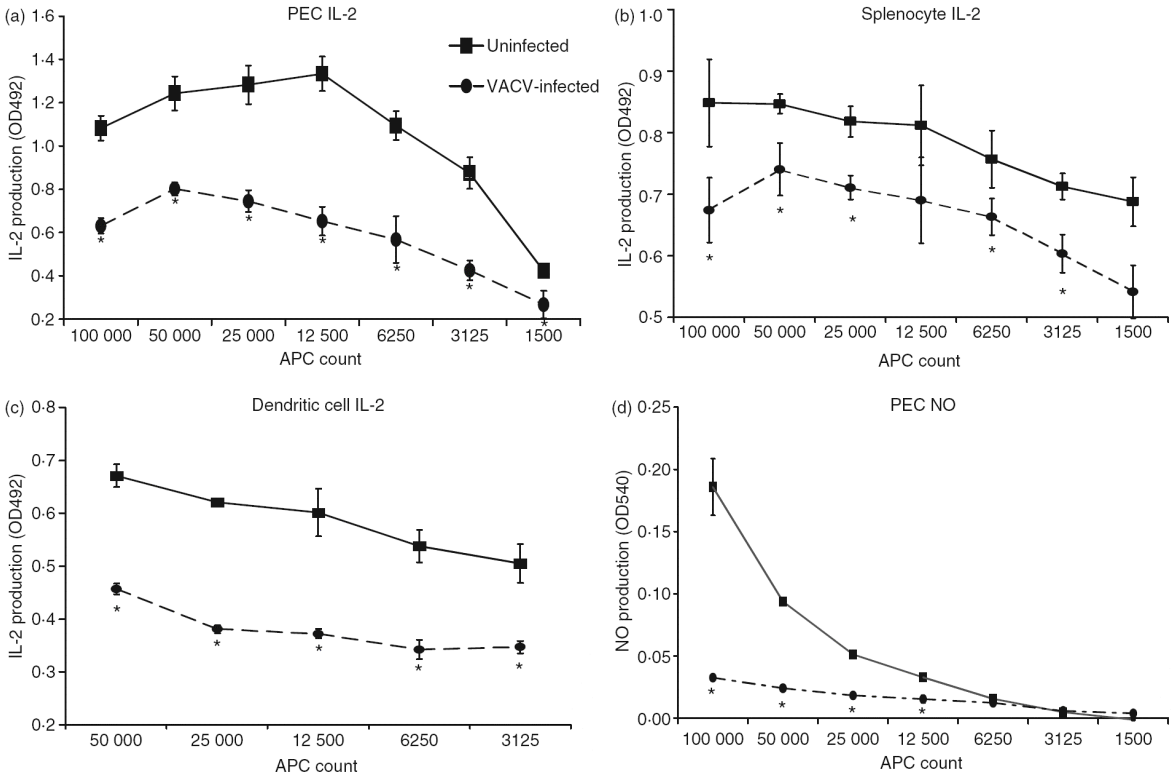

(a)
PEC IL-2
Uninfected
VACV-infected
IL-2 production (OD492)
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
100 000
50 000
25 000
12 500
6250
3125
1500
APC count
(b)
Splenocyte IL-2
IL-2 production (OD492)
1·0
0·9
0·8
0·7
0·6
0·5
100 000
50 000
25 000
12 500
6250
3125
1500
APC count
(c)
Dendritic cell IL-2
IL-2 production (OD492)
0·8
0·7
0·6
0·5
0·4
0·3
0·2
50 000
25 000
12 500
6250
3125
APC count
(d)
PEC NO
NO production (OD540)
0·25
0·20
0·15
0·10
0·05
0·00
100 000
50 000
25 000
12 500
6250
3125
1500
APC count

Figure 2: VACV does not directly affect the RsL.11 or CTLL T cells. (a) RsL.11 T cells were infected with VACV (MOI= 4) for 4 h and then incubated with decreasing numbers of antigen-pulsed PECs. After 24-48 h, supernatants (50 μl) were collected and assayed for IL-2 production using the CTLL IL-2 bioassay. (b) RsL.11 T lymphocytes were uninfected or infected for 3-4 h and then stimulated with PECs/50 nM GPMBP, 25 μg/ml Con A, or 100 nM PMA/2 μM ionomycin. Supernatants (50 μl) were collected at 20- and 40-h and assayed for IL-2. (c) RsL.11 T cells were infected with VACV for 10 h, and growth metabolism was measured by reduction of 10 μl MTS/PMS and absorbance was read at 492 nm. (d) CTLL cells were infected with VACV (MOI=5) for 4 h and then placed in media with varying amounts of an IL-2-containing supernatant. Proliferation was measured by adding 10 μl MTS/PMS.

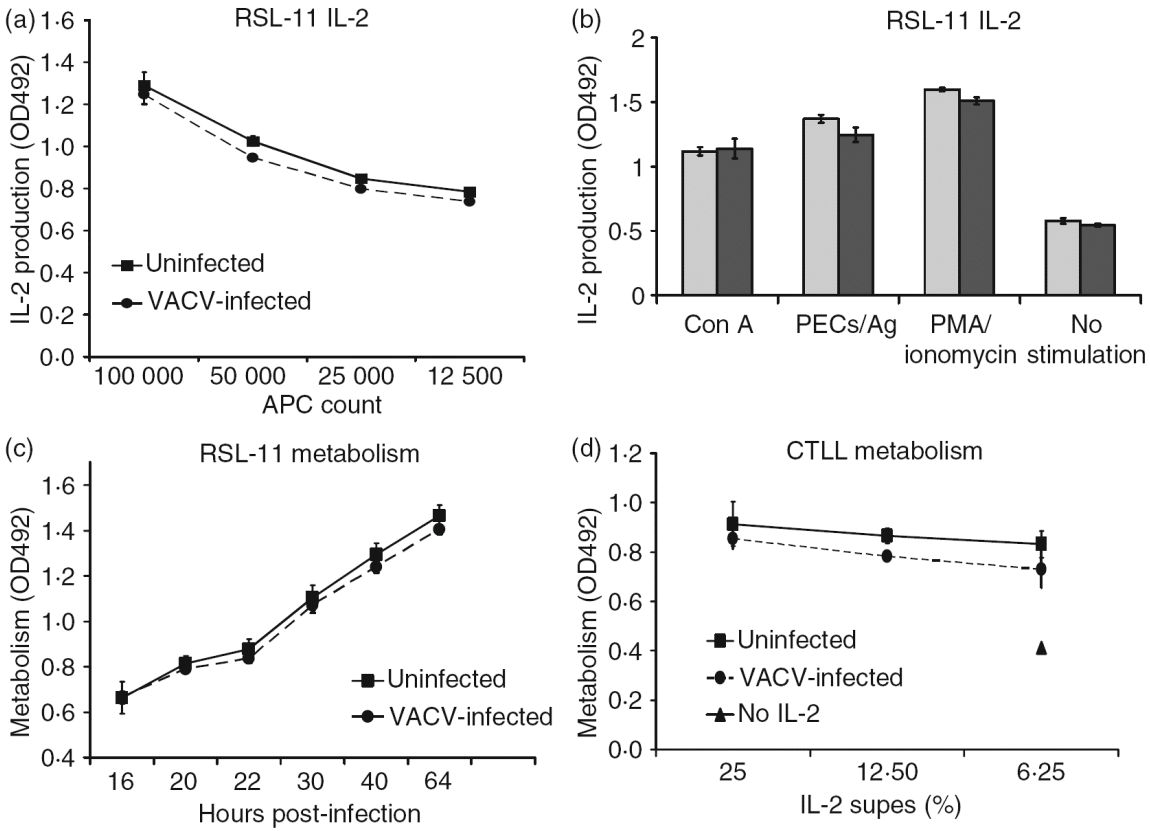

(a)
RSL-11 IL-2
IL-2 production (OD492)
1·6
1·4
1·2
1·0
0·8
0·6
0·4
0·2
0·0
Uninfected
VACV-infected
100 000
50 000
25 000
12 500
APC count
(b)
RSL-11 IL-2
IL-2 production (OD492)
2
1·5
1
0·5
0
Con A
PECs/Ag
PMA/ ionomycin
No stimulation
(c)
RSL-11 metabolism
Metabolism (OD492)
1·6
1·4
1·2
1·0
0·8
0·6
0·4
Uninfected
VACV-infected
16
20
22
30
40
64
Hours post-infection
(d)
CTLL metabolism
Metabolism (OD492)
1·2
1·0
0·8
0·6
0·4
0·2
0·0
Uninfected
VACV-infected
No IL-2
25
12·50
6·25
IL-2 supes (%)

Figure 3: VACV infection and antigen concentration. PECs were infected with VACV (MOI=2) for 4 h and then pulsed with a titration of GPMBP (50-250 nM) before being cocultured with antigen-specific T cells. Supernatants (50 μl) were collected at 24- to 48-hpi and assayed for IL-2 using the CTLL IL-2 bioassay.

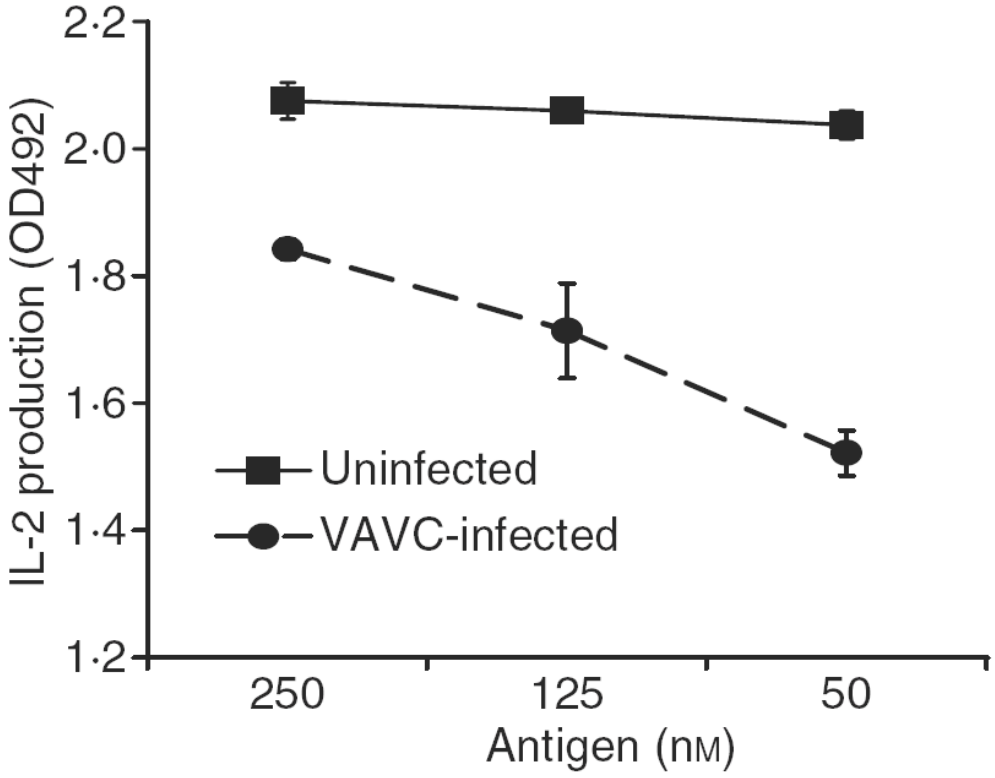
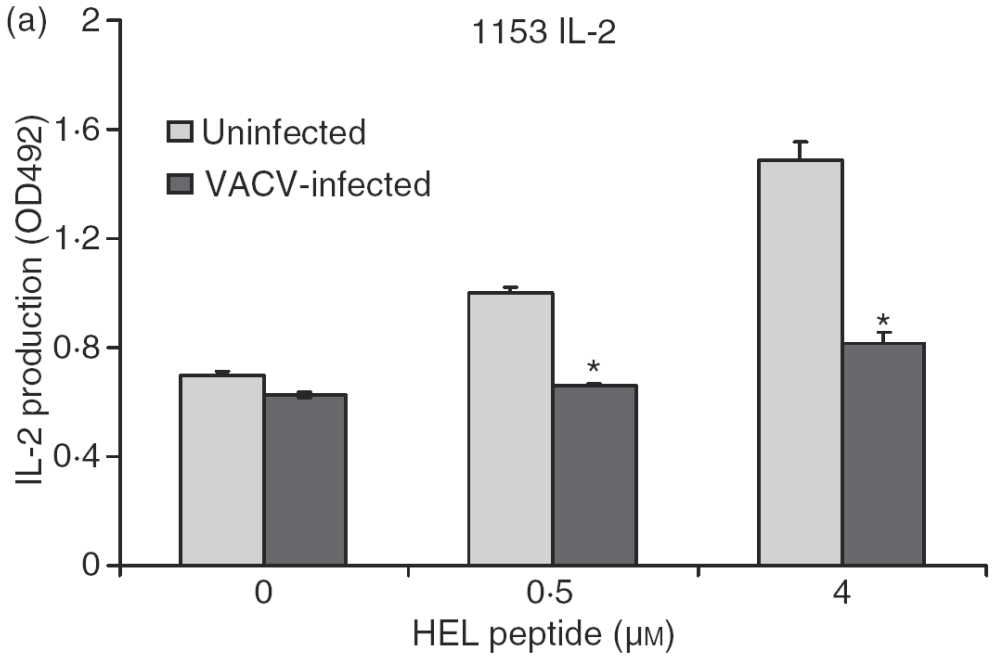

2·2
2·0
1·8
1·6
1·4
1·2
IL-2 production (OD492)
Uninfected
VAVC-infected
250
125
50
Antigen (nM)

Figure 4: VACV infection and B cell antigen presentation. (a) 25,000 1153 B cells were infected for 6 h, and then pulsed with the HEL_{74-88} antigen for 1 h, followed by the addition of 25,000 B04 T cells. After a 24- to 48-h incubation, supernatants (50 μl) were collected and assayed for IL-2 using the CTLL IL-2 bioassay. (b) 25,000 B04 T cells were infected for 24 h. MTS/PMS was then added to measure metabolism. * $p < 0.01$


(a)
1153 IL-2
Uninfected
VACV-infected
IL-2 production (OD492)
2
1·6
1·2
0·8
0·4
0
*
*
0
0·5
4
HEL peptide (μM)

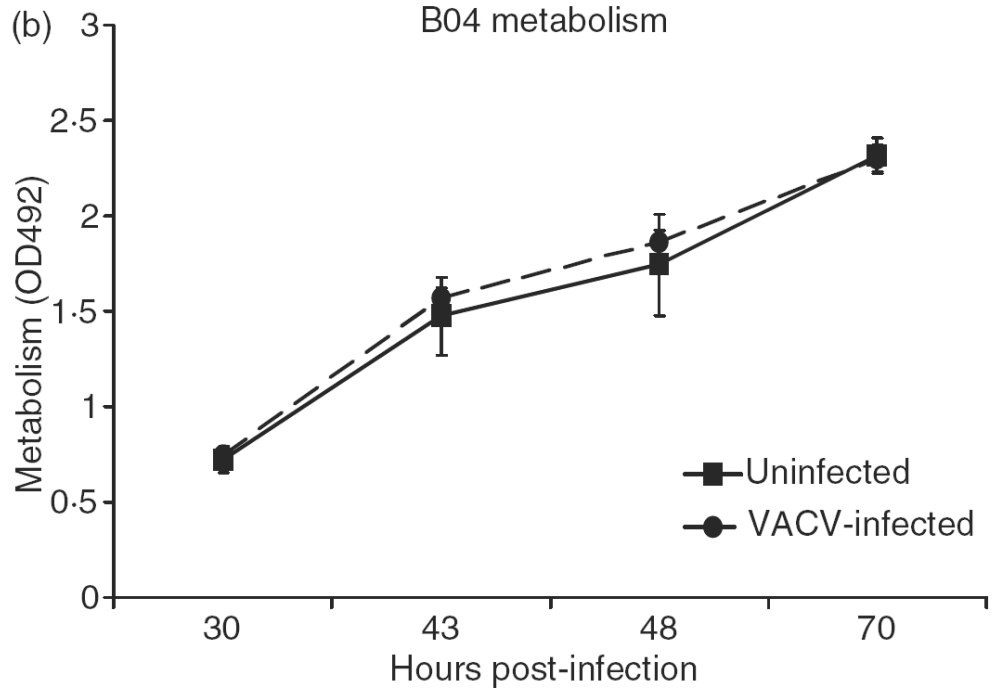

(b)
B04 metabolism
Metabolism (OD492)
3
2·5
2
1·5
1
0·5
0
Uninfected
VACV-infected
30
43
48
70
Hours post-infection

Figure 5: VACV infection and antigen-independent NO production. PEC (a) or RAW 264.7 macrophages (b) were infected at an MOI of 1 for 4 h, and were then stimulated to produce NO by the addition of LPS, IFN-γ, or a combination of both. Supernatants (50 μl) were collected at 24-48 h and then assayed for NO production by the addition of Greiss reagent. Absorbance was read at 540 nm. * $p<0.05$

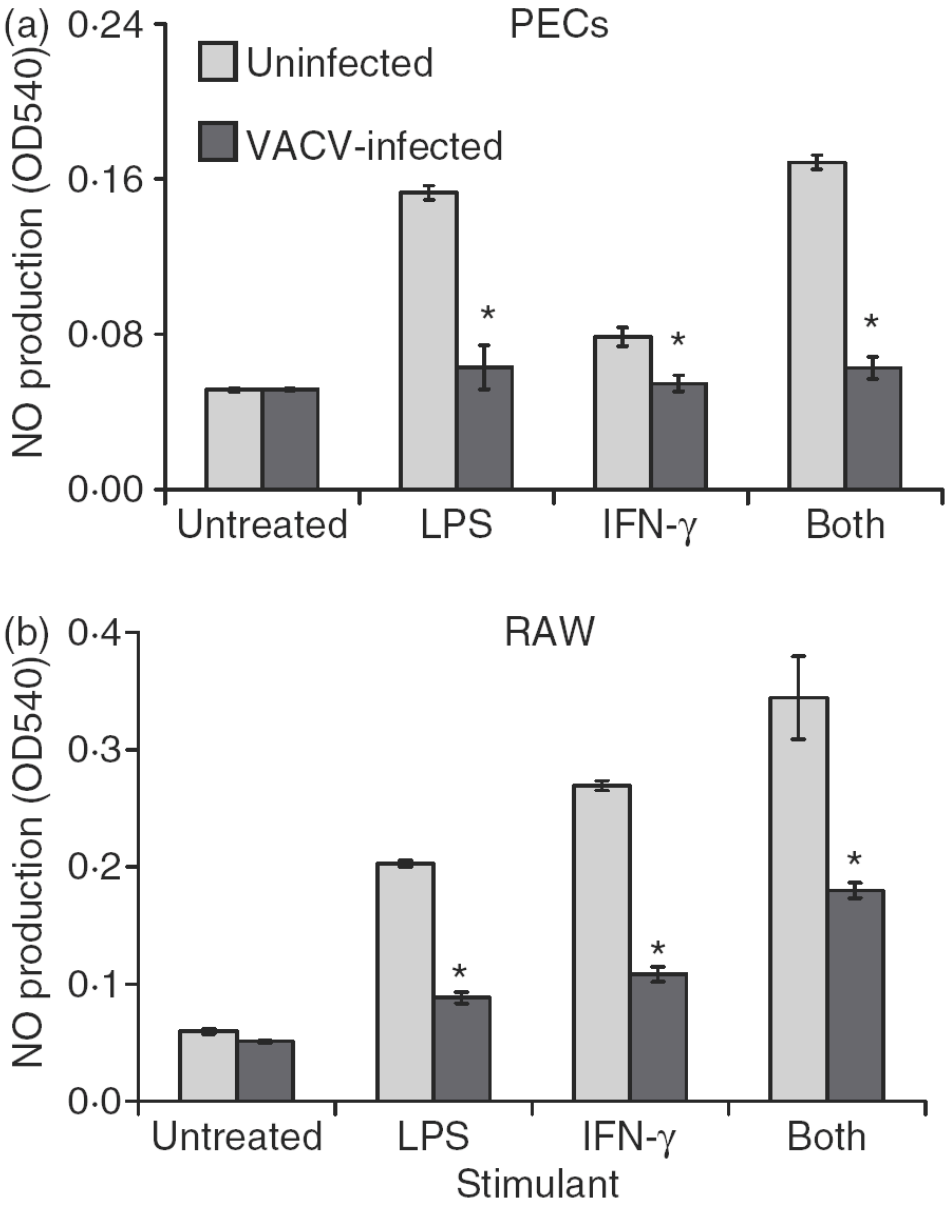

(a)
PECs
Uninfected
VACV-infected
NO production (OD540)
0·24
0·16
0·08
0·00
*
*
*
Untreated
LPS
IFN-γ
Both
(b)
RAW
NO production (OD540)
0·4
0·3
0·2
0·1
0·0
*
*
*
Untreated
LPS
IFN-γ
Both
Stimulant

Figure 6: VACV reduces cytokine synthesis as a result of antigen presention. PECs were harvested from Lewis rats, infected with VACV (MOI=2) for 5 h, pulsed with 50 nM guinea pig myelin basic protein (GPMBP), and then co-cultured with the cognate CD4+ RsL.11 T cell line. Supernatants (50 μl) were collected at 24 hpi and assayed for the production of cytokines using the LincoPlex 24 rat cytokine/chemokine Luminex bead immunoassay kit. Interleukin (IL)-2 and interferon (IFN)-α values were divided by 20 and 100 (hatch marks), respectively, to fit to scale. * $p < 0.05$.

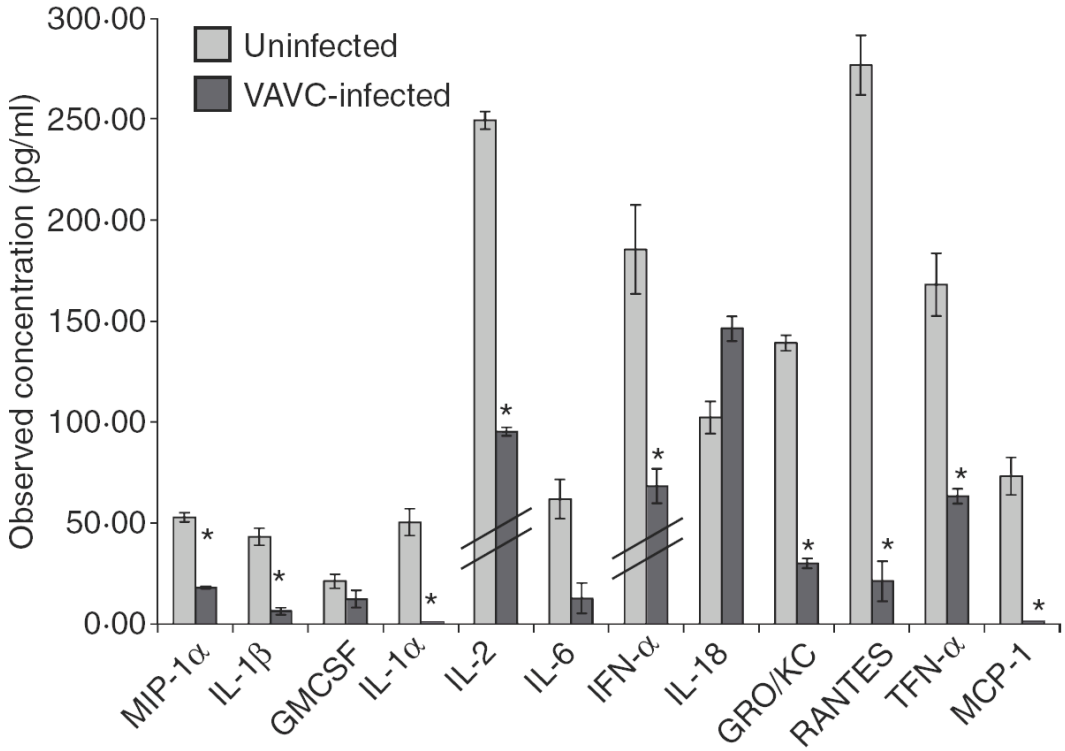

Uninfected
VAVC-infected
Observed concentration (pg/ml)
300·00
250·00
200·00
150·00
100·00
50·00
0·00
MIP-1α
IL-1β
GMCSF
IL-1α
IL-2
IL-6
IFN-α
IL-18
GRO/KC
RANTES
TFN-α
MCP-1

Figure 7: VACV decreases MHC class II expression. PECs or 1153 B cells were infected with VACV for 5 h, washed, and incubated with no primary antibody (MFI for 1153: uninfected cells, 8.4 +/- 0.59; VACV-infected cells, 5.4 +/- 0.44; PEC: uninfected cells 2.35 +/- 0.06; VACV-infected cells 2.38 +/- 0.48), anti-class II MHC (MFI for uninfected cells, 145 +/- 13.4; VACV-infected cells, 76 +/- 2.8), or anti-CD45 and then fluorescein isothiocyanate (FITC)-conjugated secondary antibody and analyzed by flow cytometry.

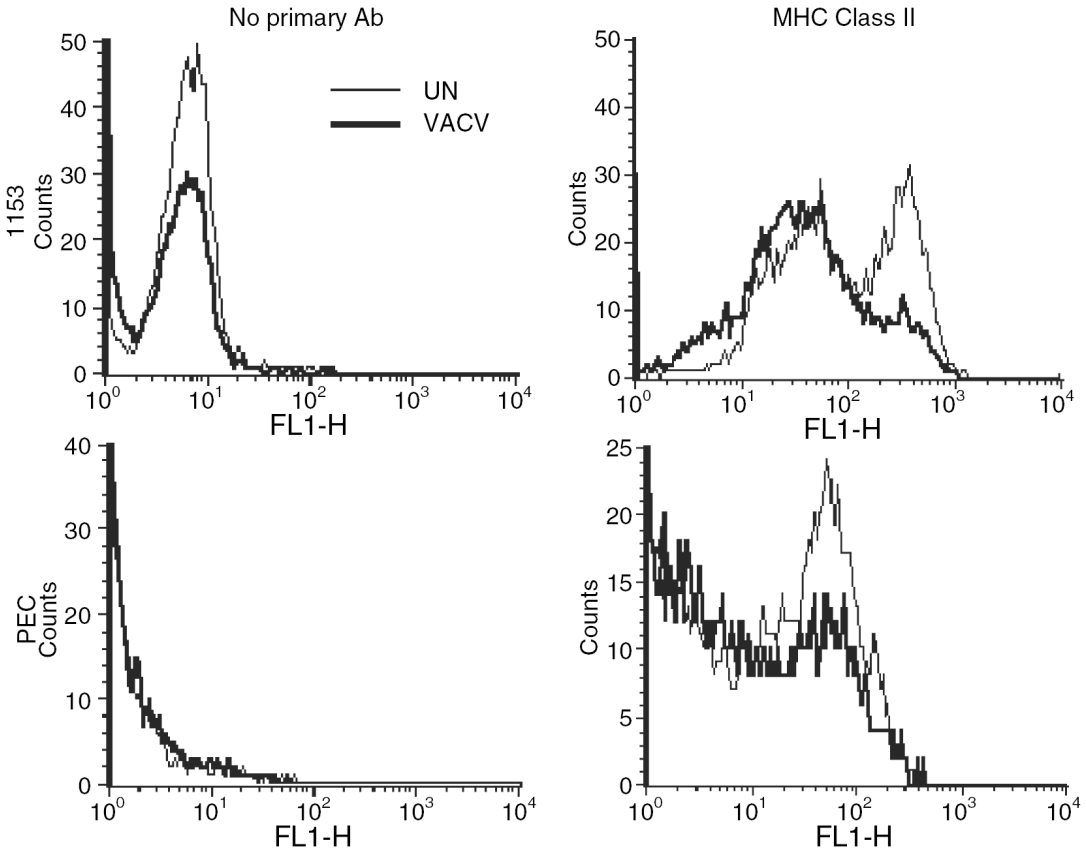
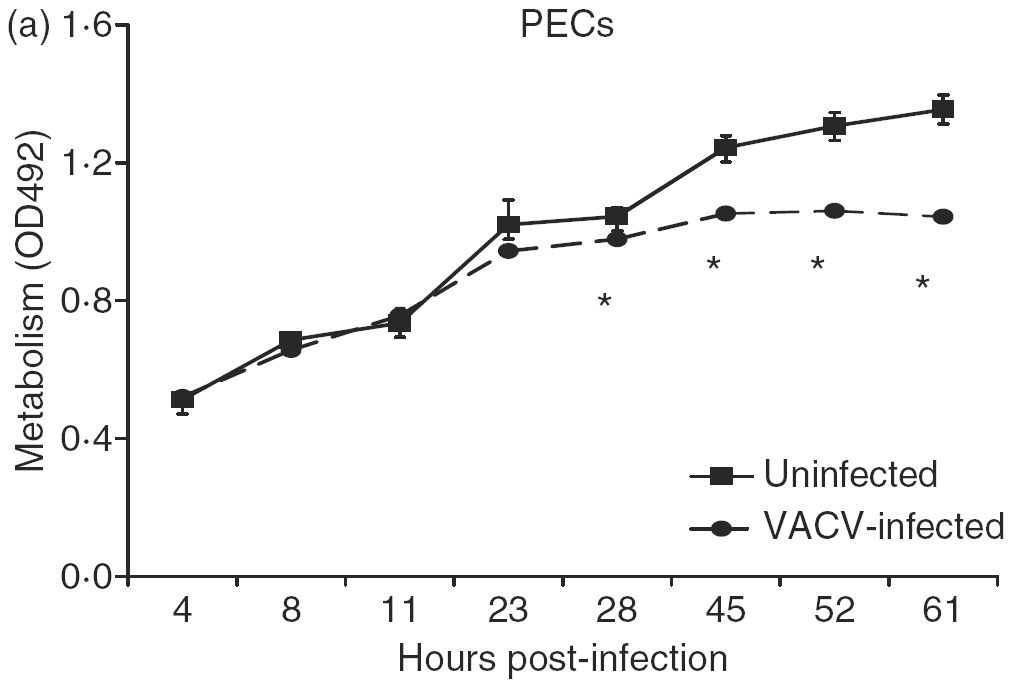

No primary Ab
MHC Class II
UN
VACV
1153
Counts
PEC
Counts
FL1-H

Figure 8: VACV inhibits the metabolism of macrophages. PECs (a) were infected with VACV (MOI=2) and 1153 B cells (b) were infected at an MOI of 5, and metabolism was measured by the reduction of 10 μl of MTS/PMS. Standard deviation bars are shown on all figures but are too small to be visible on some graphs. * $p < 0.05$.


(a) PECs
Metabolism (OD492)
1·6
1·2
0·8
0·4
0·0
4
8
11
23
28
45
52
61
Hours post-infection
Uninfected
VACV-infected
*
*
*

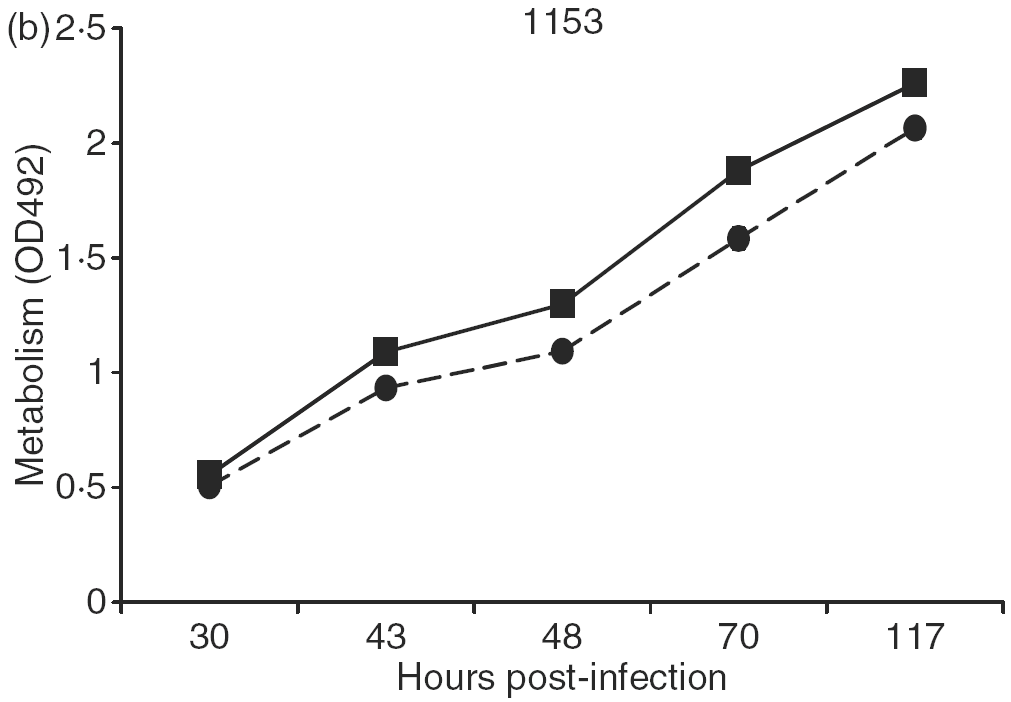

(b) 1153
Metabolism (OD492)
2·5
2
1·5
1
0·5
0
30
43
48
70
117
Hours post-infection

Figure 9: VACV induces apoptosis in macrophages. PECs or RAW 264.7 macrophages were infected with VACV for 4 h (MOI=2). 1153 B cells were infected for at an MOI=5 for 6 h. Apoptosis was assessed by staining cells with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) and analyzed by flow cytometry. Variation was not higher than 2% between duplicate samples.

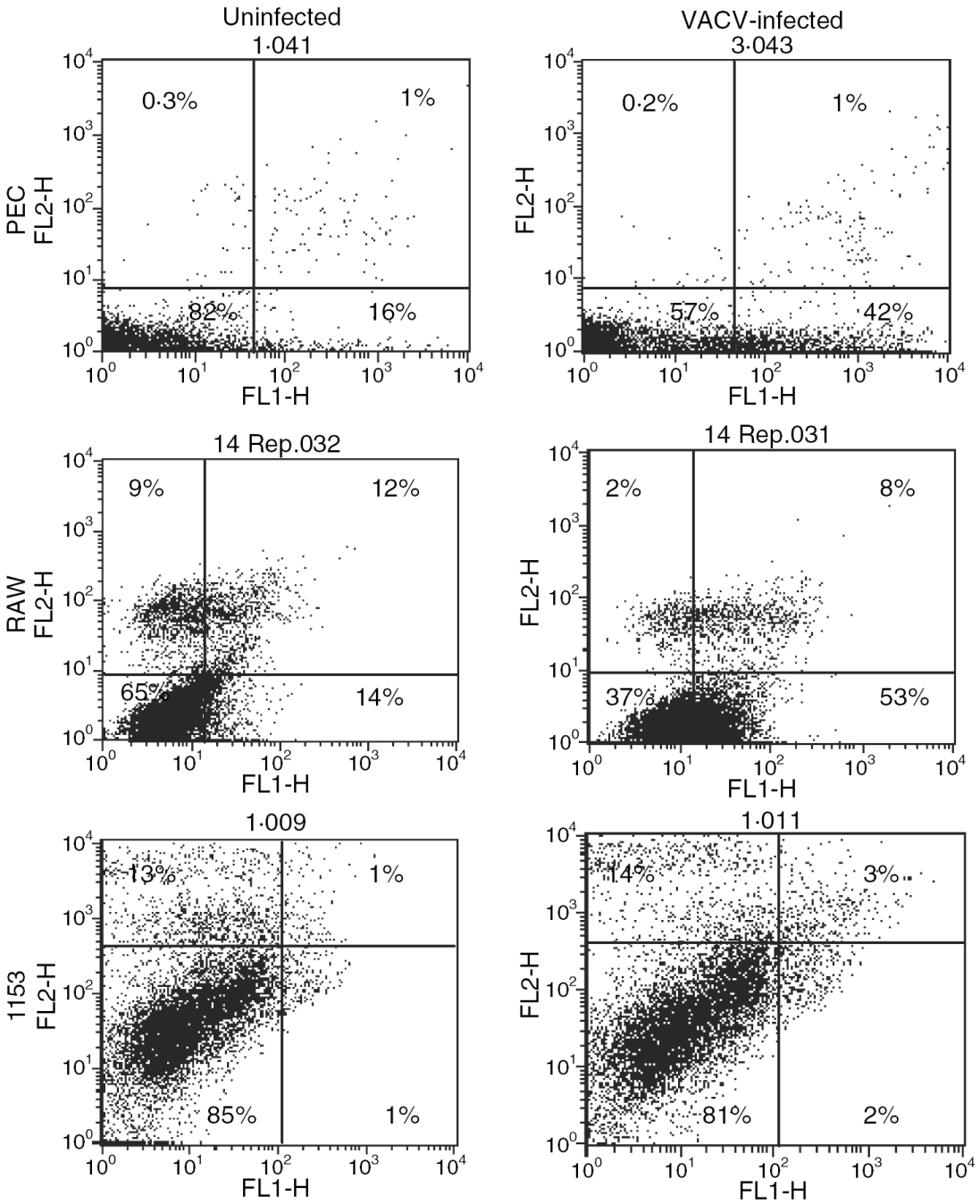

Uninfected
1·041
0·3%
1%
82%
16%
PEC
FL2-H
FL1-H
VACV-infected
3·043
0·2%
1%
57%
42%
FL2-H
FL1-H
14 Rep.032
9%
12%
65%
14%
RAW
FL2-H
FL1-H
14 Rep.031
2%
8%
37%
53%
FL2-H
FL1-H
1·009
13%
1%
85%
1%
1153
FL2-H
FL1-H
1·011
14%
3%
81%
2%
FL2-H
FL1-H

Figure 10: VACV does not replicate to high titers in PECs. BS-C-1 cells or PECs were infected with VACV (MOI=10). Cells were collected at designated times post-infection. Cell-associated virus was titrated on BS-C-1 monolayers. PFU, plaque-forming units.

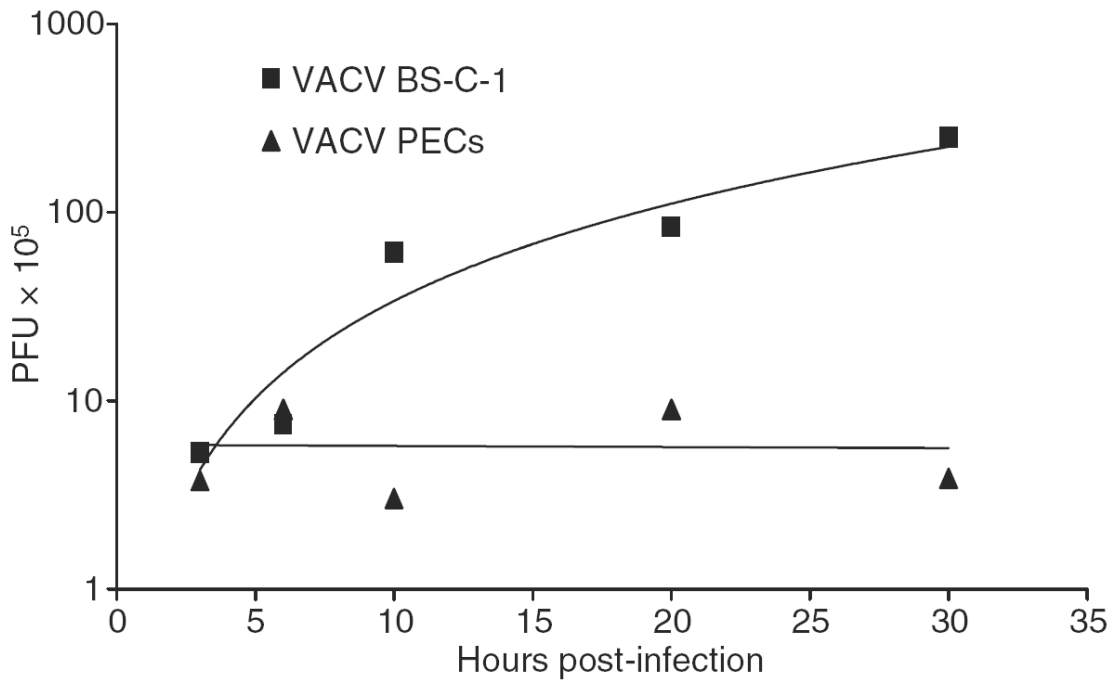
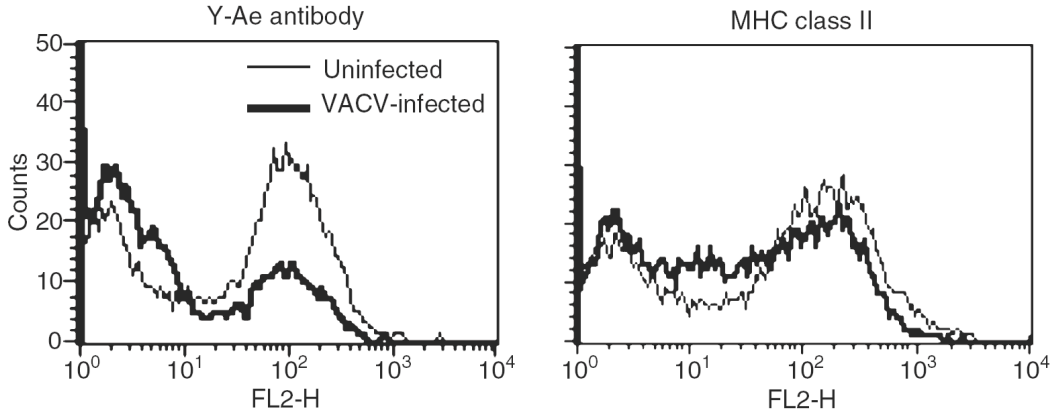

1000
100
10
1
PFU × 10^5
VACV BS-C-1
VACV PECs
0
5
10
15
20
25
30
35
Hours post-infection

Figure 11: VACV decreases peptide-MHC II complexes. Spleens were harvested from B10.A-H2^i5 H2-T18^a/(5R)SgSnJ mice, and uninfected or VACV-infected with purified virus for 3 h (MOI=10), and then incubated with biotin-conjugated Y-Ae and streptavidin-RPE. C57BL/6 mice were used as a negative control and showed no staining above background.


Y-Ae antibody
Uninfected
VACV-infected
Counts
50
40
30
20
10
0
10^0
10^1
10^2
10^3
10^4
FL2-H
MHC class II
10^0
10^1
10^2
10^3
10^4
FL2-H

CHAPTER 3: VACCINIA VIRUS A35R INHIBITS MHC CLASS II ANTIGEN PRESENTATION

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Abstract

The VACV gene A35R (Copenhagen designation) is highly conserved in mammalian-tropic poxviruses and is an important virulence factor, but its function was unknown. We show herein that A35 does not affect viral infectivity, apoptosis induction, or replication; however, we found that A35 significantly inhibited MHC class II-restricted antigen presentation, immune priming of T lymphocytes, and subsequent chemokine and cytokine synthesis. A35 localized to the endosomes and reduced the amount of a model antigenic peptide displayed in the cleft of class II MHC. In addition, A35 decreased VACV-specific T cell responses *in vivo*. Thus, this is the first report identifying a function for the A35 protein in virulence as well as the first report identifying a VACV gene that inhibits peptide antigen presentation.

Introduction

Poxviruses are large, double-stranded DNA viruses (~200 kb) that are capable of infecting a wide variety of animals (141). The most infamous poxvirus is Variola virus, the causative agent of smallpox. Smallpox is estimated to have killed over 500 million people in the 20th century before it was declared eradicated by the World Health Organization in 1980 (124). Smallpox now presents itself as a danger in biowarfare and bioterrorism. Other poxviruses can also cause significant disease in humans. In 2003, a Monkeypox outbreak occurred in the United

States, infecting over 70 people (34). Fortunately, this was an attenuated strain, and there were no human fatalities (34). A widespread monkeypox outbreak would be more difficult to contain than smallpox, due to the fact that monkeypox can replicate and persist in animal reservoirs. Other emerging zoonotic poxviruses include buffalopox in Asia (100), tanapox in Africa, Europe and the United States (52, 198), cowpox in humans and primates (causing widespread fatalities) (129, 132, 197), and Cantagalo in South America (45). Molluscum contagiosum virus is commonly seen in children and AIDS patients, accounts for nearly 300,000 doctor visits each year in the United States (140), and is emerging as a sexually transmitted disease.

The eradication of smallpox was the result of the most successful vaccination program in history. Routine vaccination was stopped in most countries in the early 1970's due to the poor safety record of the vaccine, live VACV. Vaccination is contraindicated for many individuals, including those who have inflammatory skin conditions, such as eczema, and those that are immunocompromised or pregnant (111). In a recent vaccine trial, it was reported that 1 out of every 450 of those vaccinated had a serious adverse vaccine reaction (29), and vaccine trials were stopped after reports of increased adverse cardiac events following vaccination (60, 211). In recent years, attenuated and/or non-replicating VACV strains have been increasingly employed to reduce risks, and many VACV strains are being used as a platform in the development of vaccines for other diseases, such as HIV, malaria, and cancer (56, 177, 203, 217). Thus it is crucial to elucidate the virulence mechanisms in VACV and particularly those that interfere with the development of a robust immune response. An increased understanding of poxvirus pathogenesis will aid both in antiviral drug development and in the design of improved vaccines.

While VACV elicits a strong immune response, it also encodes a plethora of

immunosuppressive genes and many of unknown function (213). Gene 158 of VACV Western Reserve (WR) strain, commonly referred to as the A35 gene by the VACV Copenhagen designation, is highly conserved in mammalian-tropic poxviruses and vaccine strains (170, 213), but has no similarity to non-poxvirus proteins, giving no clues as to its function. A mutant A35 deletion virus (A35Δ) replicated normally in several tissue culture cell lines, but was highly attenuated (100-1000 fold) in the intranasal (i.n.) and intraperitoneal (i.p.) mouse challenge models (164, 170). A rescue virus was constructed that returned the wild type A35 gene to the mutant, and this virus construct restored virulence, confirming that the viral phenotype mapped to the A35 locus. While it is difficult to compare the virulence of various gene deletion mutants because many have not been tested in the intranasal model measuring weight loss (intracranial inoculations or measures of viral titers in organs have often been performed instead), available data in the i.n. model indicate that the A35Δ virus is more attenuated than the A46R deletion mutant (195), similar in virulence to the IL-18 binding protein knock out virus (159), slightly less attenuated than the thymidine kinase (TK) knock out (113), and less attenuated than the E3L deletion, which causes a 1000 fold attenuation (216). The A35Δ virus produced similar quantities of the various morphogenic forms of virus compared to the parental wild type WR virus, with similar kinetics, and virus particles were similarly infectious on a per particle basis (170). We showed that this gene encodes a non-glycosylated, non-secreted 23 kD protein expressed early during the virus life cycle, but its mechanism of action was unknown. Here, we show that VACV A35 gene product blocks immune priming of T lymphocytes by interfering with MHC class II-restricted antigen presentation.

Materials and Methods

Cells and Virus

VACV Western Reserve (WR) strain and A35Δ mutant virus stocks were propagated using BS-C-1 cells as previously described (170). For titrations of VACV, BS-C-1 monolayers were fixed and stained with 0.1% crystal violet in 20% ethanol. All cells were grown in media containing 10% FBS. CD4+ RsL-11 T cell clones were derived and maintained as previously described (127). CTLL-2 (ATCC # TIB-214) were maintained in RPMI supplemented with 0.4% IL-2-containing baculovirus supernatant (127). RAW 264.7 (ATCC # TIB-71), a mouse macrophage cell line, was maintained in DMEM. The mouse B cell line 1153 (kind gift from Janice Blum) was maintained in RPMI (116).

Cell Tropism Replication

The cell type of interest was split into two groups of equal number for infection. One group was infected with WR and the other with A35Δ. The cells were infected for 20 h at MOI=1 followed by three freeze/thaw cycles to release newly formed virus particles. Replication was measured by titering the cell lysates on a monolayer of BS-C-1 cells and counting the plaques that formed 40 h later. For early replication in mouse organs, groups of mice were intranasally challenged with WR (170) and A35Δ at 10^4 pfu/mouse, and nose, lungs, blood, brain and spleen were harvested on day 1, 2 and 3 (6 mice on each day) post challenge. Organs were freeze/thawed three times, homogenized, sonicated, and titered as above.

Peritoneal Macrophage Isolation and Antigen Presentation Assays

Lewis rats (bred and maintained at the AAALAC-certified Brody School of Medicine animal care facility at East Carolina University) were injected i.p. with 200 μg of inactivated *C. parvum* in 5 ml of HBSS. Two to three days later, the rats were sacrificed and peritoneal exudate

cells (PEC) were harvested by washing the peritoneum in cold HBSS. PEC (>95% CD45+ hematopoietic lineage) were infected for 5 h with either WR or A35Δ, and placed into 96-well plates. PEC were incubated with 50 nM guinea pig myelin basic protein (GPMBP) for 30 minutes, followed by addition of 25,000 Lewis rat CD4+ RsL.11 clones specific for GPMBP (127). The antigen presentation plates were incubated for 24-48 h at 37ºC, 5.0% CO_2. After incubation, 50 µl of supernatants were transferred into an empty 96-well plate and frozen for the following assays. All animal experiments were in compliance with NIH guidelines.

CTLL IL-2 Bioassay

IL-2 was measured using previously described methods (127). Briefly, 10,000 CTLL clones were washed, resuspended in RPMI, and added to the collected supernatants. The plates were incubated for 48 h at 37ºC, 5.0% CO2, followed by the addition of 10 µl of MTS/PMS (2.0 mg/mL MTS, Promega; and 0.1 mg/mL PMS, Sigma). The absorbance was read at 492 nm. Media only was used to define the background control level and known IL-2 containing supernatants were used as positive control.

NO Measurement

50 µl of the harvested supernatants were transferred into a separate 96-well plate followed by the addition of 50 µl of Griess Reagent (1% sulfanilamide–0.1% N-[1-naphthy] ethylenediamine in 2.5% phosphoric acid) into each well. The absorbance was read at 540 nm (27).

Cytokine Measurement

50 µl of the harvested supernatants were analyzed using the LincoPlex 24 Rat Cytokine/Chemokine Luminex Bead Immunoassay Kit according to the manufacturer's instructions (Linco Research). The supernatants were incubated with a panel of anti-cytokine

antibodies immobilized on Luminex beads (Bio-Rad Laboratories). The following cytokines were analyzed: IL-1α, IL-1β, IL-2, IL-6, IL-17, IL-18, MIP-1α, GM-CSF, IFN-α, growth regulated oncogene alpha/keratinocyte attractant (GRO/KC), RANTES, TNF- α, MCP-1, eotaxin, G-CSF, IL-4, IL-9, IL-13, IL-5, and IL-10. Reagents for IFNγ were not available at the time. Samples were run according to the manufacturer's instructions (Bio-Rad) and analyzed on the BioPlex protein array reader (Bio-Rad) in the Duke University Human Vaccine Institute Immune Reconstitution Core Facility (Durham, NC).

RsL-11 Stimulation Assay

CD4+ RsL.11 T lymphocytes were washed and resuspended in RPMI. The cells were infected for 3-4 h, plated in a 96-well format, and stimulated with PEC pulsed with 50 nM GPMBP, 25 μg/ml Con A (Sigma), or 100 nM PMA/2 μM ionomycin (Sigma). The plates were incubated at 37^{o} C, 5% CO_2. 50 μl of the harvested supernatants were collected at 20- and 40-h and assayed for IL-2 using the CTLL bioassay already described.

Metabolism/Survival Assays

Cells of interest were infected with VACV (MOI=2). Metabolism was measured by the addition of 10 μl MTS/PMS 4-24 hpi, and analyzed similarly to the method described above for the CTLL IL-2 Bioassay. Absorbance was read at 492 nm at various times post infection. Tetrazolium salts such as MTS are reduced to colored formazan products during cellular respiration. As only live cells respire, the MTS assay can be used to measure cell survival.

Flow Cytometry

Cells were infected for 4 hours and then washed in cold PBS containing 1% heat-inactivated FBS and 0.1% sodium azide. 3 x 10^5 cells were stained with an anti-MHC II concentrated supernatant (Y3P, AF6120, and MKS4), OX1 anti-CD45, or human anti VACV

(Cangene VIG) for 1 h on ice, washed once, and then incubated with a FITC-conjugated goat anti-mouse IgG (Southern Biotech) or anti human-phycoerythrin (PE). For measurement of apoptosis, cells were infected and then stained with annexinV-FITC/propidium iodide (PI) (BD Pharmingen) per the manufacturer's instructions. Expression was measured using a FACScan (Becton Dickinson) and analyzed with Cell Quest software.

Peptide-MHC Association

The Y-Ae antibody specifically detects a complex of peptide 52-68 from I-E^d MHC class II bound in the cleft of MHC class II I-A^b (84, 173). This complex is formed in mice that express both alleles. Spleens were harvested from B10.A-H2^i5 H2-T18^a/(5R)SgSnJ mice (Jackson Labs); C57BL/6 mice were used as a negative control. Unfractionated splenocytes were infected with sucrose gradient purified virus for 3 h (MOI=10) and then incubated with biotin-conjugated Y-Ae (eBioscience). Cells were washed and incubated with streptavidin-RPE (Southern Biotech). Goat polyclonal IgG antibody to CLIP (P-15, Santa Cruz) and donkey anti-goat IgG FITC (Jackson Labs) were used to measure CLIP peptide bound in MHC class II on the surface of cells. Samples were analyzed using a FACScan equipped with Cell Quest software.

Immunofluorescence Microscopy

PEC were harvested as described above and grown on coverslips for 4 h. The cells were then infected at an MOI=10 for 2 h, fixed and permeabilized in 3% paraformadehyde and 0.1% Triton X-100, respectively, and then stained with a rabbit anti-A35 antibody 1:4000 (170) and one of the following polyclonal goat anti-endosome antibodies (Santa Cruz Biotechnology) at 1:80 diluted in 0.1% Triton X-100/PBS: Lamp-1 (C-20), Rab 7 (A-16), Rab 5B (C-12), or RhoB (M-19). The coverslips were then washed and incubated with Alexa Fluor 633 (Invitrogen) and a FITC-conjugated anti-rabbit IgG (Sigma) for 45 m, followed by a final round of washes. The

cells were visualized with a Zeiss confocal microscope and pictures were taken with the 100x objective.

One-Step Growth Curve

A one-step growth curve was performed as previously described (170).

IFNγ Elispot

To enumerate cells secreting IFNγ, 96-well ELISA plates (Immulon H2B Thermo Electron) were coated overnight with rat anti-mouse IFNγ (1 mg/ml Pharmingen). Plates were washed with blocking buffer (PBS 1% FBS) before adding murine splenocytes in RPMI 1640. Stimulation of splenocytes was achieved by the addition of virus (MOI=2) followed by incubation for 40 h at 37°C. Plates were then washed and incubated with 0.4 μl biotinylated rat anti-mouse IFNγ (Pharmingen 0.5 mg/ml). Plates were washed and incubated with streptavidin AP for 1 hr at 37°C. Plates were developed with agarose/BCIP/AMP buffer mixture. Spots were counted using a dissection microscope.

Statistical Analysis

Experiments were repeated at least three times and representative data are shown. A two-tailed Student's t test was used to compare groups. p values < 0.05 were considered significant.

Results

A35 Effects on Replication

We have shown previously that the A35Δ virus is attenuated in a mouse challenge model, but that the mutant virus replicated and spread similar to the WR parental strain in eight cell lines (RK-13, HeLa, CV-1, BS-C-1, A549, MRC-5, TK-, BHK-21) derived from human, rabbit, monkey and hamster (170). Still it was possible that the A35Δ was attenuated *in vivo* because the A35 gene was required for replication in certain cell types or tissues, such as immune cells,

neural tissue (since the VACV-WR strain is neurovirulent) (186), or heart because VACV causes cardiac inflammation (7, 60). Various cell preparations listed below (or BS-C-1 cells as control permissive cells) (170) were infected with WR or A35Δ mutant at an MOI of 1 for 1 day, and then virus titers were measured. The presence of the A35 gene in WR did not reproducibly enhance viral replication more than 2 fold relative to the A35Δ in any of the cells or tissues tested, including: Lewis Rat Peritoneal Exudate Cells (PEC), splenocytes from a young and old rat, rat thymocytes, activated and resting RSL-11 T-cells, R1-T T-cells, rat alveolar macrophage line NR8383, mouse CTLL, PC-12 neuronal cells (NGF-differentiated and undifferentiated morphologies), Heldtilt Mouse primary brain and heart cell suspensions, human fetal fibroblasts, and HEK (human kidney line) cells (data not shown). We also assessed the effects of A35 on early replication in tissues of mice i.n. challenged with WR and A35Δ at 10^4 pfu/mouse (170), and found no significant difference in replication in nose, lungs, blood, brain and spleen on days 1, 2 and 3 post challenge. Thus, there was no evidence for A35 involvement in host range or tissue tropism, suggesting that A35 is not required for any steps in replication, morphogenesis, or spread of virus.

A35 Inhibits IL-2 and NO Production

Because the A35 gene was not required for replication but did affect virulence *in vivo*, we suspected that A35 might be involved in regulating aspects of the host immune response. To test this hypothesis, we used a model *in vitro* MHC class II-restricted antigen presentation system (127) and measured responses of both the T lymphocytes and the macrophage APC. Rats are naturally infected with orthopoxviruses (129), and rat peritoneal exudate cells (PEC) are a rich source of normal primary macrophage. PEC were infected in triplicate with WR virus or the A35Δ virus, pulsed with antigen (guinea pig myelin basic protein, GPMBP), and incubated in

varying numbers with a cognate CD4+ T cell line, RsL.11 (127). Supernatants were collected, and IL-2 production was determined as a measure of the T cell response to the antigen presentation. T cells in the uninfected groups were able to secrete more IL-2 than either the T cells in the A35Δ-infected or the WR-infected groups (Fig 12a). The WR-infected PECs caused the T-cells to secrete significantly ($p < 0.05$) less IL-2 than A35Δ-infected cells 15-72 hpi, indicating that A35 down-regulates MHC class II-restricted antigen presentation responses *in vitro*. A similar A35-mediated down-regulation of IL-2 production was observed when resting PECs and unfractionated rat splenocytes were used as APC (data not shown). No IL-2 was produced in the absence of antigen or either cell type indicating that IL-2 production is a specific response to antigen presentation.

Since production of IL-2 is a measure of the T cell response, we also wanted to measure nitric oxide (NO), which is produced by the macrophage and has been shown to be an important anti-viral defense (94, 123). NO production was measured in supernatants collected from the *in vitro* antigen presentation system described above. Upon antigen presentation stimulation, uninfected macrophages produced significantly more NO than infected cells, while WR-infected macrophages produced significantly ($p < 0.05$) less NO than the A35Δ-infected macrophages (Fig 12b). NO was not produced in the absence of antigen or either cell type indicating that this is a specific antigen presentation response. These data indicate that A35 inhibits both the T lymphocyte and APC responses to antigen presentation.

A35 Reduces Cytokine Responses Induced By Antigen Presentation

We next wanted to determine whether A35 specifically reduced the amount of IL-2 and NO produced as the result of antigen presentation (Fig 12), or if the presence of A35 in WR broadly affected cytokine responses. Similar to experiments above, PEC were infected in

triplicate with WR or A35Δ, pulsed with antigen, and added to RsL.11 T cells. Supernatant cytokines were measured using an antibody-bead-based fluorescent assay. Results showed a broad and significant reduction in multiple cytokines and chemokines (Fig 13). The A35Δ virus evoked significantly ($p < 0.05$) higher levels of MIP1α, IL-1β, GMCSF, IL-1α, IL-2 (confirming our previous IL-2 bioassay results), IFN-α, 1L-17, GRO/KC, RANTES, and TNFα, (but not IL-18) than WR, suggesting that the presence of A35 inhibits production of these cytokines and chemokines and generally dampens all antigen presentation-induced responses.

A35 Does Not Affect the RsL.11 or CTLL T Cells Directly

In the antigen presentation experiments described above, PEC were pre-infected prior to addition of antigen or T cells. However, it remained possible that the T cells also became infected during the co-incubation, as some T cells can be infected by VACV (30, 175). To determine if VACV and A35 had any direct effects on the responding T cells, we performed a similar assay but with *infected* T cells and *uninfected* PEC. We determined if VACV and A35 affected the ability of the RsL.11 T cell to produce IL-2. RsL.11 T cells were incubated with either WR or A35Δ for 3 h, washed, and then incubated with a titration of uninfected antigen-pulsed PEC. Supernatants were harvested 24-48 hpi, similar to experiments in Fig 12 and 13, and tested for IL-2. Infection with either virus did not significantly affect the response of the T cell compared to uninfected T cells (Fig 14a). We also found that VACV did not affect RsL.11 responses to mitogen (ConA) or chemical stimulation (PMA/ionomycin) (Fig 14b).

We next wanted to assess whether RsL.11 T cells were permissive to VACV infection. We incubated VACV with the known permissive cell line, BS-C-1, and RsL.11 T cells. Levels of infectious virus increased 2 logs in the BS-C-1 culture but did not increase in the RsL.11 T cell culture (data not shown), and infection had no significant effect on cellular metabolism up to

64 hpi (Fig 14c). Together these data indicate that the RsL.11 T cells are refractory to VACV infection.

To assess viral affects on the CTLL cells used in the IL-2 bioassay, CTLL were incubated with WR or A35Δ for 5 h and then placed in IL-2-containing media to stimulate their proliferation. IL-2 increased the response of the uninfected CTLL compared to media containing no IL-2, and VACV did not significantly alter this response up to 48 hpi (Fig 14d). These data suggest that VACV (with or without the A35 gene) has little effect on either the RsL.11 or CTLL under these assay conditions and that A35 exerts its influence directly on the APC instead. In order to further test this hypothesis, we performed an experiment using a paraformaldehyde fixed infected human B cell line to present antigen in triplicate as previously described (116). WR infected (MOI=10) fixed B cells presented 30% as well as uninfected fixed B cells, and significantly less than the A35Δ infected fixed B cells, which presented 68% of the levels of uninfected fixed B cells. These data mirror what we have seen in the rodent system using unfixed antigen presenting cells where A35 potentiates VACV inhibition of antigen presentation. Together these data indicate that the APC are directly affected by A35 prior to interaction with T cells. Thus we further explored the effects of A35 on APC.

A35Δ is as Infectious as WR Virus

We had previously shown that A35Δ mutant virus particles were equally infectious on a per particle basis as wild type WR parental virus (170) in BS-C-1 cells, however it was possible that the A35Δ virus infected immune cells (PEC) less well than the wild type, thus allowing WR to be more inhibitory. In order to assess viral infectivity in these cells, PECs were incubated with WR and A35Δ for 10 h, and viral antigens were measured by FACS analysis using a human polyclonal anti-VACV hyperimmune serum. In three experiments, the A35Δ showed as much

viral antigen staining as wild type virus (Fig 15a), indicating that the A35Δ is as infectious as wild type virus in these cells and proceeds normally through viral protein expression up to 10 hpi. Uninfected cells stained with anti-VACV hyperimmune serum and VACV infected cells stained with secondary antibody alone gave comparable negative results.

A35Δ Replicates Equal to WR in PECs

We also assessed the replication of both viruses in PEC using a one-step growth curve. We infected PEC and BS-C-1, a known permissive cell line, with WR or A35Δ, and harvested supernatants and cells for titers at various time points. WR and A35Δ were able to replicate similarly in BS-C-1 cells increasing approximately 2 logs by 30 hpi (Fig 15b), while neither virus replicated well in PEC. We also measured virus released into supernatants (not shown), and showed that WR and A35Δ had very similar supernatant titers. These data indicate that A35 does not alter viral replication in PEC.

A35 Does Not Induce Apoptosis of PEC

One possible explanation for the A35 mediated reduction in antigen presentation was that A35 might reduce the viability of the APC since VACV is known to induce apoptosis in many APC types (22, 62, 80, 95). To determine this, PEC were infected with WR or A35Δ, and metabolism/viability was measured. While virus infection reduced metabolism/survival of both cell types by 24 hpi, there was no difference in metabolism between the WR- and A35Δ-infected groups, suggesting that A35 does not promote cell death (Fig 16a). Similar experiments were carried out in the murine RAW 264.7 macrophage line, and similar results were obtained: VACV decreased metabolism of RAW, but A35 had no effect. Apoptosis induction was also measured in PEC using annexin V and propidium iodide staining at 4, 6 and 28 hpi. VACV infection induced apoptosis as previously reported, from 17% to 43% in Fig 16b, but A35 had no effect at

any time point measured, indicating that the A35-induced reduction in antigen presentation was not likely due to the increased induction of cell death.

A35 Does Not Affect Antigen-Independent NO Production

Since A35 acts on APC, and A35 caused an inhibition of NO production by PEC in antigen presentation assays with T lymphocytes (Fig 12), we tested the effects of A35 on NO production in response to purified LPS and IFNγ in PEC and the murine RAW 264.7 macrophage cell line. We infected cells for 4 h with either WR or A35Δ, stimulated the cells with IFNγ, LPS, or both, and then measured NO production in supernatants 24 hpi. NO was produced by uninfected PEC and RAW in response to each stimulus (Fig 17), and VACV infection significantly reduced the amount of NO produced as previously reported (13), however the presence of A35 had no effect on this outcome. We performed this experiment with a higher MOI of 5 and a lower MOI of 1 to assess whether an A35 effect might be seen at lower virus infectious dose, but no effect could be detected. Thus, A35 does not always decrease macrophage NO responses, but inhibits NO production specifically as the result of antigen presentation interactions with T cells (Fig 12). These data suggested that A35 specifically blocks the interaction between the PEC and the RsL.11.

A35 and MHC Class II and B7.2 Expression on APC

Since data suggested that A35 might block interactions between APC and T cells, we measured the effect of A35 on surface expression of B7.2/CD86 and MHC class II, proteins important in antigen presentation. Several labs have now shown that VACV can inhibit MHC class II and CD86 expression on APC *in vitro* and *in vivo* under various conditions (62, 86, 95, 116, 227). PEC were infected for 4 h at an MOI of 10 and analyzed by flow cytometry. Uninfected PECs were 43% bright positive for MHC class II, A35Δ-infected PECs were 39%

and WR-infected PEC were 29% (data not shown), indicating that the A35 protein plays a role in inhibiting the highest expression of MHC II on the surface of PECs. We also looked at how A35 affected the MHC II expression on the 1153 murine B cell line. Following a similar approach to that described for the PEC, uninfected 1153 B cells were 38% bright positive (arrow shown) for MHC II while WR-infected 1153 B cells were 15% (Fig 18), and A35Δ-infected cells were 23%, indicating that the presence of A35 in WR mildly reduced MHC II surface expression. In the same experiments, there was no difference in CD45 (leukocyte common antigen) expression between the groups, indicating that VACV and the presence of the A35 gene mildly but specifically decrease MHC class II on the surface of APC. CD28 stimulation through B7 is required for protection from poxvirus infection (65), and B7.2 expression was also measured. PEC and the mouse 1153 B cell line were infected at an MOI of 5 for 4 hours and incubated with primary antibodies that recognize B7.2/CD86 costimulatory molecules. Surface expression of B7.2 costimulatory molecule was decreased from 16% in uninfected and A35Δ infected cells to 10% in WR infected cells, but infection did not decrease surface expression of CD45. These data suggest that A35 may modestly downregulate surface proteins required for antigen presentation.

A35 Decreases the Amount of Peptide Presented in MHC II

One level of immune regulation, both in homeostasis and infection, lies in controlling the association of peptides with the MHC molecules that present them (82). We next sought to determine whether A35 affected the association of peptides with MHC II on the cell surface. To do this, we used the Y-Ae antibody, which specifically detects peptide 52-68 from I-E^d MHC class II bound in the cleft of mouse MHC class II I-A^b (84, 173). MHC class II molecules frequently present self peptides from surface proteins that recirculate through endosomes and are degraded, similar to exogenous antigens that are internalized. The Y-Ae antibody recognizes a

major determinant (approximately 12% of MHC molecules) in mice expressing both of these class II molecules (173, 200). We measured the amount of peptide bound in the cleft of MHC class II on the surface of uninfected, WR and A35Δ infected spleen cells. As Figure 19 shows, uninfected and A35Δ infected cells were 63% positive for class II MHC molecules complexed with this I-E peptide, while infection with WR reduced this to 30% 3 hpi, with only a slight decrease in MHC class II expression. These data indicate that A35 interferes specifically with the loading of this model peptide into the cleft of MHC class II molecules or expression of such molecules on the cell surface. As surface expression of antigenic peptide was decreased by A35 in this model system, we tested whether MHC class II bound to the remnant chaperone invariant chain peptide CLIP was transported to the cell surface. CLIP peptide was detectable on approximately 14% of uninfected cells, 24% of A35Δ infected cells and 33% of WR infected cells (Fig 19) suggesting that A35 enhances the binding and/or transport of CLIP peptides and thus may interfere with presentation of a wide variety of naturally occurring MHC class II presented antigenic peptides.

To determine whether A35 affects VACV stimulated immune responses to VACV antigenic peptides during natural infection, we infected groups of 5 mice with 1000 pfu VACV i.n. as previously described (170) and enumerated VACV specific IFN γ secreting spleen cells on day 8 post infection. WR infected mice produced significantly fewer IFNγ secreting cells compared to A35Δ infected mice and produced significantly lower levels of VACV specific antibody, suggesting that A35 also acts *in vivo* to suppress immune responses to VACV (164). PBS-vaccinated mice and vaccinated mouse cells without virus stimulation were used as negative controls and produced no signal.

A35 Localizes to the Endosomes

Since MHC and B7 proteins recycle in endosomes, and MHC class II is loaded with peptides in the endosomes, we determined the intracellular localization of A35 in APCs. We had published previously that A35 localizes intracellularly in viral factories in BS-C-1 fibroblast cells, in which there is no A35 phenotype (170). Since our data suggest that A35 functions in APC early after infection, we determined the localization of A35 in PECs at a time point when an A35 phenotype is seen in antigen presentation. PEC were infected with either WR or A35Δ, and A35 was visualized using fluorescent microscopy. The presence of A35 in the WR-infected PEC could be seen as diffuse punctuate staining throughout the cell (Fig 20), which is consistent with the staining of endosomal structures, where many aspects of MHC II antigen processing and presentation occur. In order to verify endosomal localization, we co-localized the A35 protein with known endosomal markers, Rab 5 (plasma membrane and early endosomes), Rho B (multivesicular bodies), Rab 7 (late endosomes)), and Lamp-1 (late endosomes and lysosomes) (33, 66, 119, 167). A35 showed partial co-localization with these endosomal markers, although least with Rab 5 (Fig 20). In each case there were endosomal structures that had both A35 and the marker (yellow colocalization), but also punctate staining for each antibody alone (green or red). Thus, A35 localizes generally throughout the range of endosomes in the cell, but is not found in all endosomes and does not partition to any distinct endosomal type. The presence of A35 in these structures suggests that A35 protein may interact with or affect proteins in endosomes including class II MHC, although further research is required to elucidate with which proteins A35 interacts specifically.

Discussion

We showed previously that the VACV A35 gene is an important virulence factor,

increasing virulence by approximately 100-1000 fold (164, 170), but its function was unknown. We have shown here that the VACV A35 gene product is not required for replication in a variety of cell types and tissues in six different mammals. Furthermore, A35 does not control early replication in mouse tissues on days 1-3 post infection before specific immunity develops. However, we have shown that A35 affects the development of specific immune responses *in vitro*, specifically acting on APC. A35 contributes to VACV-induced reduction in MHC II antigen presentation (Fig 12) and subsequent cytokine synthesis (Fig 13) using *primary* macrophages as APC, even though the mutant virus was able to infect the APC as efficiently as wild type (Fig 15). A35 did not affect metabolism, permissiveness, apoptosis of PEC nor NO production induced by immunostimulators in rat PEC or the murine RAW macrophage cell line. These data suggested that A35 did not have a major systemic effect on macrophage, but rather that it specifically interfered with the interaction between APC and T cells since those assays specifically showed an A35-dependent phenotype.

We subsequently found that A35 mildly affected the expression of MHC class II on the surface of the APC (Fig 19). While several labs have now shown that VACV can inhibit MHC class II and CD86 expression on APC *in vitro* and *in vivo* under various conditions, there has been much debate about the circumstances under which this regulation occurs and its significance (116). Some laboratories have associated the reduction in surface molecules with induction of apoptosis (95), while others have concluded that VACV does not block basal B7.2 or class II MHC expression but that it blocks activation-induced B7.2 or class II MHC expression (62 2000). We have found that early after infection, VACV and the A35 gene can mildly decrease expression of these surface markers both in rat PEC and in a murine B cell line. Whether a small decrease in MHC class II levels will have physiologic significance is unknown,

however it is known that MHC class II antigen presentation is crucial to protection from poxvirus infection. In studies with knockout mice, it was found that the absence of MHC class I molecules or CD8 T cell response did not diminish protection but that decreases in CD4 or MHC class II expression caused a loss of protective immunity (221, 224).

VACV genes B1R and H5R have been shown to inhibit CD1d-mediated non-classical lipid antigen presentation, although the importance of this function was difficult to ascertain because of the pleiotropic effects of these genes (220). Several laboratories have shown that VACV also blocks MHC class II antigen presentation in a number of assays using different APC, model antigens (exogenous, virus encoded and cellular endogenous), and response measures (163). VACV-infected primary Langerhans cells were deficient in presenting KLH peptide to a cognate T cell line, as measured by production of IFNγ (51). Primary rodent bone marrow derived macrophages infected with VACV were deficient in presenting lysozyme peptide to T cells, as measured by stimulation of IL-2 production (126). Li et al found that VACV inhibited MHC class II antigen presentation in *in vitro* infected human and rodent APC, including B cell lines, dendritic cells, macrophage and fibroblasts presenting several antigens (116). The accumulation of data indicates that VACV generally inhibits MHC class II antigen presentation; however, no viral gene had previously been identified as blocking antigen presentation. We have shown here that A35 interferes with antigen presentation responses to an exogenous model antigen (Fig 12, 13), as well as surface expression of an endogenous peptide in MHC class II (Fig 19). We have also found that A35 increases surface CLIP expression in MHC class II suggesting that A35 may inhibit presentation of a range of antigenic peptides. Further, we have found that A35 diminishes anti-VACV T cell responses and antibody in mice (164) suggesting that A35 may similarly inhibit presentation of virally expressed VACV antigens during infection

in vivo.

Our data suggest that VACV A35 contributes to viral inhibition of MHC class II antigen presentation by decreasing the expression of antigenic peptides in the cleft of MHC class II on the surface of infected cells. We showed this in the murine system with an MHC derived peptide as the antigen. Dr. Blum's laboratory showed that VACV decreases the amount of glutamate decarboxylase (GAD) peptide in the cleft of MHC class II in a human B cell line (116). Together these data suggest that VACV inhibition of peptide loading into the cleft is a general mechanism of immune response inhibition independent of the antigen or APC type. The fact that A35 is very highly conserved in mammalian tropic poxviruses suggests that A35 may act similarly in a number of different viruses and host species. Interestingly, Fig 19 shows that there is a population of cells in spleens that is unaffected by the presence of VACV and A35 gene (MOI of 10). Since splenocytes contain numerous cell types, these data suggest that there is a VACV resistant population in the spleen, consistent with other studies showing differing sensitivities to infection among immune cells (30, 175). It will also be important to determine the effects of A35 on lymphocytes in the target organs, especially the lungs.

In these experiments, we showed that A35 inhibited antigen presentation, suggesting that the A35Δ mutant viruses will provide improved vaccines, as well as improved platform vaccines for other infectious diseases and cancer treatment. The A35 gene is conserved in all VACV vaccine strains including MVA. While A35 inhibited antigen presentation, it was also clear that the A35 deletion mutant virus blocked antigen presentation when compared to uninfected APC (Fig 12, 13). This suggests that there are multiple poxvirus proteins that function independently to decrease antigen presentation. It is important to identify these immunosuppressive genes and test the cognate knockout viruses as improved poxvirus (or poxvirus platform) vaccines.

While we showed that both T cell and PEC cytokine production was reduced subsequent to antigen presentation interactions, our data indicated that it was the APC that were directly affected by VACV, and the T cells were indirectly affected via regulation of the APC. A35 had the effect of suppressing the production of numerous cytokines and chemokines, which are crucial in the development of an appropriate immune response. Notably, many of the cytokines that are regulated by VACV and A35 are chemotactic factors (MIP-1α, IL-1, TNF-α, GRO/KC, RANTES, and MCP-1), which aid in the migration of immune response cells to sites of inflammation and infection. Interestingly, IL-18 was not significantly reduced by VACV and A35 (Fig 13). Perhaps this is because IL-18 is stored intracellularly as pro-IL-18 and rapidly released upon stimulation (69). Since IL-18 is an important factor in the induction of anti-viral IFNγ production, its storage for rapid release may be an evolved host mechanism to contravene immunomodulation by viral gene products such as A35. The importance of IL-18 in antiviral defense is underscored by the possession of an IL-18 binding protein encoded by poxviruses (159).

There are multiple mechanisms by which A35 could decrease MHC II peptide presentation that must be further explored. Future experiments will address with which cellular proteins A35 interacts. Since A35 may affect peptide loading, it is possible that it interacts with invariant chain (Ii) that chaperones MHC class II proteins, directly with MHC class II itself, or DM molecules that are involved in the exchange of antigenic peptides. Furthermore, we will test various immune responses to the A35Δ mutant virus in comparison to wild type virus in transgenic mouse models to understand which pathways are inhibited by A35. Our hypothesis is that A35 acts through blocking MHC class II antigen presentation, and we predict that anti-viral CD4 responses will be diminished, and that this decrease in T helper cells will diminish

development of most downstream anti-viral effector cells (including CD8 cytotoxic T cells, and antibody secreting B cells). Thus far our animal model data are consistent with this hypothesis, showing a reduction in both VACV specific antibody and splenic T lymphocyte responses (164).

Figures

Figure 12: A35 inhibits the amount of antigen presentation-induced IL-2 and NO. PEC were isolated from a rat, infected with VACV (MOI=2) for 5 h, pulsed for 30 min with 50 nM GPMBP, and then incubated with the cognate CD4+ RsL.11 T cell line. Uninfected PEC served as a control. At 24- to 48-hpi, supernatants (50 μl) were collected and assayed for either IL-2 using the IL-2 dependent cell line CTLL (a) or for NO using Greiss reagent (b). For the CTLL bioassay, media only was used to define the background control level and known IL-2 containing supernatants were used as positive control. IL-2 production was determined as the mean OD value of the experimental group minus the background control level. * $p < 0.05$

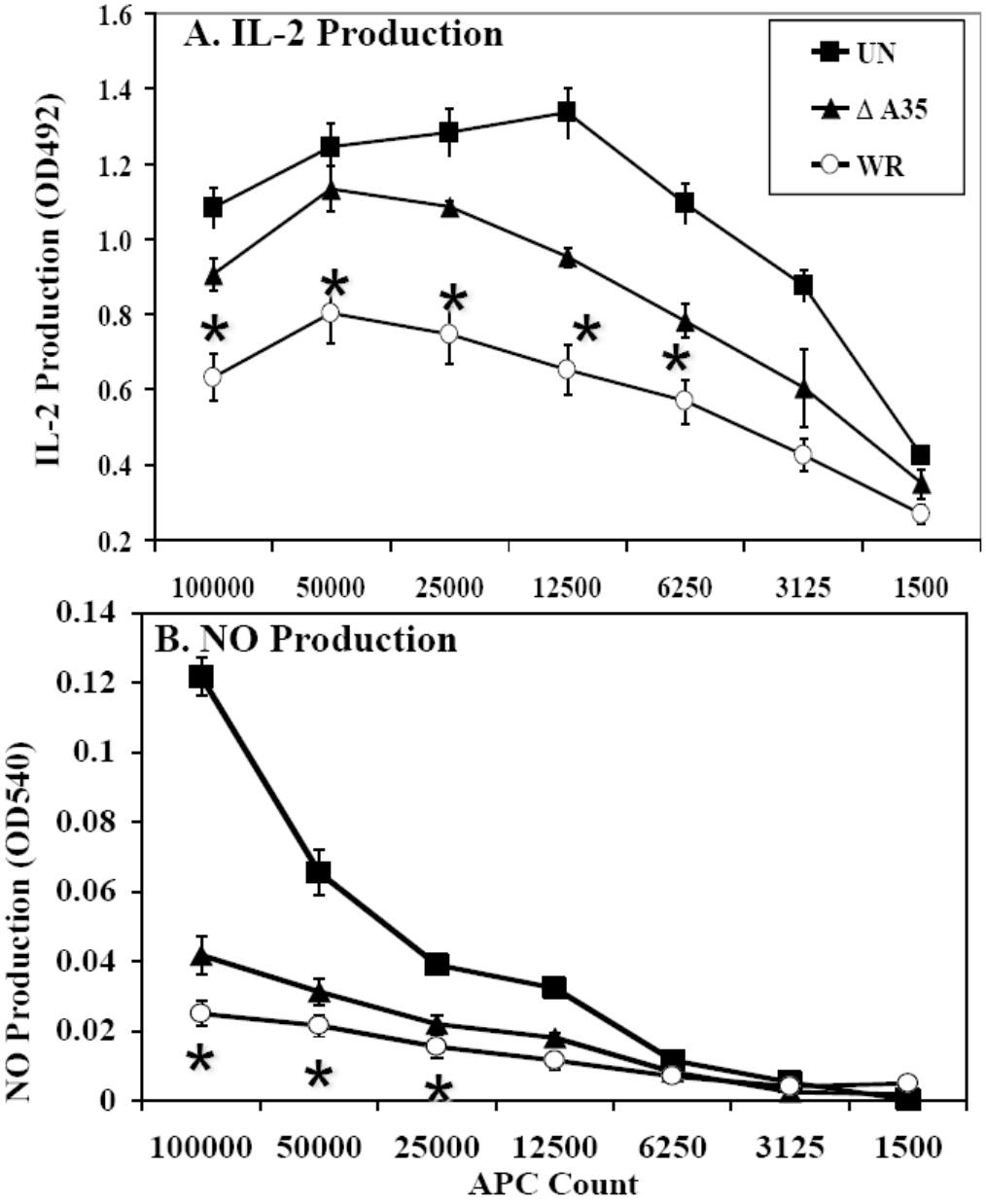

A. IL-2 Production
UN
Δ A35
WR
IL-2 Production (OD492)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
100000
50000
25000
12500
6250
3125
1500
B. NO Production
NO Production (OD540)
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
APC Count

Figure 13: A35 reduces antigen presentation-induced cytokine synthesis. PEC were harvested from Lewis rats, infected with WR or A35Δ virus (MOI=2) for 5 h, pulsed with 50 nM GPMBP, and then co-cultured with the cognate CD4+ RsL.11 T cell line. Supernatants (50 μl) were collected at 24-h post incubation and assayed for the production of cytokines using the LincoPlex 24 Rat Cytokine/Chemokine Luminex Bead Immunoassay Kit. IL-2 and IFNα values were divided by 20 and 100 respectively to fit to scale. IL-6, IL-18, and MCP-1 were not significantly reduced by the presence of the A35 gene in WR, all other differences between WR and A35Δ were $p < 0.05$.

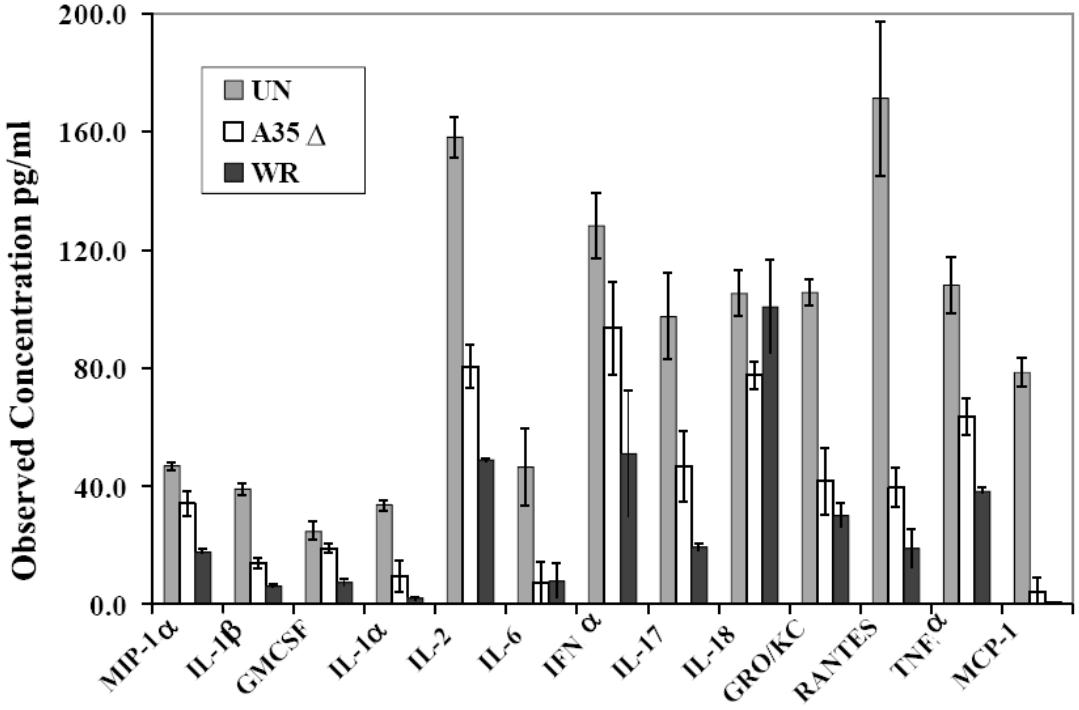

Observed Concentration pg/ml
200.0
160.0
120.0
80.0
40.0
0.0
UN
A35 Δ
WR
MIP-1α
IL-1β
GMCSF
IL-1α
IL-2
IL-6
IFN α
IL-17
IL-18
GRO/KC
RANTES
TNFα
MCP-1

Figure 14: A35 does not directly affect T lymphocytes. (a) RsL.11 T cells were infected with WR or A35Δ virus (MOI= 4) for 4 h and then incubated with decreasing numbers of antigen-pulsed PEC. After 24-48 h, supernatants (50 μl) were collected and assayed for IL-2 production using the CTLL IL-2 bioassay. (b) RsL.11 T lymphocytes were infected for 3-4 h and then stimulated with PEC/50 nM GPMBP, 25 μg/ml Con A, or 100 nM PMA/2 μM ionomycin. Supernatants (50 μl) were collected at 20- and 40-h and assayed for IL-2. (c) RsL.11 T cells were infected with VACV for 10 h, and growth metabolism was measured by reduction of 10 μl MTS/PMS. (d) CTLL cells were infected with VACV (MOI=5) for 4 h and then placed in media with varying amounts of an IL-2-containing supernatant. Proliferation was measured by adding 10 μl MTS/PMS.

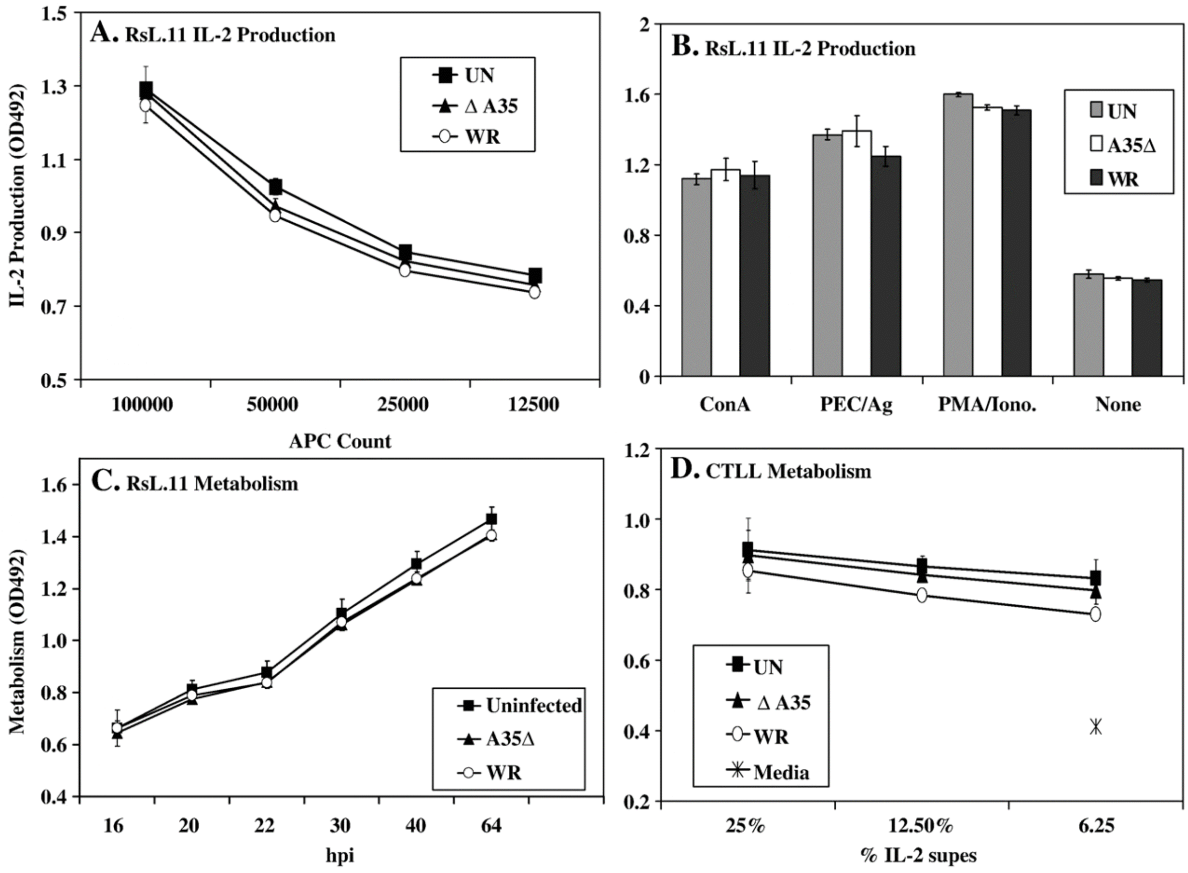
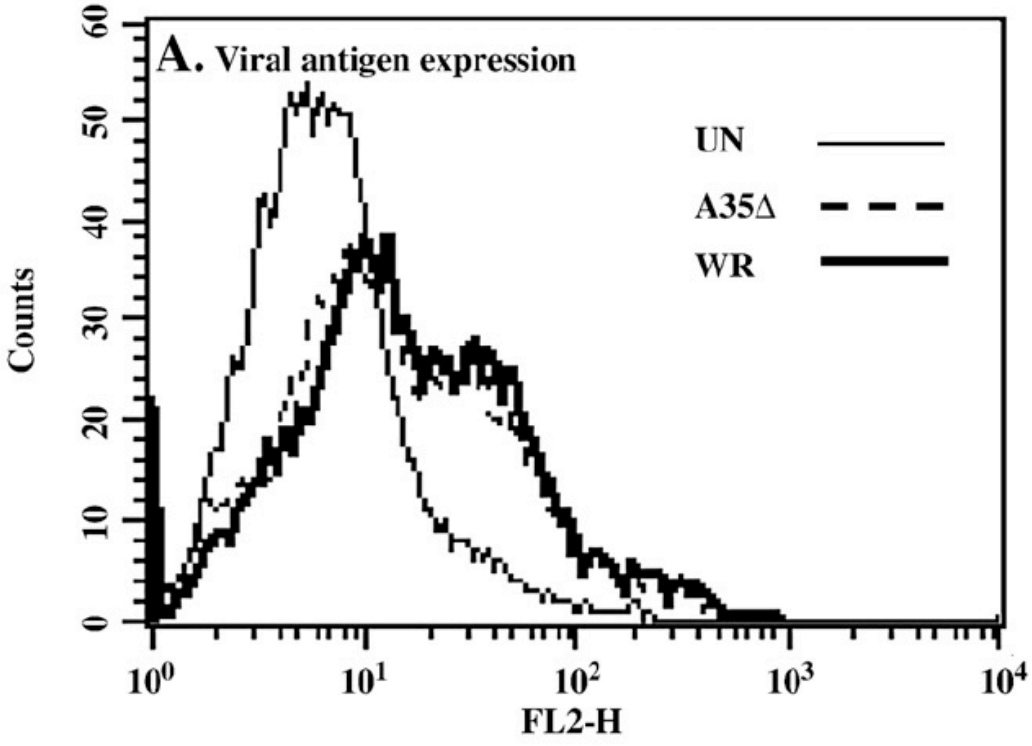

A. RsL.11 IL-2 Production
IL-2 Production (OD492)
1.5
1.3
1.1
0.9
0.7
0.5
UN
Δ A35
WR
100000
50000
25000
12500
APC Count
B. RsL.11 IL-2 Production
2
1.6
1.2
0.8
0.4
0
UN
A35Δ
WR
ConA
PEC/Ag
PMA/Iono.
None
C. RsL.11 Metabolism
Metabolism (OD492)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
Uninfected
A35Δ
WR
16
20
22
30
40
64
hpi
D. CTLL Metabolism
1.2
1.0
0.8
0.6
0.4
0.2
UN
Δ A35
WR
Media
25%
12.50%
6.25
% IL-2 supes

Figure 15: A35Δ mutant virus is as infectious as WR and replicates equally. a) PEC were incubated with WR and A35Δ at an MOI of 10 for 10 h, fixed, permeabilized, and incubated with human polyclonal anti-VACV hyperimmune sera. Viral antigens were measured by flow cytometry. b) BS-C-1 cells or PEC were infected with either WR or A35Δ mutant virus (MOI=10). Cells were collected at designated times post-infection and cell-associated virus was measured.


A. Viral antigen expression
UN
A35Δ
WR
Counts
0
10
20
30
40
50
60
10^0
10^1
10^2
10^3
10^4
FL2-H

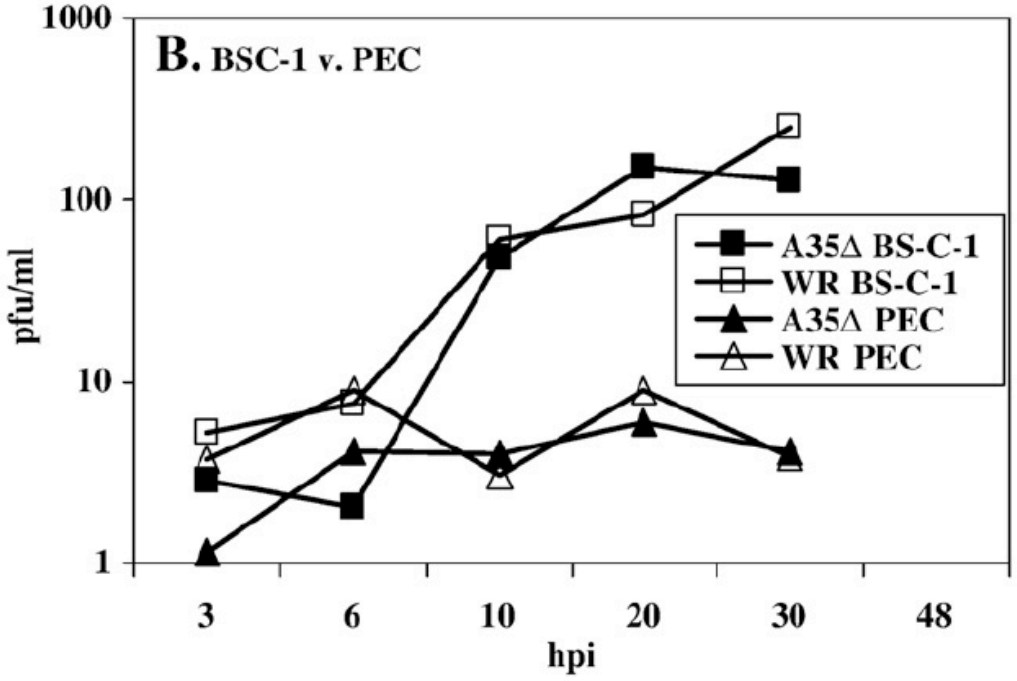

B. BSC-1 v. PEC
A35Δ BS-C-1
WR BS-C-1
A35Δ PEC
WR PEC
pfu/ml
1000
100
10
1
3
6
10
20
30
48
hpi

Figure 16: A35 and apoptosis. (a) PEC were infected with A35Δ or VACV WR (MOI=2) and metabolism was measured by the reduction of 10 μl MTS/PMS. (c) PEC apoptosis was measured using annexin V and propidium iodide and flow cytometry following a 4 h infection.

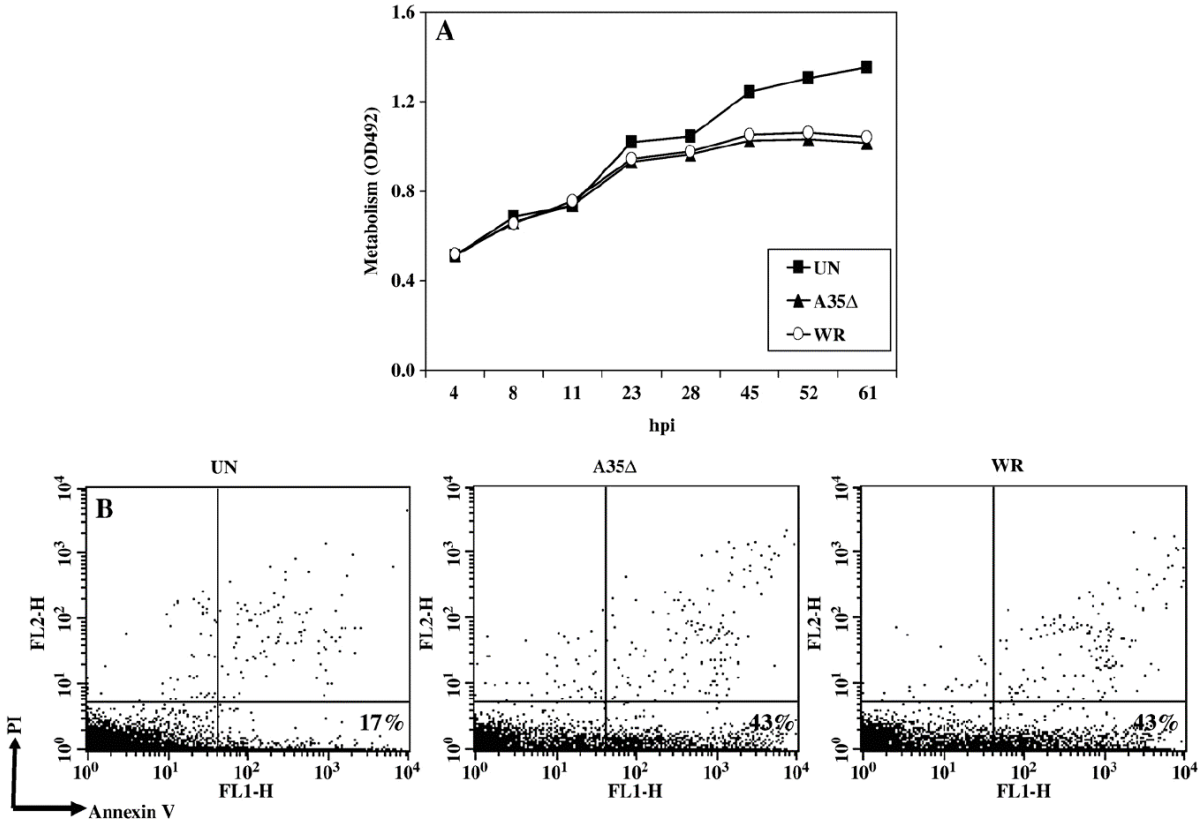

A
Metabolism (OD492)
1.6
1.2
0.8
0.4
0.0
4
8
11
23
28
45
52
61
hpi
UN
A35Δ
WR
B
UN
A35Δ
WR
FL2-H
FL1-H
PI
Annexin V
17%
43%
43%

Figure 17: A35 does not block antigen-independent NO production. PEC (a) or RAW 264.7 macrophages (b) were infected at an MOI of 1 for 4 h, and were stimulated with LPS, IFN-γ, or a combination. Supernatants (50 μl) were collected at 24-48 h and then assayed for NO production.

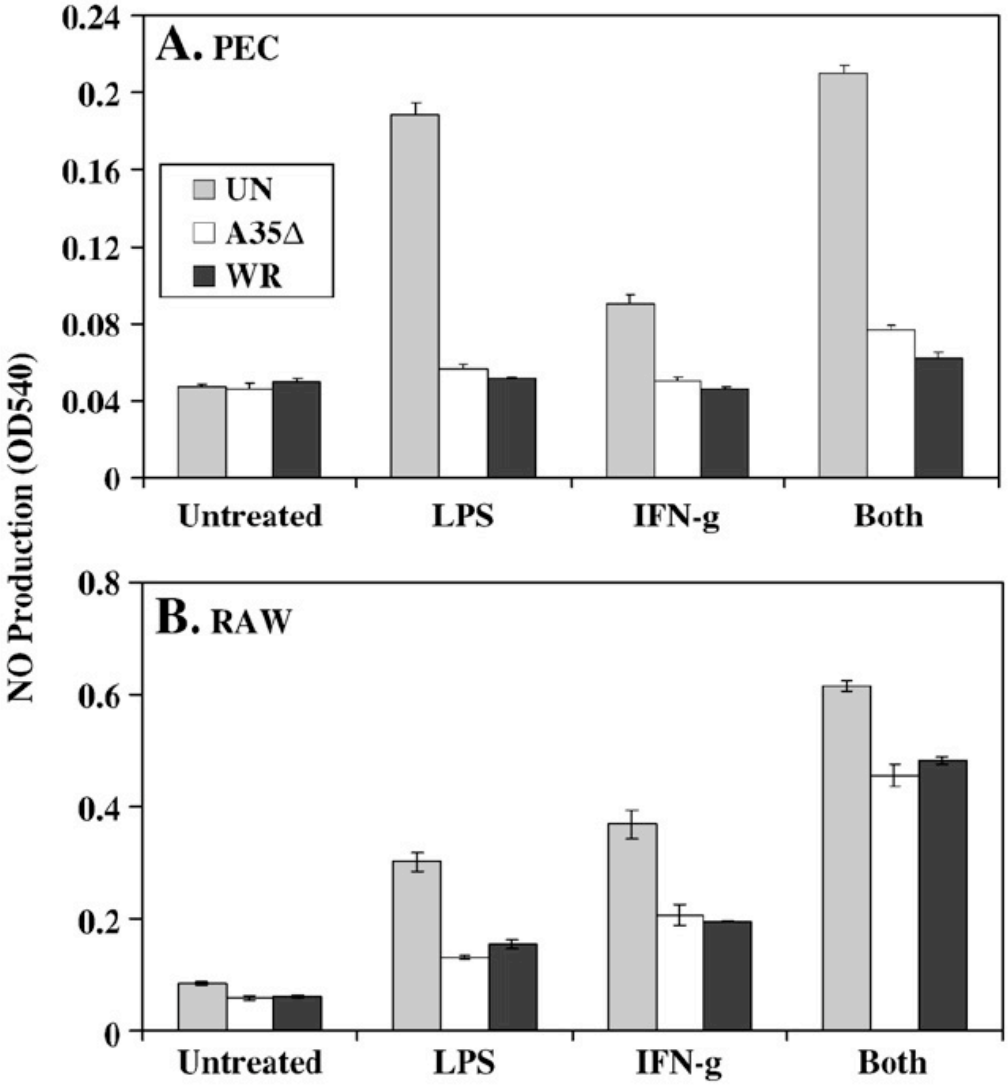

A. PEC
B. RAW
UN
A35Δ
WR
NO Production (OD540)
0.24
0.2
0.16
0.12
0.08
0.04
0
0.8
0.6
0.4
0.2
0
Untreated
LPS
IFN-g
Both

Figure 18: A35 decreases MHC class II expression. 1153 B cells were infected with WR or A35Δ virus for 5 h, washed and incubated with no primary antibody, anti-class II MHC, or anti CD45 and then FITC-conjugated secondary and analyzed by flow cytometry. Mean fluorescence intensities (MFI) for MHC class II were: UN 154, A35Δ 111, and WR 77.

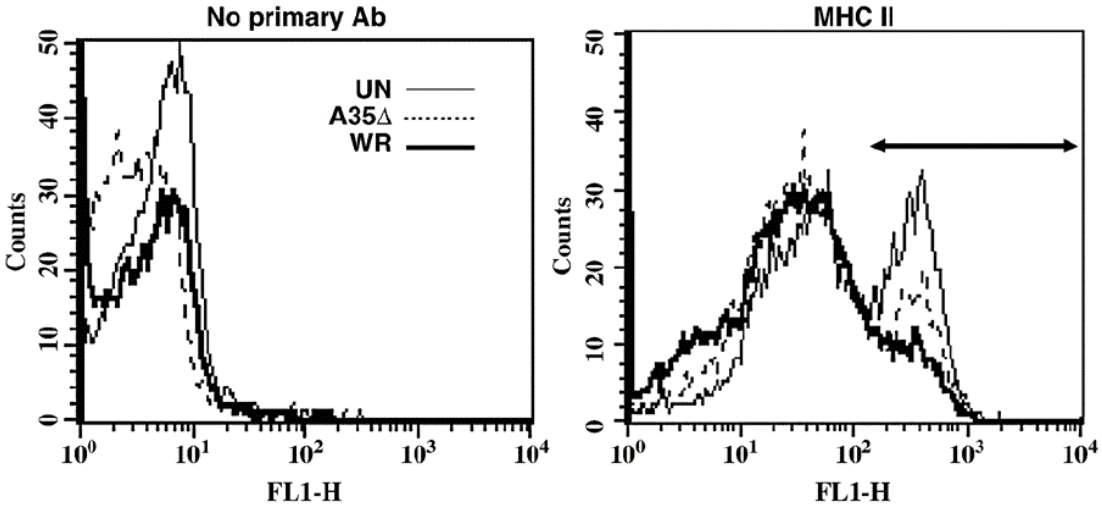

No primary Ab
UN
A35Δ
WR
Counts
FL1-H
MHC II
Counts
FL1-H

Figure 19: A35 decreases peptide-MHC class II complexes. Splenocytes from B10.A-H2^i5 H2-T18^a/(5R)SgSnJ mice (Jackson Labs) were infected for 3 h at an MOI=10, washed, and then incubated with no primary antibody, antibodies to MHC class II or CLIP, or biotin-conjugated Y-Ae anti-peptide in MHC antibody followed by streptavidin-RPE. Samples were analyzed by flow cytometry, and MFI for Y-Ae were: UN 85, A35Δ 76, and WR 36. MFI for CLIP were UN 5.7, A35 Δ 8.2, and WR 13.9.

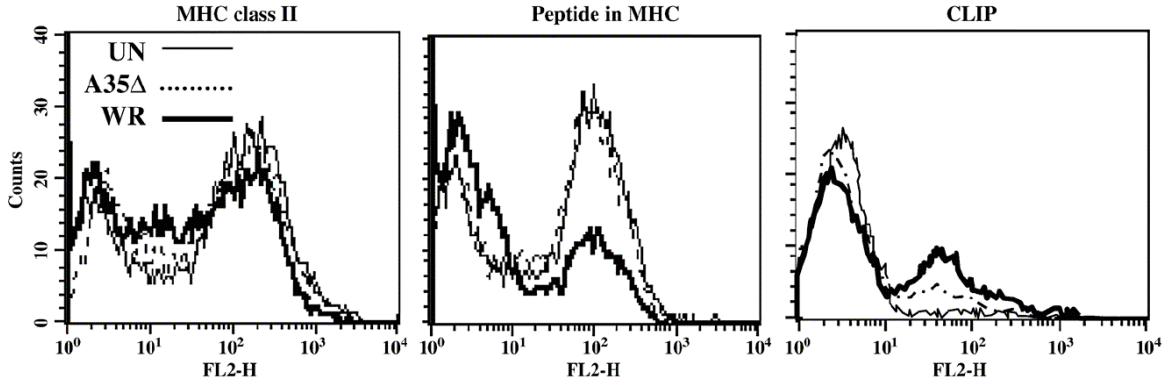
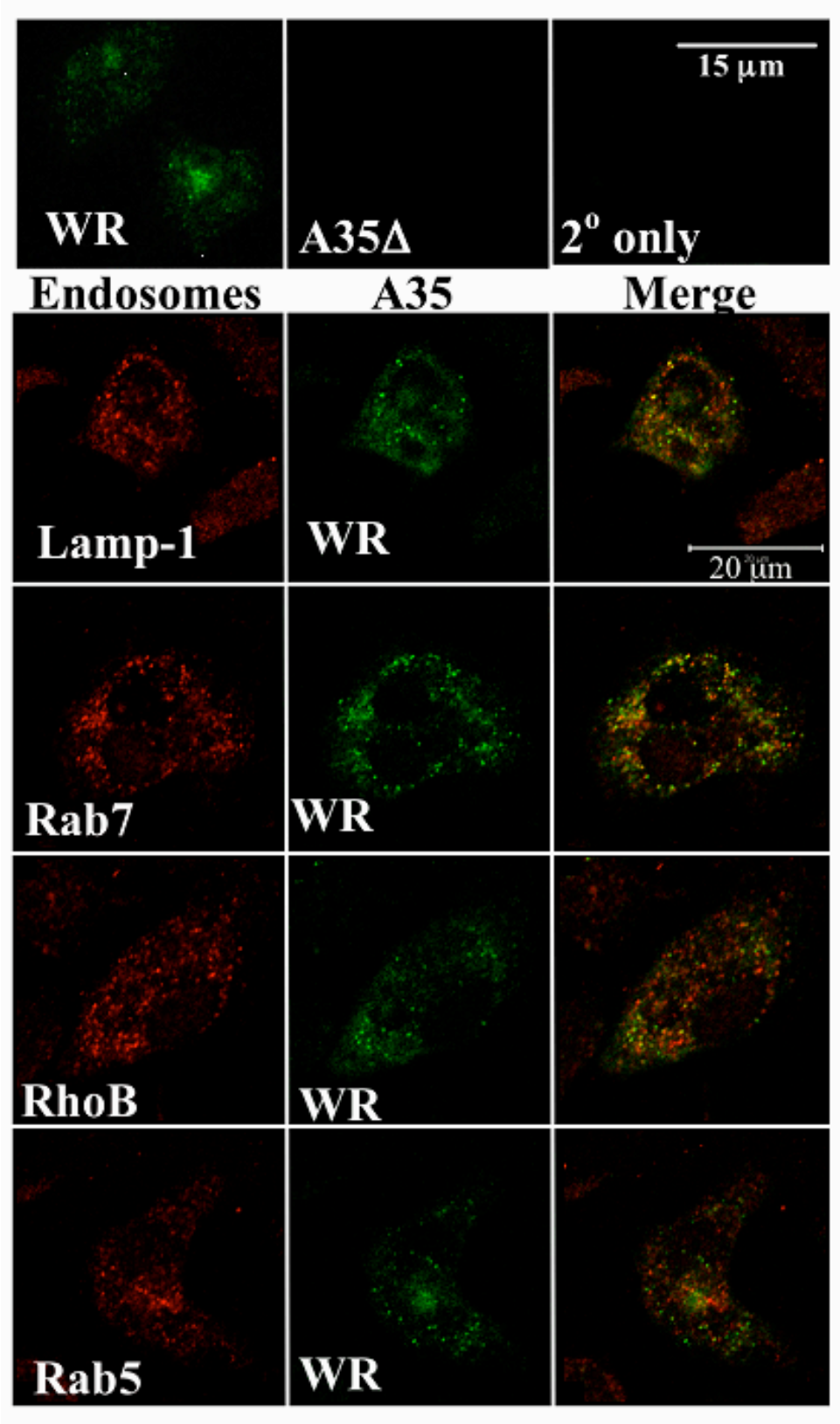

MHC class II
Peptide in MHC
CLIP
UN
A35Δ
WR
Counts
FL2-H
FL2-H
FL2-H

Figure 20: A35 localization in PEC. PEC were grown on coverslips for 4 h and then infected with WR or A35Δ virus at an MOI=10 for 2 h. The cells were then fixed and permeabilized, and stained with a rabbit anti-A35 antibody and one of the following: goat anti-LAMP, goat anti-Rab7, goat anti-RhoB, goat anti-Rab7. The A35 protein was visualized with a FITC-conjugated secondary antibody, and the endosomal markers were visualized with Alexa Fluor 633.


WR
A35Δ
2° only
15 µm
Endosomes
A35
Merge
Lamp-1
WR
20 µm
Rab7
WR
RhoB
WR
Rab5
WR

CHAPTER 4: THE POXVIRUS A35 PROTEIN IS AN IMMUNOREGULATOR

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Abstract

It has been shown previously that the highly conserved VACV A35 gene is an important virulence factor in respiratory infection of mice. We show here that A35 is also required for full virulence by the intraperitoneal route of infection. A virus mutant in which the A35 gene has been removed replicated normally and elicited improved antibody, IFNγ-secreting cell and cytotoxic T lymphocyte responses compared to wild type virus, suggesting that A35 increases poxvirus virulence by immunomodulation. The enhanced immune response correlated with improved control of viral titers in target organs after the development of the specific immune response. Finally, the A35 deletion mutant virus also provided protection from lethal challenge (1000 x LD_{50}) equal to that of the wild type virus. Together these data suggest that A35 deletion viruses will make safer and more efficacious vaccines for poxviruses. In addition, the A35 deletion viruses will serve as improved platform vectors for other infectious diseases and cancer and will be superior vaccine choices for post-exposure poxvirus vaccination as they provide improved kinetics of the immune response.

Introduction

Poxviruses are large, complex viruses with a broad host range and worldwide distribution (141). Members of the family Poxviridae include Variola virus, the causative agent of smallpox, which induced a fatality rate of approximately 30% and killed hundreds of millions of people

before its eradication in 1980 (124). Currently the most dangerous extant human-infecting poxvirus is monkeypox virus, which is commonly found in African rodents. Monkeypox virus causes a smallpox-like illness with a 10% fatality rate. A recent study showed that 1.7% of people in the Likouala region in Africa had monkeypox specific IgM, indicating a significant ongoing infection rate (112). An outbreak of a low virulence strain of monkeypox occurred in the United States in 2003, causing more than 80 human infections and several hospitalizations (34). This outbreak raises concern that monkeypox virus could establish itself in wild rodent populations in North America (152), thus creating a local zoonotic reservoir for this emerging pathogen. Of further concern are the facts that monkeypox is spreading more efficiently in humans, (81, 110, 144) and that the current poxvirus vaccine is not universally protective against monkeypox infection (117). Both Variola and monkeypox viruses are considered bioterrorism and biowarfare concerns and are Category A select-agent pathogens. There are also other poxvirus infections that sporadically cause human outbreaks, including Cantagalo in South America (45, 185), buffalopox virus in India (100), and incidence of Tanapox appears to be increasing (52, 198). Molluscum contagiosum poxvirus accounts for approximately 300,000 doctor visits each year in the United States alone (140). Thus, the study of virulence mechanisms in this group of viruses is important.

The eradication of smallpox was accomplished through the use of the related VACV as a live virus vaccine. Despite its phenomenal success, the public vaccination program was discontinued because of the high incidence of complications due to the virulence of wild type VACV. It is estimated that approximately 25% of the population should not receive this vaccine because of immunodeficiency, eczema, pregnancy, or heart disease (60, 96, 211). Safer vaccines are necessary to protect against emerging or released poxviruses. In addition, poxviruses are

being used as platform vaccines for other diseases, such as human immunodeficiency virus, malaria, and cancer, because they induce a robust immune response and accommodate the insertion of large pieces of foreign DNA. It is therefore of great importance to identify poxvirus virulence genes in order to develop safer and more effective poxvirus vaccines. Replication-defective strains, such as Modified Vaccinia Ankara (MVA), have been used in an effort to reduce the risks associated with vaccination (41, 221), but production of these viruses can be challenging, they require higher doses of vaccine, and their protective efficacy against poxvirus infections in humans is unknown. As poxviruses inhibit activation of antigen presenting cells and antigen presentation (116, 163), another way to construct a safer vaccine is to develop replication-competent vaccine strains that exclude immunosuppressive genes (19) while retaining protective antigenic epitopes (79, 148). We show herein that the A35 gene is an excellent candidate for removal from vaccine strains.

The VACV A35 gene is highly conserved in mammalian-tropic poxviruses, and a sequence identity search has revealed that the protein has little similarity to any other poxvirus protein or any non-poxvirus protein, suggesting that this gene has an important and novel function (170). We have shown that the A35 gene is not required for viral replication *in vitro*, but is required for full virulence in the mouse model (170). We therefore tested the effects of A35R on immune responses during infection in the mouse model, and tested its protective efficacy against virulent challenge.

Materials and Methods

Cells and Virus

VACV Western Reserve (WR) strain and A35Δ mutant virus stocks were propagated using BS-C-1 cells in MEM containing 10% FBS as previously described (170). P815 cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM).

Mouse Infection and Sample Collection

Four 5-week old BALB/c mice were anesthetized using isoflurane and infected intranasally (i.n.) as previously described (170) with purified virus in 18 μl (100 μl for i.p. infection), or mock infected with PBS. Titers were confirmed each experiment using the dilution used to infect mice that day. Mice were weighed and monitored daily for signs of illness and were euthanized if 20% weight loss occurred. For experiments assessing immune responses, five mice from each group were sacrificed, and the spleens were collected in ice cold RPMI medium. Splenocytes were obtained as previously described and incubated in RPMI (36). Blood was collected using cardiac puncture, followed by centrifugation to separate plasma from red blood cells. All experimental protocols were approved by the Animal Care and Use Committee of East Carolina University.

ELISA

To determine antibody response, 96-well ELISA plates (Immulon H2B Thermo Electron) were coated overnight with 0.1 μl/well (100 μl) crude WR virus in ELISA coating buffer (10.3 g H_2BO_4, 7.31 g NaCl, 1 L double distilled H_2O, pH 8.5) at 4°C. Plates were blocked with 1% FBS/PBS at room temperature for 30 min. Plates were washed with ELISA wash buffer (1x PBS, 0.02% Tween 20, 0.1% NaN3) and a titration of mouse sera was added. Plates were incubated at room temperature for 2 h and washed. Goat anti-mouse IgG-alkaline phosphatase (Southern

Biotech) was added and incubated at room temperature for 1 h. Plates were washed three times, developed (BioRad Alkaline Phosphate Substrate Kit) and absorbance was read at 405 nm. Anti-B5R ELISAs were similarly performed by coating plates with purified baculovirus-expressed B5R protein prepared by C-PERL (the kind gift of Stuart Isaacs).

Measurement of Neutralizing Antibody

VACV-specific neutralizing antibody was measured by incubating 25 μl of sera from PBS, WR or A35Δ-infected mice (n=5) with WR virus (200 pfu) in 100 μl of media containing 10% heat inactivated FBS for 1 h on ice. The virus/serum mixture was then added to confluent monolayers of BS-C-1 cells, and plaques were counted 40 h later.

IFNγ Enzyme-Linked Immunospot Assay (Elispot)

Numbers of IFNγ secreting spleen cells were enumerated similarly to methods described previously (42, 70, 180). Ninety six-well plates (Immulon H2B Thermo Electron) were coated overnight with 0.2 μl anti-mouse IFNγ (1 mg/ml Pharmingen) at 4°C. Plates were washed with blocking buffer before adding a titration of murine splenocytes in RPMI 1640 medium. Stimulation of splenocytes was achieved by either the addition of WR virus only (MOI=2) or with the use of 50,000 WR-infected (MOI=3) P815 stimulator cells, followed by incubation for 40 h at 37°C. Plates were then washed and incubated with 0.4 μl biotinylated rat anti-mouse IFNγ (Pharmingen 0.5 mg/ml) for 2 h at 37°C. Plates were washed again and incubated with streptavidin-alkaline phosphatase (AP) for 1 h at 37°C. Plates were developed with agarose-BCIP (5-bromo-4-chloro-3-indolylphospahte)-AMP mixture, and spots were counted using a dissecting microscope.

CTL Assay

Cytotoxic T lymphocyte (CTL) activity was measured as the release of lactate dehydrogenase (LDH) into supernatants from WR virus-infected P815 cells using Promega Cytotox kit. P815 target cells were infected (MOI=5) for 3 h and added to a titration of splenocytes from the vaccinated mice in round-bottom 96-well plates at 37^{o} C. After 6 h, 50 μl of supernatant were collected and assayed for LDH by adding an equal volume of substrate and reading the absorbance at 492 nm. Incubation of splenocytes with uninfected P815 cells was used to measure non-specific lysis, which was subtracted from each experimental group to yield specific lysis.

Cytokine Measurement

Sera were analyzed using the LincoPlex Mouse Cytokine/Chemokine Luminex bead immunoassay kit according to the manufacturer's instructions (Linco Research). Sera were incubated with a panel of anti-cytokine antibodies immobilized on Luminex beads (murine 23-plex, Bio-Rad Laboratories), run according to the manufacturer's instructions, and analyzed on the BioPlex protein array reader (Bio-Rad) at the Duke University Human Vaccine Institute Immune Reconstitution Core Facility (Durham, NC).

Virus Titrations

Groups of mice (n=5) were sacrificed at various days post infection (dpi), and the organs were placed into 1 ml of ice cold RPMI medium. The organs were then freeze/thawed three times, homogenized, and sonicated. Viral replication was evaluated by titration of the organ on BS-C-1 monolayers and staining with 0.1% crystal violet in 20% ethanol 40 h later.

Statistical Analysis

Experiments were repeated at least three times and representative data are shown. A two-tailed Student's t test was used to compare groups. p values < 0.05 were considered significant.

Results

Virulence Studies

We had shown previously that the VACV A35 gene is not required for replication and is a major virulence factor in the mouse i.n. challenge model, increasing virulence almost 100 fold (170). It was unknown whether A35 would also play a role in infection via other challenge routes, or if perhaps A35 was required for replication in certain tissues such as nasal or lung tissue. We therefore challenged groups of mice (n=5) intraperitoneally (i.p.), bypassing the respiratory route, with the wild type parental VACV WR strain, the A35Δ virus, or PBS as control. We found that VACV (WR and A35Δ) injected i.p. did not cause any weight loss at 10^5 and 10^6 pfu/mouse, but that at 10^7 pfu/mouse, the WR virus caused significant weight loss and death in 2 of 5 mice, while the A35Δ virus was attenuated, causing only mild weight loss and no mortality (Fig 21). These data indicate that the A35 gene is required for virulence in mice infected by either the i.n. or i.p. route.

Since A35 is a virulence factor independent of the route of infection, and VACV can block MHC class II antigen presentation *in vitro* (116, 163), we wanted to measure the effect of A35 on immune responses *in vivo*. We anesthetized and i.n. infected groups of mice ($n \geq 5$) with the VACV WR strain, the A35Δ virus, or PBS as control. Since we wanted to evaluate the immune responses in the presence or absence of the A35 gene without any confounding effects of overall systemic illness in the animals, we titrated the infectious dose to identify a dose of virus such that the more virulent WR strain did not cause significant weight loss in animals (Fig

22). We found that 10^5 to 10^4 pfu/mouse caused significant weight loss and mortality in >80% of WR infected mice (approximate LD_{50} of 5 x 10^3 pfu (161)) by day 8, while all A35Δ infected mice survived (LD_{50} of 5 x 10^5 pfu). At 10^3 pfu/mouse, WR caused significant weight loss while A35Δ did not, and 11/12 WR infected mice and all A35Δ infected mice survived. At 500 pfu/mouse, no weight loss was noted at any time point for either virus (Fig 22); therefore, we chose 500 pfu/mouse as our infection dose to study immune responses to these viruses.

Spleen Enlargement

Visual observation during necropsy suggested that spleens of infected/vaccinated mice were larger than spleens of uninfected mice, and that spleens of A35Δ- infected mice were larger than spleens of WR-infected mice. These observations suggested that A35Δ virus might generate a superior immune response with robust lymphocyte proliferation. B and T lymphocytes proliferate in response to antigen, and a spike of T cell proliferation has been reported in the spleens and lymph nodes of mice days 5 to 7 after VACV infection was previously reported (223). To begin to understand the effects of A35 on the immune response, mice were infected i.n. with 500 pfu of WR or the A35Δ virus. In all experiments, on at least one day (days 6-8 post infection), the spleens from A35Δ infected mice were significantly ($p<0.05$) larger than those from WR-infected mice (Fig 23a). Enlarged spleens correlated with increased numbers of splenocytes isolated from spleens. This difference was reproducible, but not significant. A35Δ-infected mice had higher numbers of splenocytes than WR-infected mice; however the difference was lost by 9 dpi (Fig 23b).

Antibody Response

The effects of A35 on VACV specific immunoglobulin (Ig) production in mice were assessed. The generation of anti-VACV specific antibody was previously shown to be crucial in

protecting mice from poxvirus infections (14, 32, 151). VACV-specific Ig was not detected above background levels on days 5 and 6 pi (i.n.). By day 7, there was measurable anti-VACV Ig in the serum, and mice vaccinated with the A35Δ virus produced significantly more VACV-specific Ig than mice infected with WR (Fig 24a), and this difference was maintained to 34 dpi (Fig 24b), the latest time point tested. These data reveal that A35 inhibits the production of Ig during a specific immune response, and that even a highly attenuated virus can induce an equal, or even superior, antibody response compared to the wild type vaccine strain. The B5R antigen is the major target of virus-neutralizing Ig in anti-VACV serum (12); therefore, we also measured Ig recognizing B5R protein in the sera of VACV-infected mice. A35Δ infected mice also produced higher titers of B5R specific Ig, but the difference was not significant (Fig 24c).

Since A35Δ-infected mice produced higher VACV-specific antibody titers, we next compared the levels of neutralizing antibody present in the sera from WR and A35Δ-infected mice on day 34 pi. Sera from WR-infected mice reduced plaque formation by an average of two-fold compared to sera from mock-infected mice (data not shown). Impressively, sera from the A35Δ-infected mice were able to reduce plaque formation nearly six-fold compared to sera from mock-infected mice and three-fold compared to the sera from WR-infected mice. Together, these data indicate that infection with A35Δ not only generated larger amounts of total VACV-specific Ig, it also lead to the production of larger amounts of neutralizing Ig.

IFNγ-Secreting Splenocytes

The ELISPOT assay allows enumeration of cells secreting IFNγ a cytokine known to be protective against VACV, in response to stimulation by VACV antigen presented in the context of MHC (42, 70, 180). We tested how the presence of the A35 gene in the virus infecting mice i.n. affects the induction of IFNγ secreting cells in the spleens using two methods for antigenic

stimulation of IFNγ: the addition of VACV infected P815 cells (70) or the addition of infectious virus directly to the splenocytes (42). As shown in Fig 25, splenocytes were stimulated to produce IFNγ by the addition of infectious virus directly to the cells, and the presence of the A35 gene in the WR virus significantly reduced the number of VACV-specific IFNγ-secreting splenocytes developed by the mouse, in some cases by 10 fold. Similar results were obtained when virus-infected P815 cells were used to stimulate the splenocytes (data not shown). A35 dependent differences were seen at early times pi (days 5 to 8), were significant on days 7 to 8, and were not consistently significantly higher at later times as the immune response waned (no significant differences were seen at day 9, 14 or 34). No IFNγ production was detected in PBS mock-infected mice or in any group without addition of virus or infected P815 cells as stimulators.

CTL Activity

Since cytotoxic T lymphocytes (CTL) are important in killing virally infected target cells and have been shown to be important in the defense against poxviruses (32, 88, 224), we measured the effect of A35 on CTL generated in spleens of i.n. infected mice. Splenocytes from PBS-, WR-, or A35Δ–infected mice were incubated with VACV-infected P815 target cells at various ratios, and specific lysis of the P815 cells was measured through the release of LDH into the supernatants. As shown in Fig 26, A35Δ-infected mice developed significantly stronger VACV-specific cytolytic activity than WR-infected mice. Differences were significant only on days 6 and 7 pi. Together the immune data suggest that an A35Δ virus induces a significantly stronger B and T cell immune response especially early after infection/vaccination compared to wild type virus.

Cytokine Production

In order to determine whether a cytokine signature could be identified for effective immune responses against poxvirus infection, and which cytokines might be mediating immune response differences in WR- and A35Δ-infected mice early after infection, we infected mice and harvested sera day 2 and 3 pi and measured 23 cytokines. As shown in Fig 27, VACV infection altered the serum cytokine composition on day 2 pi compared to PBS mock-infected mice, increasing levels of IL-6, G-CSF, and MIP-1α significantly. However, there was no significant difference between WR- and A35Δ-infected mouse sera that might explain the later differences in immune responses to these virus infections. Sera levels of IL-2, IL-3, IL-4, IL-5, IL -10, IL -12 p70, and eotaxin were below the standard curve values and are not shown. Day 3 data (not shown) were similar to day 2 with smaller differences between groups. Thus, it was not possible to predict the effectiveness of an immune response based on early post infection serum cytokine levels. We also assessed cytokine levels day 6 and 7 pi. On day 6 pi, differences were small; however, on day 7 pi, serum levels of MCP-1, IFNγ, and G-CSF were elevated at least two-fold in WR- compared to A35Δ-infected mice, however as discussed below, viral titers were much higher in WR-infected mice at this time point, suggesting that these cytokines might be elevated in response to viral infection rather than immune response per se.

Viral Titers in Organs

We had shown previously that the A35 gene was not required for replication in numerous tissue culture cell lines (170), but it was possible that A35 increased virulence by allowing VACV to replicate in certain animal tissues. We therefore wanted to compare virus replication levels in tissues both at early and late times after infection i.n. with WR and A35Δ viruses. Furthermore, since A35 inhibits the generation of an anti-VACV immune response in terms of

VACV-specific antibody production, IFNγ secretion, and CTL activity, we assessed viral titers in the organs at time points after development of the specific immune response (later than day 6) to assess whether the immune response was more effective in controlling virus in A35Δ infected mice. On days 1, 2 and 3 pi, there were no reproducible significant differences in WR and A35Δ virus titers in the tissues infected early after infection: lung, brain, nose, blood and spleen (data not shown). However, on days 6-9 pi, WR infected mice usually had approximately 2-to 10-fold higher viral titers in most organs (sometimes more than 100-fold) than A35Δ (Fig 28), suggesting that the improved immune response in A35Δ infected animals was better controlling viral replication. Peak titers were reached on day 7 for most organs, and after day 9 in these sublethally infected animals, titers dropped precipitously in the organs from both WR- and A35Δ-infected mice.

Protection from Lethal Challenge

We wanted to determine whether the A35Δ virus would act as a protective vaccine. Since immune recognition of A35 epitopes has not been found in the analysis of human or mouse B (antibody) or T cells (88, 153), we hypothesized that its deletion would not harm vaccine efficacy. Further, the improved immune responses (Figs 23-26) suggest that, in addition to being safer (Figs 21-22), the A35Δ virus might be a more efficacious vaccine. Mice were infected/vaccinated i.n. with a low dose of WR or A35Δ virus (10^3 pfu/mouse) or mock-vaccinated with PBS. Four weeks later, the mice were challenged with a high dose (LD_{50} x 450) of virulent WR virus. Mock-vaccinated mice rapidly lost weight, and all died by 4 days post challenge (Fig 29a). However all WR- or A35Δ-vaccinated mice survived to 3 weeks with only slight weight loss immediately after challenge. The A35Δ virus mutant virus delivered i.p. also protected mice from virulent WR challenge (LD_{50} x 600) as well as the WR virus vaccination

(Fig 29b). We tested vaccination over a range from 10^4 to 10^7 i.p., and 10^3 to 10^5 i.n., with challenge up to (LD_{50} x 1000), and found that in every case the A35Δ-vaccinated mice were fully protected from weight loss and death, and protected as well as the surviving WR-vaccinated mice. We therefore predict that A35Δ mutant viruses will be superior vaccines.

Discussion

We have previously shown that the A35 gene is required for full virulence in the mouse respiratory infection model and that the protein is not required for viral replication (170). Here we demonstrate that the A35 protein is also important for virulence when introduced i.p. In the i.n. mouse model, our data here, together with our previous data (170), show that 10^6 pfu of A35Δ-mutant virus is required to reach the same level of weight loss caused by 10^3 pfu of WR virus, a 1000-fold difference. It is difficult to compare the virulence of various gene deletion mutants because many have not been tested in the i.n. model measuring weight loss; intracranial inoculations or measures of viral titers in organs have often been performed instead. Available data indicate that the A35Δ virus is similar in virulence to the IL-18 binding protein knock out virus (159), more attenuated than the A46R deletion mutant (195), and less attenuated than the E3L deletion, which causes a 1000-fold attenuation (216).

The A35 gene affects development of the host adaptive immune response. Compared to WR-infected mice, mice infected with the A35Δ virus had larger spleens, generated higher titers of VACV-specific Ig and neutralizing antibody, and developed higher numbers of VACV-specific IFNγ-secreting cells and improved CTL responses. The faster and greater magnitude immune response that developed in the A35Δ-infected/vaccinated mice correlated with reduced viral replication in tissues. It is important that viral titers 1 to 4 dpi (before development of a specific immune response) were not significantly different. Thus the control of virus replication

correlates with the enhanced immunogenicity of the A35Δ virus, suggesting that the immune response is responsible for decreases in virus titers. The relatively uniform reduction in viral load in multiple organs further implicates the immune control of virus rather than a tissue specific replication defect of the A35Δ mutant.

Interestingly, the improved immune response seen in the A35Δ-infected/vaccinated mice was most evident on early days (days 6 to 8) pi, suggesting that A35 delays the generation of virus-specific immunity allowing the virus an advantage in establishing a generalized infection. We have shown (Fig 22) that differences in weight loss between WR- and A35Δ virus-infected mice occur at the time when the specific adaptive immune response is developing in A35Δ-infected mice and delayed in WR-infected mice. This A35 mediated delay may allow enough time for the virus to replicate throughout the host organs and establish an infection in the mouse, resulting in weight loss and death. In contrast, the superior immune response in the A35Δ-infected mice results in a reduction in viral replication, clearance of the infection, and survival. While A35 seemed to delay T cell responses early, and the differences waned later during infection, the antibody response remained elevated in the A35Δ-infected mice to day 34 pi (the latest time point tested), indicating that the immune response is not only delayed by A35 in WR, but also persistently diminished even a month after infection. Impressively, this enhanced antibody response in A35Δ-infected mice occurs despite the increased antigen load and persistence of antigen in WR infected mice (demonstrated by viral titer data for days 6 to 8 pi).

Sublethal viral infection in a host may be simplistically considered in several overlapping steps; i) viral replication in tissues initially infected, ii) viral stimulation of and interaction with the innate immune system, iii) development of the specific immune response, iv) viral replication in target organs, and v) control of virus by immune mechanisms. Our data suggest that A35

interferes with the development of the specific immune response, because early viral replication up to day 4 is not diminished in the A35Δ, and the difference seen between A35Δ and WR occur with the same kinetics as the development of specific immunity. Since both B and T lymphocyte immunities are impeded by A35, it seems likely that A35 acts at an early stage in the initiation of a specific immune response, or potentially at the interface between innate and specific immunity. VACV is known to block MHC class II antigen presentation to CD4+ T lymphocytes (116). This would be predicted to result in smaller spleen size, which is indicative of decreased lymphoctye proliferation in this organ, and may also be expected to decrease Ig and CD8+ T lymphocyte responses that are dependent on CD4+ T helper cells. CD4+, CD8+ and B lymphocytes are known to be important for protection from poxvirus infections (14, 32, 68, 151, 224).

The fact that the A35Δ mutant virus is highly attenuated and more immunogenic suggests that deletion of A35 will create superior vaccine strains. Indeed, in a lethal i.n. challenge model, the A35Δ virus vaccine, even at low doses, was as efficacious as WR in multiple challenge experiments up to 1000 x LD_{50}. It is important not to delete viral genes that contain protective epitopes from vaccine strains. Several groups have identified the CD4+ (26) and CD8+ (55, 88, 97, 148, 208) T cell epitopes that confer protection to poxviruses. None of these studies has identified A35 as a T cell determinant. Other groups have analyzed human vaccinia immune globulin in an effort to determine the VACV antigens that it recognizes (48, 91). Again, A35 was not among the reactive VACV antigens. Thus removal of the A35 gene is anticipated to improve vaccine safety, while having no detrimental effect on the generation of a protective immune response in mammals. Indeed the immune response is enhanced in A35Δ-infected animals. This is important for the development of poxvirus vaccines, especially for post-

exposure vaccination, which requires a rapid immune response for protection. In addition, poxvirus platform vaccines for other infectious diseases and cancer will likely benefit from removal of the A35 gene.

Figures

Figure 21: Intraperitoneal infection of mice. Groups (n=5) of female BALB/c mice were infected i.p. with 10^7 pfu/mouse of WR or A35Δ virus, or mock-vaccinated with PBS, and weighed (g $\pm$SEM) at various time points pi. * Two mice died in the WR infected group.

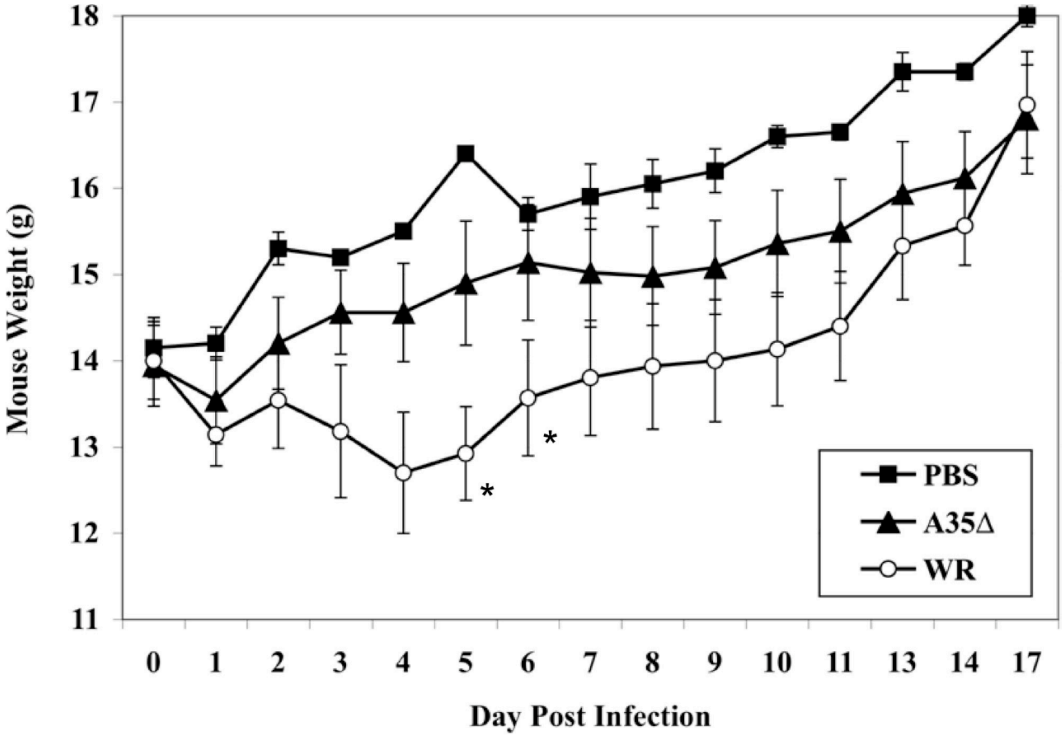

Mouse Weight (g)
18
17
16
15
14
13
12
11
0
1
2
3
4
5
6
7
8
9
10
11
13
14
17
Day Post Infection
PBS
A35Δ
WR
*
*

Figure 22: Intranasal infection of mice. Groups of mice were infected i.n. with 500 to 10^5 pfu/mouse of WR or A35Δ virus, or mock-vaccinated with PBS and weighed (g $\pm$SEM) for 4 weeks. Weights increased after day 9. *All mice (5/5) died in the group infected with 10^5 pfu WR, 6/7 mice died in the group infected with 10^4 pfu WR, and 1/11 mice died in the group infected with 10^3 pfu WR. No A35Δ-infected mice died.

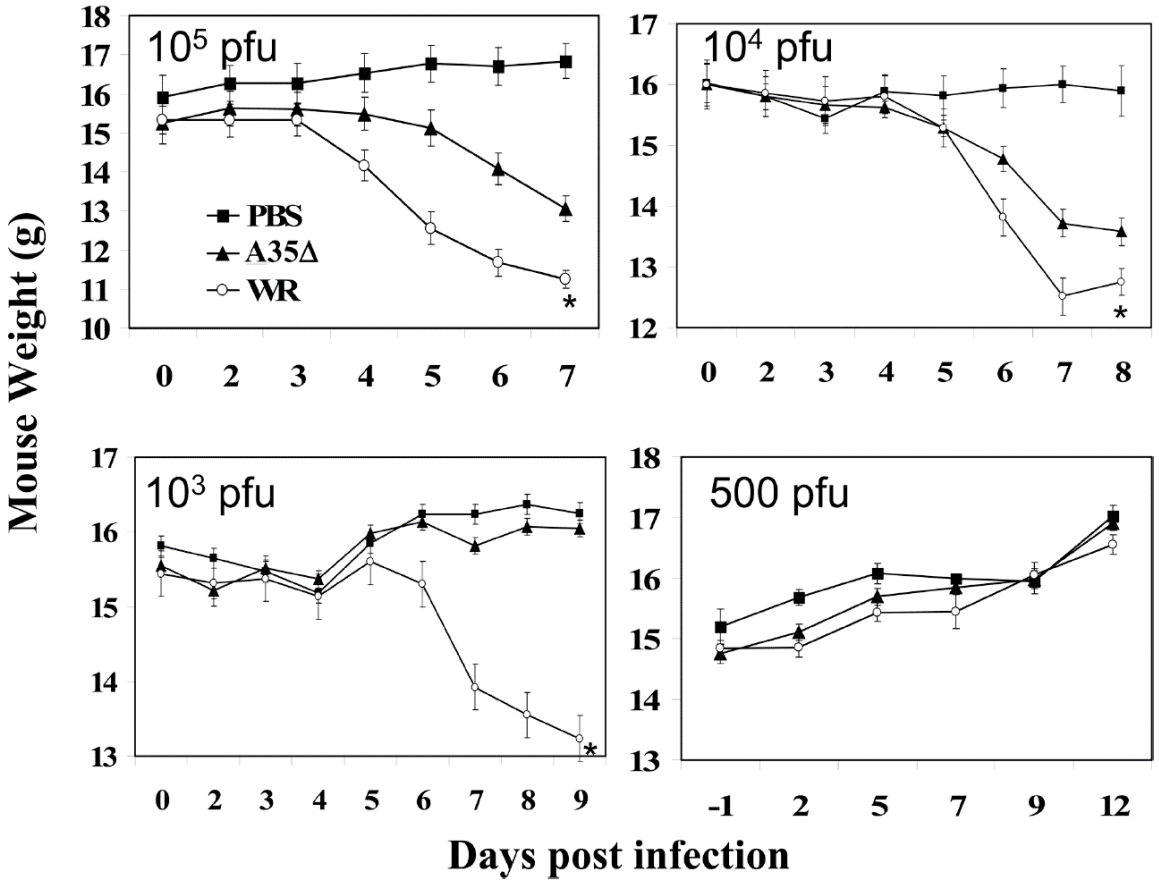

Mouse Weight (g)
10^5 pfu
10^4 pfu
10^3 pfu
500 pfu
PBS
A35Δ
WR
*
Days post infection

Figure 23: Spleen weights and counts. Mice were infected with WR or A35Δ virus i.n. on day 0 with 500 pfu/mouse. Five mice per infection group were sacrificed, their spleens were weighed on day 7 (a), and total splenocytes were counted (b). Data show the average (+/- SEM).

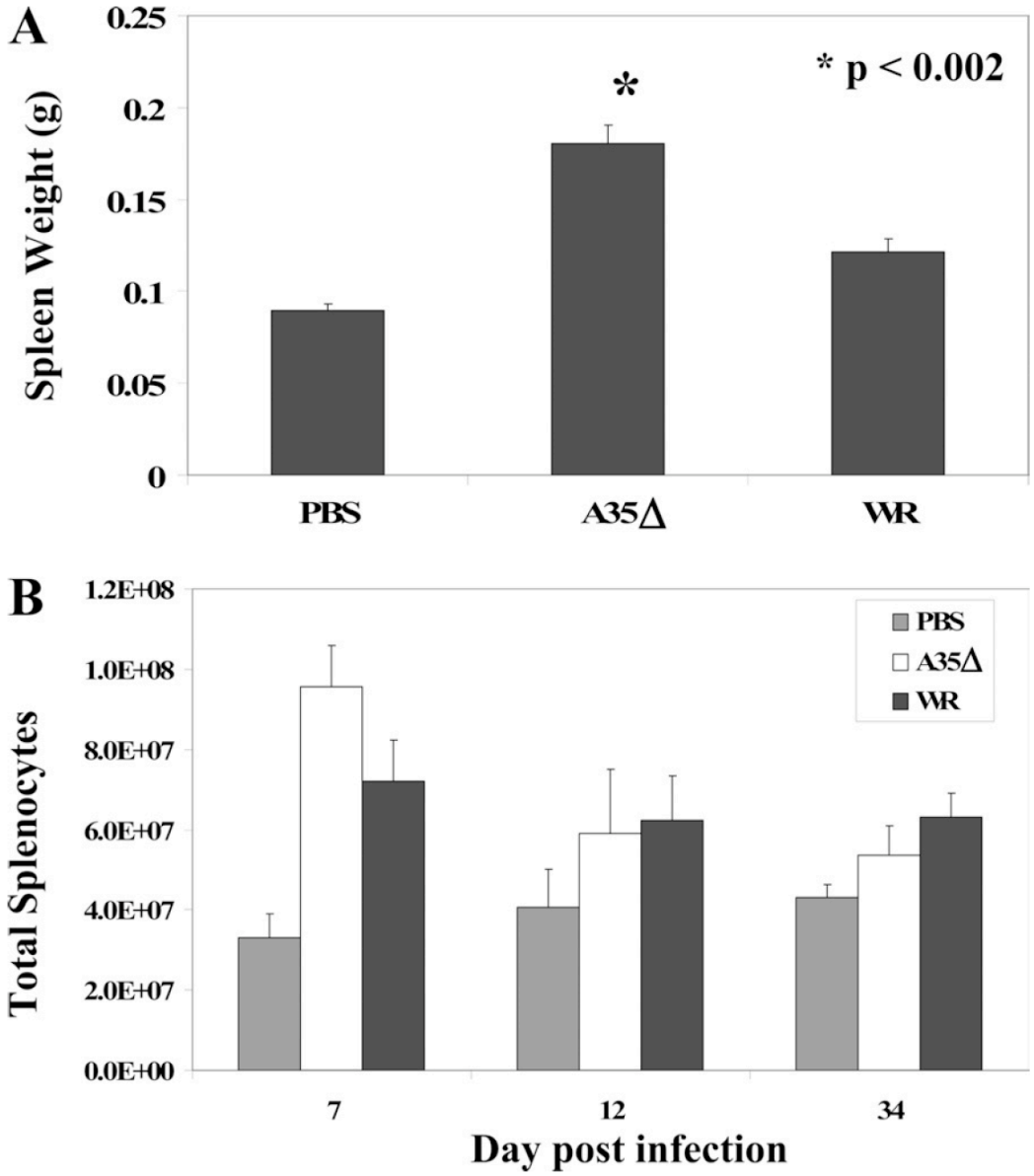

A
Spleen Weight (g)
0.25
0.2
0.15
0.1
0.05
0
*
* p < 0.002
PBS
A35Δ
WR
B
Total Splenocytes
1.2E+08
1.0E+08
8.0E+07
6.0E+07
4.0E+07
2.0E+07
0.0E+00
PBS
A35Δ
WR
7
12
34
Day post infection

Figure 24: VACV- and B5R-specific antibody response. Mice (n=5) were infected i.n. with WR or A35Δ virus, and blood was collected on various days pi. Serum reactivity was measured by ELISA on VACV-coated plates for day 7 (a) or day 34 (b) sera, or on purified B5R-coated plates (c) for day 7 pi sera. Data show the average absorbances (+/- SEM). OD405, optical density at 405 nm.

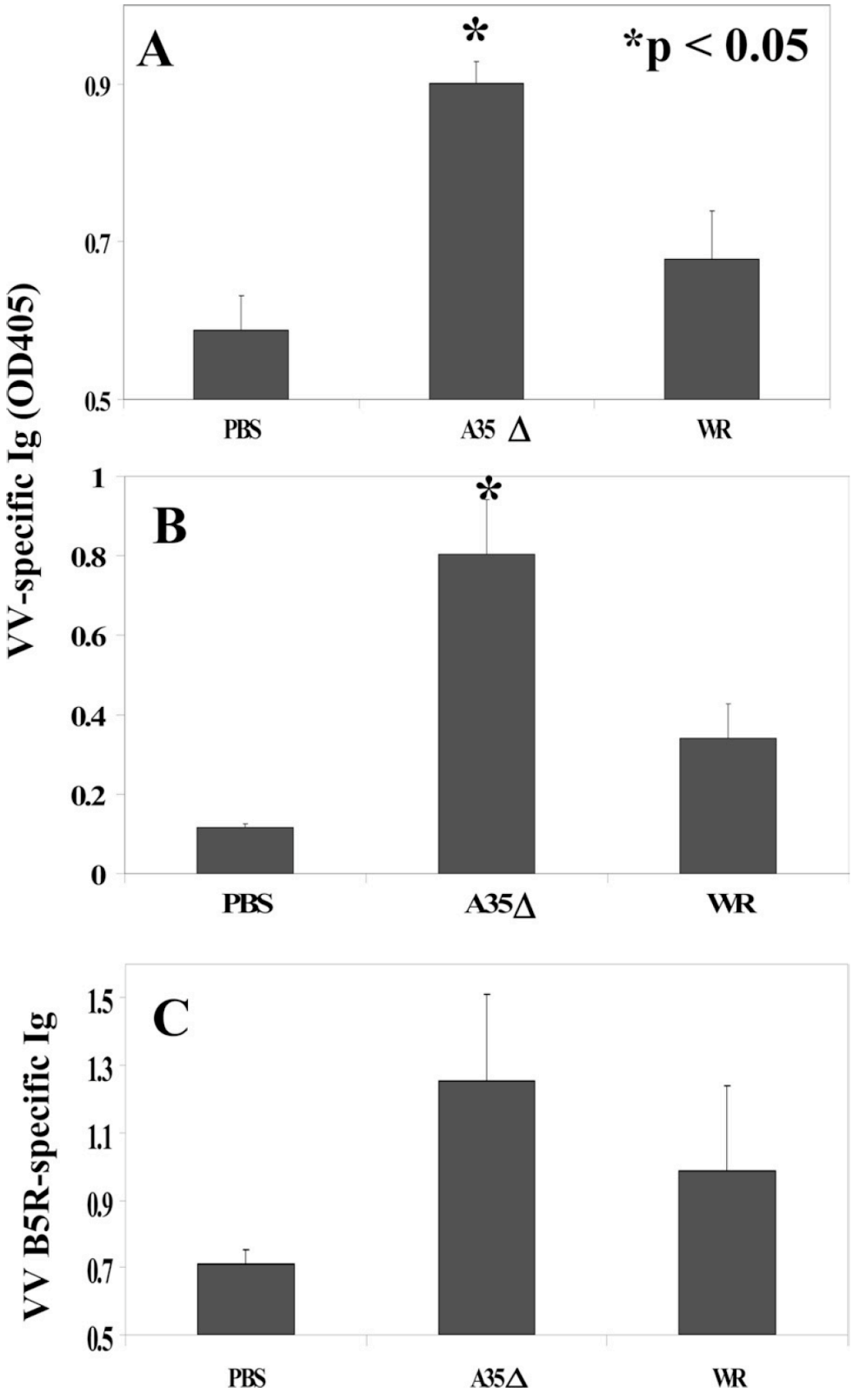

A
*
*p < 0.05
0.9
0.7
0.5
PBS
A35 Δ
WR
B
*
1
0.8
0.6
0.4
0.2
0
PBS
A35Δ
WR
VV-specific Ig (OD405)
C
1.5
1.3
1.1
0.9
0.7
0.5
PBS
A35Δ
WR
VV B5R-specific Ig

Figure 25: VACV-specific IFNγ-producing cells. On days 6, 7 and 8 pi (i.n.), the spleens from five mice/group were harvested and splenocytes were analyzed by ELISPOT for virus-specific IFNγ production 48 h after stimulation with VACV-WR virus. Data show the average number of spots (+/- SEM).

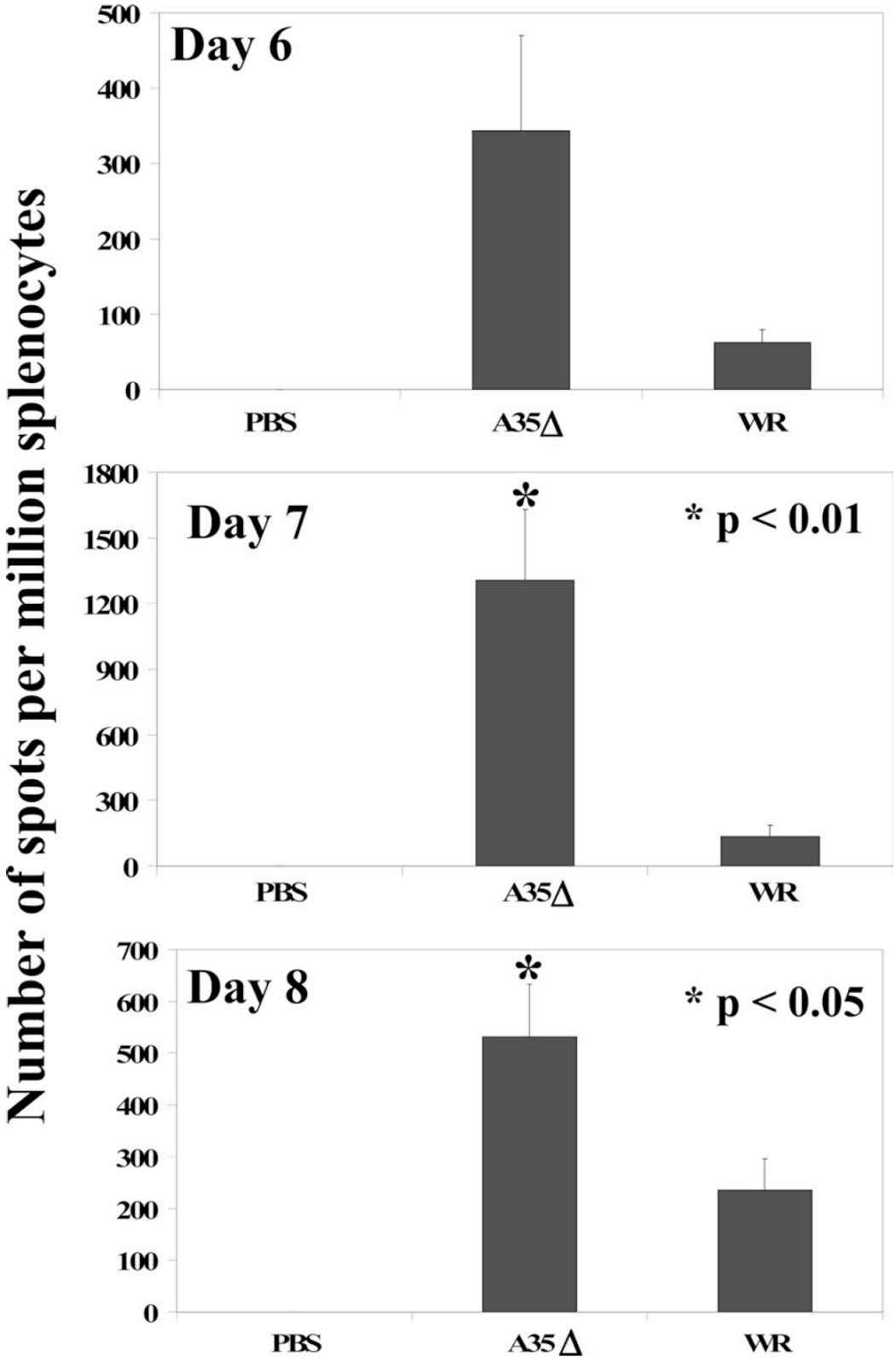

Number of spots per million splenocytes
Day 6
500
400
300
200
100
0
PBS
A35Δ
WR
Day 7
*
* p < 0.01
1800
1500
1200
900
600
300
0
PBS
A35Δ
WR
Day 8
*
* p < 0.05
700
600
500
400
300
200
100
0
PBS
A35Δ
WR

Figure 26: Cytotoxic T lymphocytes. Mice were infected i.n. on day 0 with 500 pfu/mouse of WR or A35Δ virus. On day 7 pi, the spleens from five mice/group were harvested and the splenocytes were incubated with WR-infected (MOI=5 for 3 h) P815 cells as targets. After 6 h, supernatants were collected and analyzed for the presence of LDH. Data show the average lysis (+/- SD). OD492, optical density at 492 nm.

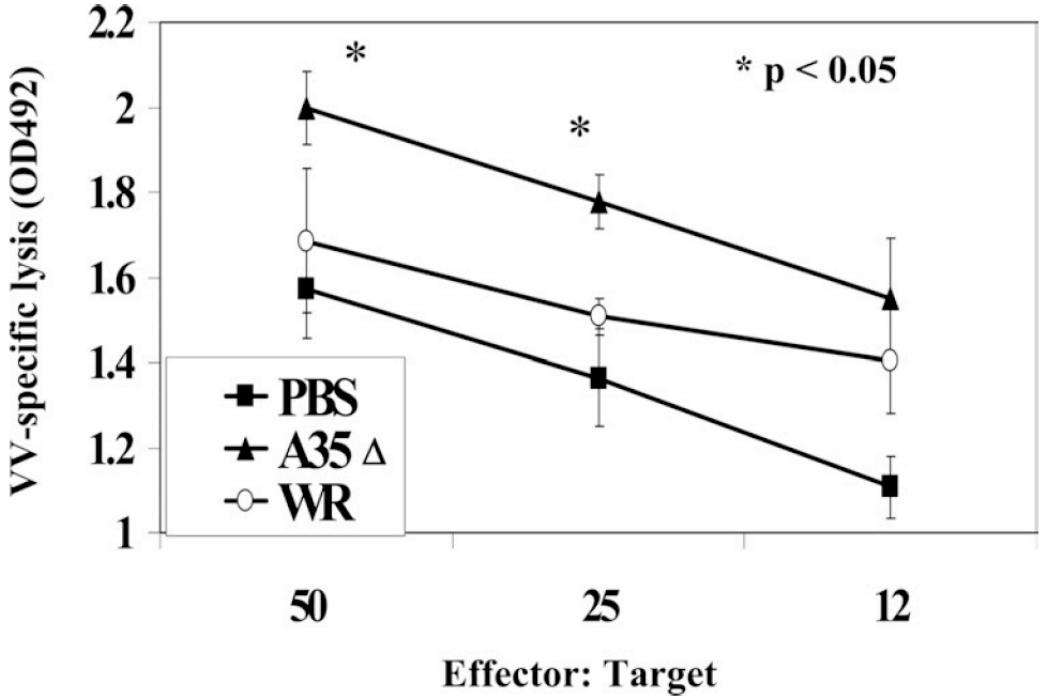

2.2
2
1.8
1.6
1.4
1.2
1
VV-specific lysis (OD492)
*
*
* p < 0.05
PBS
A35 Δ
WR
50
25
12
Effector: Target

Figure 27: Cytokines in blood. Mice were infected i.n. on day 0 with 500 pfu/mouse of WR or A35Δ virus or PBS, and sera were collected on day 2 pi and analyzed for cytokine quantities (+/- SEM) by using a Luminex Bioplex assay. TNFα values were divided by 20 to better show all data.

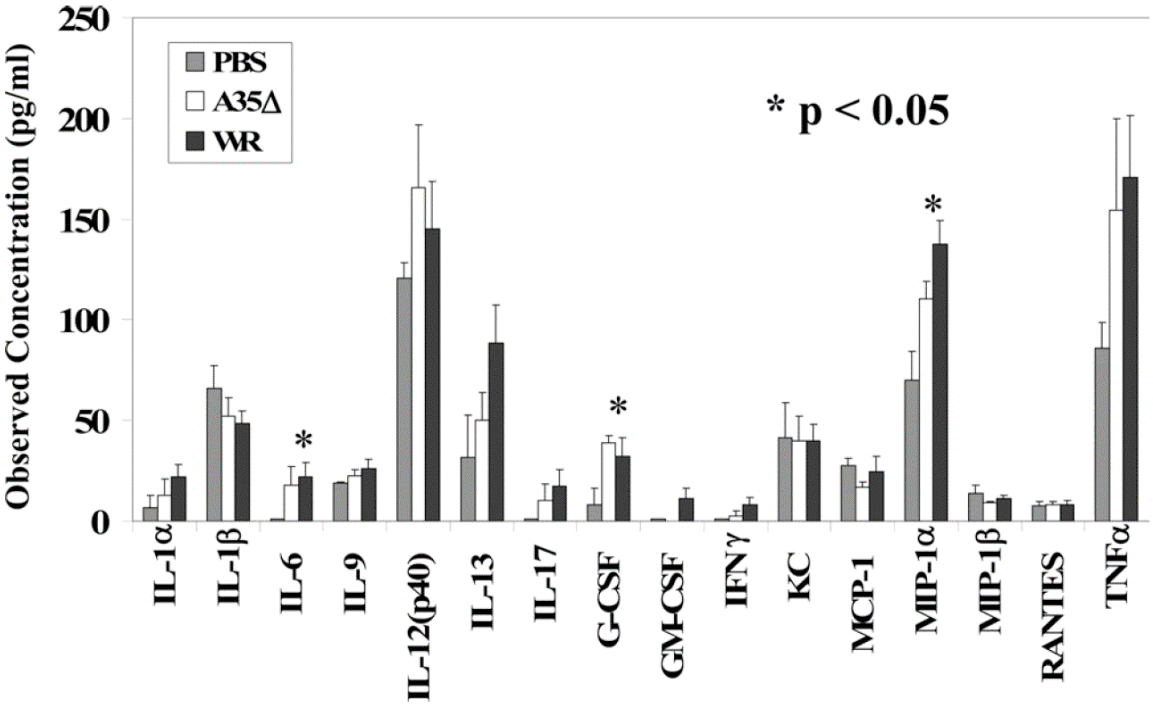

Observed Concentration (pg/ml)
250
200
150
100
50
0
PBS
A35Δ
WR
* p < 0.05
*
*
*
IL-1α
IL-1β
IL-6
IL-9
IL-12(p40)
IL-13
IL-17
G-CSF
GM-CSF
IFNγ
KC
MCP-1
MIP-1α
MIP-1β
RANTES
TNFα

Figure 28: Viral titers in organs. Mice were infected i.n. with 500 pfu/mouse of WR or A35Δ virus, and organs were harvested from five mice per group per day into 1 ml cold RPMI medium. To quantify viral replication, organs were freeze/thawed three times, homogenized, and sonicated before titration on BS-C-1 cells. Monolayers were stained ~40 h later with crystal violet and the plaques were counted. Data show the average (+/- SEM). Lung and nose titers were divided by 2, and heart, liver, kidney were multiplied by 20, 50, and 100 respectively in order to make data more visible.

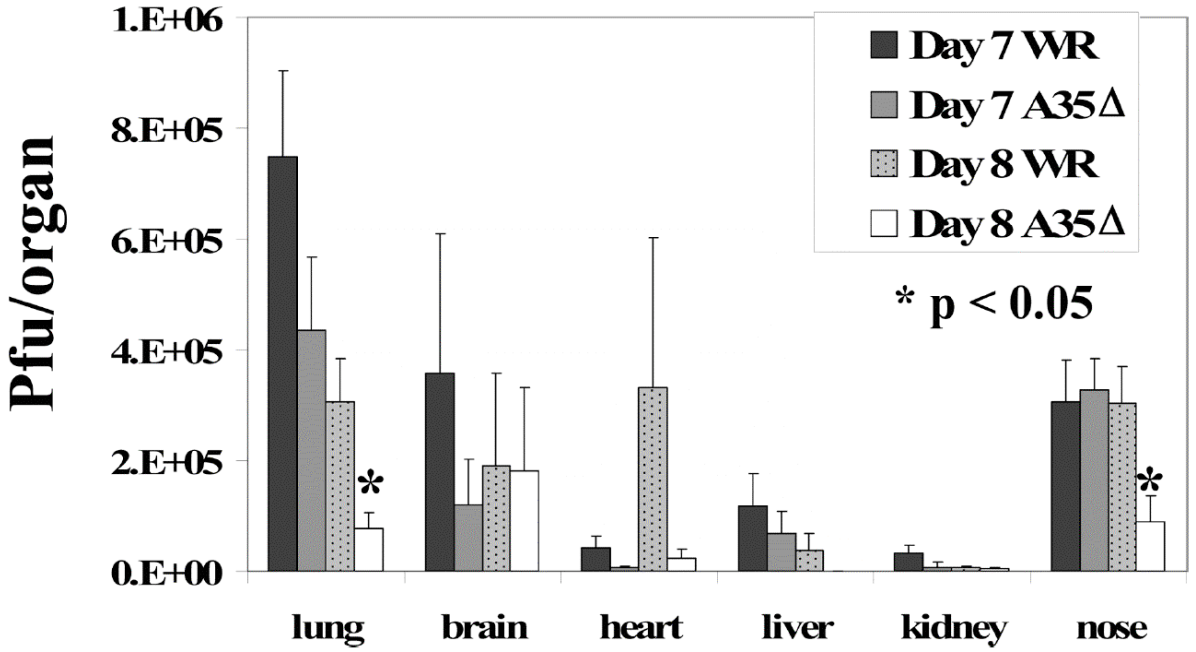
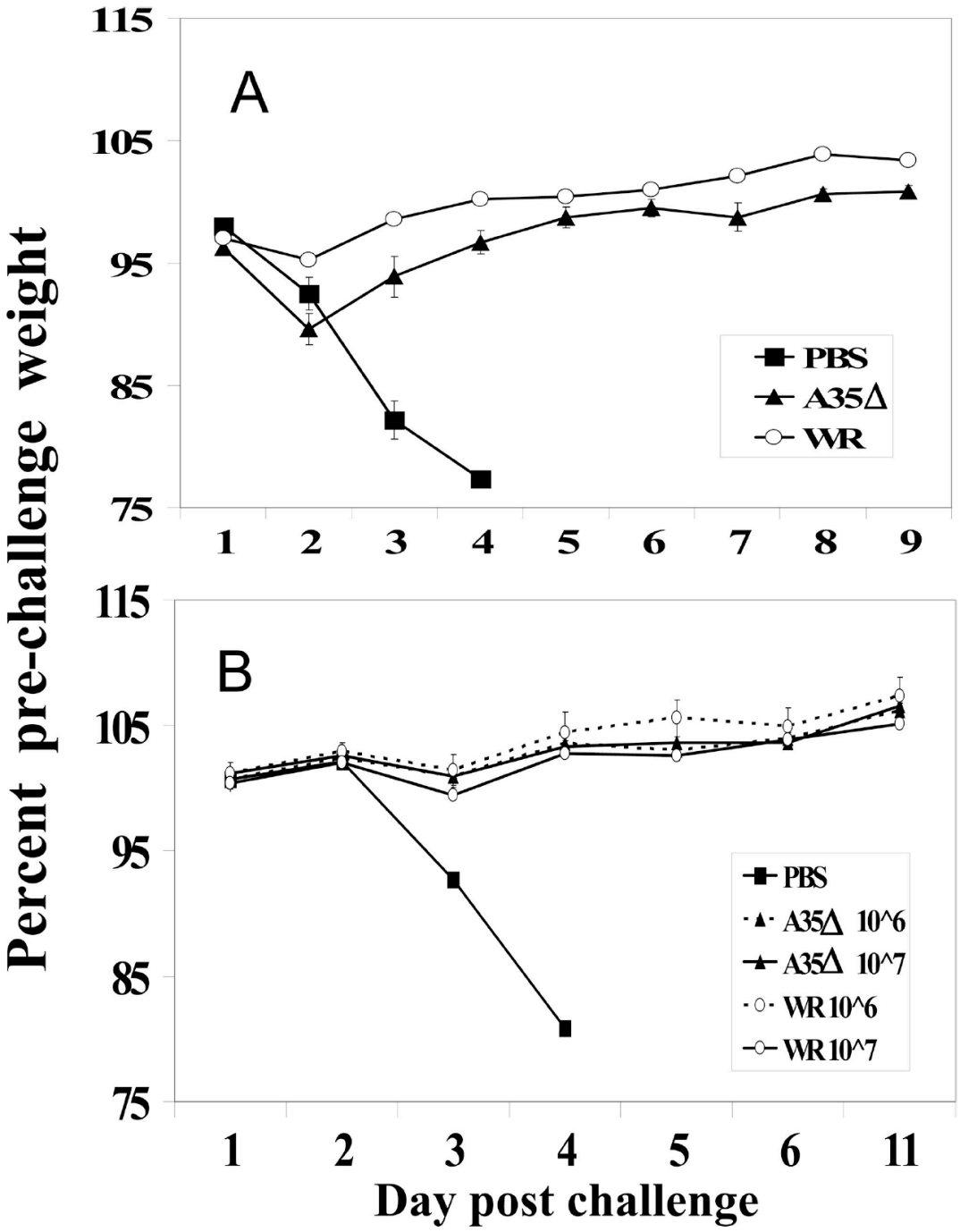

Pfu/organ
1.E+06
8.E+05
6.E+05
4.E+05
2.E+05
0.E+00
Day 7 WR
Day 7 A35Δ
Day 8 WR
Day 8 A35Δ
* p < 0.05
*
*
lung
brain
heart
liver
kidney
nose

Figure 29: Protection from lethal challenge. (a) Mice were vaccinated i.n. on day 0 with 1000 pfu of WR or A35Δ virus, or mock-vaccinated with PBS. Four weeks later, the mice were challenged i.n. with LD_{50} x 450 virulent WR virus. (b) Mice were vaccinated i.p. with 10^6 or 10^7 pfu and challenged 4 weeks post vaccination with LD_{50} x 600 WR.


A
B
Percent pre-challenge weight
Day post challenge
115
105
95
85
75
1
2
3
4
5
6
7
8
9
11
PBS
A35Δ
WR
A35Δ 10^6
A35Δ 10^7
WR 10^6
WR 10^7

CHAPTER 5: DELETION OF THE A35 GENE FROM MODIFIED VACCINIA ANKARA INCREASES IMMUNOGENICITY, ISOTYPE SWITCHING, AND CREATES AN EFFECTIVE POXVIRUS VACCINE

Abstract

The Vaccinia virus (VACV) A35 gene (Copenhagen designation) is highly conserved in mammalian tropic poxviruses and is an important virulence factor in the mouse model, but it is not required for replication in the VACV Western Reserve strain. Futhermore, the protein mediates suppression of *in vitro* MHC class II-mediated antigen presentation and suppresses immune responses in mice. The Modified Vaccinia Ankara (MVA) virus has been used as a safer alternative poxvirus vaccine; however, concerns about its immunogencity and efficacy have been raised. Herein, we show that A35 is not required for the normal viral spread and replication of MVA in BHK-21 cells, and that infection of mice with an MVA A35 deletion mutant (MVA35Δ) virus enhanced B and T lymphocyte responses compared to MVA. Specifically, mice infected with the MVA35Δ virus had enhanced class switching to IgG isotypes and increased numbers of virus-specific IFNγ- secreting splenocytes. Both MVA and the MVA35Δ virus were able to protect mice equally from a lethal challenge (500 x LD_{50}). Our data suggest that the deletion of the A35 gene may be an effective strategy to enhance the immunogenicity of MVA-based vaccines without decreasing efficacy.

Introduction

Vaccinia virus (VACV) was used as the live virus vaccine during the smallpox eradication campaign, and its use ultimately led to the eradication of this fatal disease (124). Despite being phenomenally successful, vaccination of the general public was discontinued due to the unacceptably high rate of adverse events, including disseminated vaccinia, progressive

vaccinia, eczema vaccinatum, and encephalitis (111). It has been estimated that 25% of the current population should not be vaccinated due to immunosuppression, inflammatory skin conditions, pregnancy, or heart abnormalities (60, 96, 211), and 1 out of every 450 people vaccinated will have a serious adverse reaction resulting in hospitalization (29).

Since the discontinuation of widespread poxvirus vaccination, there has been an increase in the incidence of human poxvirus infections throughout the world, including buffalopox in India (100), Cantagalo virus in South America (45, 185) and cowpox in Europe (107). The most dangerous emerging poxvirus is monkeypox, which is a zoonotic poxvirus that causes a smallpox-like illness and has a 10% fatality rate in humans. Monkeypox is endemic to Africa, but in 2003 there was an outbreak in the United States that was associated with the importation of African rodents (34, 112). This outbreak resulted in approximately 80 cases, but because this was an attenuated strain, no deaths occurred (34, 115). In addition to the threat of emerging poxvirus infections, there is also concern that the smallpox virus could be used as a biowarfare agent, resulting in widespread sickness and mortality in a now largely unvaccinated population. Thus, safer poxvirus vaccines are needed in the face of emerging disease and bioterrorism.

A safer alternative to the traditional poxvirus vaccine is the use of attenuated, replication-deficient strains, such as Modified Vaccinia Ankara (MVA). Attenuation of this virus occurred after >570 serial passages in chicken embryo fibroblasts (CEF), resulting in deletion of approximately 25 kb of the VACV genome (136), including genes encoding immunomodulatory proteins (17), and the inability to replicate in most mammalian cells (17). MVA is being tested as a vaccine to protect against poxviruses (57, 67, 202), and is also being used as a platform vaccine for other infectious diseases and cancer (59, 105, 166). Poxviruses make good platform vaccines because they are easy to produce and stable, induce robust, long lasting cellular and

humoral immune responses, and can accommodate the insertion of large pieces of foreign DNA (169). MVA has been shown to be safe when administered to immunodeficient mice (221), non-human primates (201) and humans (125, 199). Recent vaccine trials have shown that MVA can be given safely to those infected with HIV (41) and those with atopic dermatitis (90), two populations that cannot receive the traditional vaccine. Thus, this attenuated VACV shows promise as a safer alternative poxvirus vaccine and also as a platform vaccine.

While MVA is clearly safer, there are concerns about its immunogenicity and efficacy (8). In studies comparing the immunogenicity of MVA to traditional poxvirus vaccines, MVA was unable to elicit the same levels of both antibody and IFNγ-producing cells as traditional poxvirus vaccine strains, even when 10-100 times more virus was administered, although both viruses offered similar levels of protection (14, 67, 221). The immunogenicity of MVA may especially be a concern in populations with an already weakened immune system, such as cancer patients. Data available suggest that multiple doses of MVA may need to be administered in order to achieve the level of immunogenicity obtained with traditional replication-competent strains (14). One strategy that can be used to enhance the immunogenicity of MVA is to identify and exclude genes that are immunosuppressive. The Copenhagen A35 gene (Western Reserve strain gene 158, MVA gene 146R) is one such gene.

We have previously shown that the poxvirus A35 gene is highly conserved in all sequenced mammalian-tropic poxviruses, including MVA (170). It is an important virulence factor in the mouse model, increasing virulence by nearly 1000-fold, but is not required for replication of the VACV Western Reserve (WR) strain (164, 170). In addition, we have shown that A35 suppresses *in vitro* MHC class II-restricted antigen presentation (162), and, following intranasal (i.n.) infection in mice, the protein mediates immunosuppression, significantly

decreasing virus-specific antibody production, IFNγ-secreting T cells, and CTL killing, resulting in the inability to control viral replication in target organs and thus contributing to morbidity and mortality of the host (164). Here, we characterize the A35 gene function in the background of the MVA genome.

Materials and Methods

Cells and Virus

VACV Western Reserve (WR) stocks were propagated using BS-C-1 cells in MEM containing 10% FBS as previously described (170). MVA and MVA35Δ stocks were propagated using BHK-21. P815 and BHK-21 cells (ATCC) were grown in DMEM containing 10% FBS.

Immunostaining of Virus Infected Cells

Immunostaining was performed as previously described (171). Briefly, BHK-21 cells were infected with a titration of either the MVA or MVA35Δ virus for 72 h. Cells were then incubated with a 1:1000 dilution of polyclonal VACV rabbit antiserum (BEI Resources NR629) for 1 h at 4^{o} C, followed by incubation with a 1:1000 dilution of goat anti-rabbit IgG-horseradish peroxidase (HRP) for 1 h at 4^{o} C. 1 ml of the following substrate solution was then added to the wells to visualize virus-infected cells: 12 μl of 30% H_2O_2 and 240 μl of dianisidine-saturated ethanol in 12 ml of PBS.

Construction of the MVA35Δ Mutant Virus

The DNA segment containing the A35 flanking regions and the *E. coli* xanthine guanine phosphoribosyl transferase (*gpt*) gene was amplified from the existing vA35Δ mutant made from WR (170) using the primer pair TCGTGTTCATGATCTTGTTC and TTGCCTAGACCGGATACTA. The PCR product was then transfected with *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methyl-sulfate (DOTAP; Roche) into BHK-21

cells infected with the MVA strain. The mutant viruses were selected in media containing mycophenolic acid (Sigma). Recombinant viruses (two independent mutant lines) were plaque purified three times by using the immunostaining protocol described above and crude stocks were propagated in BHK-21 cells containing 10% FBS using previously described methods (170). Crude virus lysates were prepped for PCR by incubation with an equal volume of 2x PCR buffer (0.9% IGEPAL, 0.9% Tween-20, 20 mM Tris-HCl, pH 8.3, 3 mM $MgCl_2$, 100 mM KCl) and 0.3 mg/mL Proteinase K for 30 m at 45^o C, followed by heat-inactivation of the Proteinase K for 10 m at 94^o C. The recombinant viruses were then PCR screened for the absence of A35 gene using the primers described above. All immunologic experiments were performed using both independently derived MVA35Δ mutant virus isolates to confirm that any difference seen compared to MVA was due to the deletion of the A35 gene.

Western Blotting

Western blots were performed as previously described using polyclonal rabbit anti-A35 sera (170).

One-Step Growth Curve

A one-step growth curve was performed to measure viral replication as previously described (170). Virus foci were enumerated using the immunostaining protocol already described above.

Mouse Vaccinations

Female BALB/c mice (n=5) were infected intramuscularly (i.m.) with 10^7 pfu MVA or one of the two MVA35Δ viruses in 100 μl, or mock infected with PBS. Titers were confirmed on the day of infection with the virus dilution used to infect the animals. For challenge experiments, mice were vaccinated i.m. as described above and then infected intranasally (i.n.) 4

weeks later with 500 x LD_{50} of virulent WR (164). Mice were weighed and monitored daily for signs of illness, and were humanely euthanized using isoflurane overdose if 20% weight loss occurred. For experiments assessing the immune response, five mice from each virus group were sacrificed and the spleens were collected in ice cold RPMI. Splenocytes were obtained using previously described methods (36, 164). Blood was collected using a cardiac stick, followed by centrifugation to separate out the antibody-containing serum from the RBCs. All experimental protocols were approved by the Animal Care and Use Committee of East Carolina University.

Enzyme-Linked Immunosorbent Assay (ELISA)

Antibody response was measured similar to our previous work (164). Ninety six-well ELISA plates (Immulon H2B Thermo Electron) were coated overnight with 0.1 μl/well (100 μl) crude WR virus in ELISA coating buffer (10.3 g H_2BO_4, 7.31 g NaCl, 1 l ddH_2O, pH 8.5) at 4°C. Plates were blocked with 1% FBS/PBS at room temperature for 30 min. Plates were washed with ELISA wash buffer (1x PBS, 0.02% Tween 20, 0.1% NaN3) and a titration of mouse sera from MVA or MVA35Δ-vaccinated mice (n=5) was added. Plates were incubated at room temperature for 2 h and washed. Alkaline phosphatase (AP)-conjugated goat anti-mouse Ig, IgM, IgG, IgG1, or IgG2a (Southern Biotech) was added and incubated at room temperature for 1 h. Plates were washed 3 times, developed (BioRad Alkaline Phosphate Substrate Kit) and absorbance was read at 405 nm.

IFNγ Elispot

Numbers of IFNγ secreting spleen cells were enumerated similar to our previous work (164). Ninety six-well plates (Immulon H2B Thermo Electron) were coated overnight with 0.2 μl anti-mouse IFNγ (1 mg/ml Pharmingen) at 4°C. Plates were washed with blocking buffer before adding a titration of murine splenocytes in RPMI 1640. Stimulation of splenocytes was achieved

by either the addition of WR virus only (multiplicity of infection (MOI)=2) or with the use of 50,000 WR-infected (MOI=3) P815 stimulator cells, followed by incubation for 40 h at 37°C. Plates were then washed and incubated with 0.4 μl biotinylated rat anti-mouse IFNγ (Pharmingen 0.5 mg/ml) for 2 h at 37°C. Plates were washed again and incubated with streptavidin-AP for 1 h at 37°C. Plates were developed with agarose/BCIP/AMP mixture, and spots were counted using a dissecting microscope or color intensity was read at an absorbance of 492 nm.

Flow Cytometry

Cell types present in spleens were analyzed by flow cytometry. Splenocytes (10^6) from MVA or MVA35Δ-vaccinated mice (n=5) were fixed in 3% paraformaldehyde on ice for 5 min, washed, and then incubated for 15 min with an anti-Fc block (BD Pharmingen) to block non-specific binding of antibodies to Fc receptors. The following anti-mouse allophycocyanin (APC)-, Pacific Blue (PacBl)-, phycoerythrin (PE)-, or phycoerythrin-cyanine 7 (PE-Cy7)-conjugated monoclonal antibodies were then added to the samples for 45 min on ice: CD8a/Lyt-2 (clone 53-6.7, Southern Biotech); CD11b (clone M1/70, eBioscience); CD11c (clone N418, eBioscience); CD45R/B220 (clone 30-F11; eBioscience); CD49b (Dx5, NK marker; BD Pharmingen); Ly-6G/Ly-6C (granulocyte marker, clone RB6-8C5; BD Pharmingen); F480 (macrophage marker; Caltag Labs); MHC II (I-A/I-E, clone M5/114.15.2; eBioscience). Samples were run on an LSR II flow cytometer and data were analyzed using the FACS Diva software.

Statistical Analyses

Experiments were repeated at least 2 times and representative data are shown. A two-tailed Student's t test was used to compare groups. p values < 0.05 were considered significant.

Results

Molecular Characterization of MVA35Δ

We previously have shown that A35 in VACV strain Western Reserve (WR) inhibits *in vitro* MHC II-restricted antigen presentation (162) and suppresses both the T and B lymphocyte response in mice infected i.n. (164). We next wished to determine if removal of A35 from an attenuated vaccine strain would affect replication or increase immunogenicity using the attenuated poxvirus strain MVA. To determine the role of A35 during infection with MVA, a mutant virus missing the A35 gene was constructed. Similar to our previous A35 deletion mutant in the WR strain (designated A35Δ) (170), a PCR product containing the *E. coli gpt* gene with A35 flanking regions on either side was transfected into MVA-infected cells, and recombinant viruses were selected and purified. To confirm that A35 had been successfully knocked out of MVA, PCR analysis was performed using primers in the flanking regions. As shown in Fig 30a, PCR amplification of the parent MVA virus yielded a product of 1.5 kb, and PCR analysis of two independently isolated MVA35Δ mutants resulted in an approximately 400 base pair larger product when compared to the product from MVA. This is the expected size increase as a result of the insertion of *gpt*-containing PCR fragment. There was no wild type A35 detected in the mutants suggesting that the A35 gene had been successfully removed and indicating that the mutants were purified from MVA parental virus. To further confirm the absence of A35 from the recombinant viruses, a Western blot was performed to analyze reactivity with rabbit anti-A35 polyclonal sera (170) using lysates from MVA and MVA35Δ infected cells (Fig 30b). A protein band of about 23 kD (the approximate size of A35 (170)) was recognized in the lysates from the WR and MVA-infected cells, and this protein band was absent

in the A35Δ and MVA35Δ-infected cells. Together, these data indicated that A35 was successfully knocked out of MVA in these two independently derived mutants.

MVA35Δ Virus Replicates Normally

We previously reported that an A35Δ mutant virus made from WR formed normal size plaques on the African green monkey kidney cells BS-C-1 and replicated normally in several cell lines and mouse tissues (162, 164, 170), but it was possible that the loss of A35 in WR was being compensated for by another VACV protein. Since MVA is missing ~ 25 kb of the genome of VACV, we assessed whether A35 might be required for MVA replication. The MVA35Δ virus formed normal, MVA-sized foci on BHK-21 cells (Fig 31a), indicating that the loss of A35 had no apparent effect on normal viral replication and spread in these cells. To compare the kinetics of replication of the MVA35Δ virus with the parental MVA virus, we performed a one-step growth curve (Fig 31b). BHK-21 cells were infected (MOI=10) with either MVA35Δ or MVA for 2 h, and then inocula were removed. At various times post infection, cells and media were collected and the amount of virus in each was determined by titration on BHK-21 cells. For virus produced in the cells, there was <2-fold difference between the amount of virus produced by MVA and MVA35Δ at all time points tested. Similarly, for virus in the supernatants, MVA35Δ showed no decrease in virus production. Together, these data indicate that A35 is not required for the normal replication and release of MVA in BHK-21 cells and further our conclusion that A35 is not required for VACV replication or spread from cell to cell.

Infection of Mice with MVA and MVA35Δ

The loss of A35 in virulent WR results in a 1000-fold decrease in virulence in the mouse model (164). To determine whether the deletion of A35 had any effect on the avirulence of MVA, mice were infected intramuscularly (i.m.) with 10^7 pfu MVA or one of the two MVA35Δ

mutant viruses, a typical route of administration of MVA at a dose that has been shown to elicit a measurable immune response (14, 67, 221), or mock-infected with PBS as a control. Mice were weighed and monitored daily for lesions or signs of illness. As shown in Fig 32, mice infected with either MVA or MVA35Δ gained weight similar to PBS control and had no signs of illness. Similar results were seen with infection no matter which MVA35Δ virus was used. In contrast, intranasal (i.n.) vaccination of mice with 10^3 pfu virulent WR results in significant weight loss and death (164, 170). These data confirm that the avirulence of MVA is not altered by the loss of A35 from the genome.

Antibody Response to Infection with MVA35Δ

An effective poxvirus vaccine should elicit both a humoral and a cellular immune response, as both arms of the immune system have been shown to mediate aspects of protection against poxvirus infections (14, 32, 224). Our previous data with WR indicated that removal of A35 results in an increase in both cellular and humoral immunity (164), so we hypothesized that the removal of A35 from MVA would boost its immunogenicity, but this was not known. In WR is was possible that A35 interacted with another viral protein to be functional, and since MVA is has several large deletions, this interacting protein may have been deleted, rendering A35 non-functional. To begin to understand the effects of A35 in MVA on the immune response**,** we compared the production of anti-VACV specific Ig in the sera of mice infected with either MVA or MVA35Δ (Fig 33). Mice infected with MVA35Δ had significantly greater amounts of total VACV-specific serum Ig than those infected with MVA on days 7 and 10 pi. We also analyzed the production of various antibody isotypes on days 7 and 10 pi (Fig 34), and, by day 10, mice infected with MVA35Δ virus produced significantly more VACV-specific IgG, IgG1, and IgG2a than those infected with MVA. There was no significant difference in IgM production on these

days. Infection with either mutant MVA35Δ virus yielded similar results and representative data are shown. These data suggest that A35 in MVA decreases VACV-specific antibody production and inhibits isotype switching.

T Lymphocyte Response to Infection with MVA35Δ

In order to measure the effect of A35 in MVA on the cellular immune response to infection, we used the ELISPOT assay to measure IFNγ production as a result of stimulation with VACV antigens presented in MHC (42, 70, 180). IFNg is an important anti-poxvirus cytokine. Mice were infected and on day six pi their splenocytes were harvested and stimulated to produce IFNγ by adding virus directly to the splenocytes. There was significantly more VACV-specific IFNγ production in the cells of mice infected with MVA35Δ compared to the mice infected with the parental MVA (Fig 35a). On day 10 pi, there was a three-fold increase in the number of IFNγ producing spleen cells in the MVA35Δ group compared to the MVA group, but this difference did not reach statistical significance. Similar results were seen with both MVA35Δ mutant viruses and when splenocytes were stimulated with VACV-infected P815 cells. No IFNγ was produced by splenocytes from PBS control mice or in any of the groups without addition of virus or infected P815 cells, thus demonstrating the specificity of the response. Since CD8+ T cells produce most of the IFNγ following a VACV infection (224), we also measured the percentage of CD8+ T cells in the splenocytes of infected mice on days 7 and 10 pi (Fig 35b). We found that MVA infection significantly increased the percentage of CD8+ T cells in the spleens, but there was no difference between the MVA and MVA35Δ-infected groups.

Measurement of Cellular Subsets in the Spleens

We had previously noted that VACV WR infection induces splenomegaly, presumably as a systemic specific immune response develops, and that spleen size increased most when mice were infected with a virus that is missing the immunosuppressive A35 gene (164). To understand more about the mechanism of A35-mediated immunosuppression during infection with MVA, we performed a flow cytometry experiment to measure the percentage of cellular subsets within the spleens of MVA and MVA35Δ-infected mice. On day 6 pi, there was little change in splenocyte populations (Fig 36). There was a small but significant increase in the percentage of cells containing the macrophage marker CD11b+ and a marker for granulocytes in the splenocytes of the MVA35Δ-infected mice compared to MVA-infected mice (Fig 36a). The CD11b+ difference was maintained on day 8 pi (data not shown). Also, on day 8 pi, there was a small but significant increase in the percent of DX5+ natural killer (NK) cells found in the MVA35Δ group compared to the MVA group (data not shown). Since our *in vitro* data indicate that A35 specifically affects the APC (162), we also looked at APC subsets within the spleen, including B cells (B220+CD11c-), macrophages (CD11b+CD11c-), and dendritic cells (CD11b-CD11c+), and found that on day 6 pi, there was a small but significant increase in the percent of macrophages in the spleens of the MVA35Δ-infected mice compared to those infected with MVA (Fig 36b). There was no significant difference between the groups in the percentage of total MHC class II expressing cells (Fig 36a). Thus, infection with MVA results in a small but significant increase in cells expressing CD8 (Fig 35b), and A35 modestly reduces an infection-induced increase in the percentage of granulocytes and CD11b+ expressing cells in spleens (Fig 36).

MVA35Δ Protects Mice from Lethal Challenge

It was next determined whether the MVA35Δ virus would be an efficacious vaccine and protect mice from a lethal VACV challenge. Removal of A35 from the WR strain of VACV did not reduce vaccine efficacy (164). Many studies have been performed to determine the protective antigenic epitopes of VACV (88, 91), but only the most recent has identified A35 as containing possible CD8+ T cell epitopes (89). We therefore tested whether the removal of A35 from MVA would decrease vaccine efficacy due to the loss of predicted protective epitopes or perhaps increase efficacy due to enhanced immune responses. Mice were vaccinated i.m. with 10^7 MVA or MVA35Δ, or PBS as control, and four weeks later were challenged i.n. with 500 x LD_{50} virulent WR virus (Fig 37). PBS control mice quickly lost weight and all died by 4 days post challenge. All MVA or MVA35Δ vaccinated mice survived the challenge with no significant weight loss and no signs of illness. Similar data were obtained no matter which MVA35Δ virus was used to infect the mice. Together, these data support the hypothesis that an MVA-based vaccine that excludes the A35 gene will be both protective and highly immunogenic.

Discussion

We have previously shown that the VACV A35 protein in WR is a virulence factor in the mouse model, increasing virulence by nearly 1000 fold and decreasing immunogenicity (164, 170). In this manuscript, we provide a characterization of A35 in the highly attenuated VACV strain, MVA. In WR, we have not demonstrated a role for A35 in replication (164, 170), but it was possible that its loss was being compensated for by another redundant VACV gene. Since MVA is missing nearly 25 kb of the VACV genome (136), including most immunomodulatory genes (17), we tested whether A35 was required for replication of MVA. We could not uncover

a replication function for A35 in MVA using BHK-21 cells (Fig 31); however, A35 may have been retained by MVA because it is necessary for the replication of the virus in avian cells (CEF), the cells used during its attenuation. During its > 570 passages in CEFs, MVA lost many of the immunosuppressive genes that are found in VACV, including the secreted receptor homologs for IFNγ, IFNα, IFNβ, and TNF (17). It is interesting to note that MVA did retain a few immunoevasion genes, including the IL-1β receptor homolog (17) and A35 (170). It is unknown as to why only certain immunosuppressive genes were kept by MVA since these genes would not have been necessary for the survival of the virus in CEF. Perhaps these genes serve different functions for the virus in the avian host. We are currently performing experiments to determine whether A35 may be needed for the replication of MVA in avian cells.

Due to its avirulent phenotype, MVA is being tested as an alternative poxvirus vaccine and is also being used as a platform vaccine for delivering various other antigens, including those for cancer and HIV (59, 104, 166). Unfortunately, MVA fails to elicit the same levels of B and T cell responses as the traditional replicating poxvirus vaccines (14, 67, 221). This will especially be a concern when vaccinating persons with already weakened immune systems, such as those infected with HIV, cancer patients, and transplant recipients, as these individuals will require higher or multiple doses of the vaccine compared to those with a competent immune system. It is therefore important to develop strategies to increase the immunogenicity of MVA. Our previous work with A35 in WR suggested that the removal of A35 from MVA would result in a more immunogenic vaccine (164). It was therefore important to elucidate the functional effects of the A35 gene ortholog in this attenuated VACV strain. In WR, it was possible that A35 interacted with another viral protein and that this interaction was required for A35 to be functional. Since MVA is missing nearly 25 kb of the VACV genome (136), it was possible that

any protein that interacted with A35 may have been deleted, rendering A35 ineffective. We show here that A35 does indeed function to suppress the specific immune response in the background of the MVA genome. Specifically, i.m. infection of mice with MVA35Δ virus resulted in increased antibody production, isotype switching, and IFNγ secreting T cells. This is consistent with our previous work with A35 in WR (164) and provides further evidence that that the removal of A35 from poxvirus-based vaccines will enhance their immunogenicity. In WR, the improved immune response in the absence of A35 correlated with improved control of virus replication in target organs (164), but since MVA does not replicate in mammalian tissues, we could not perform those experiments here. We also show that MVA35Δ virus can protect mice as well as MVA from a virulent challenge. While many studies have been performed in an attempt to identify the protective antigenic B and T cell epitopes of VACV (48, 88), only the most recent of these has identified A35 as containing antigenic CD8+ T cell epitopes (89). Our data presented here (and previous data with WR (164)) indicate that the removal of A35 has no measurable effect on vaccine efficacy in our challenge model, as the MVA35Δ virus protected mice as well as MVA from a virulent challenge (Fig 37).

A35 in MVA decreased the production of VACV-specific IFNγ-secreting cells (Fig 35a), but it did not affect the percentage of CD8+ T cells in the spleens of these mice (Fig 35b). NK and CD4+ and CD8+ T cells can secrete IFNγ, but studies have implicated the CD8+ T cells as the main IFNγ secreting cells during VACV infection (57, 221, 224). Our results indicate that either A35 reduces the amount of virus specific IFNγ secretion without reducing the percent of CD8+ T cells in the spleen, suggesting that A35 either decreases the amount of IFNγ secretion on a per cell basis or blocks the production of this cytokine from another subset of IFNγ-producing cells, such as CD4+ T cells or NK cells. Interestingly, we did see a small but significant

decrease in the percent of NK cells in the spleens in the MVA-infected mice compared to those infected with MVA35Δ on day 8 pi. Splenocytes from infected mice will need to be stimulated with VACV and then double stained with antibodies to IFNγ and CD8, CD4, or DX5 in order to determine specifically which IFNγ-secreting cell type A35 is affecting.

A35 also inhibited the generation of VACV-specific antibody (Fig 33-34). We measured total Ig, IgM, IgG, IgG1, and IgG2a, and found that A35 had no effect on IgM production, but did significantly decrease Ig, IgG, IgG1, and IgG2a. IgG2a and IgG1 can be used as a measure of the Th1 and Th2 immune responses, respectively, (183). Compared to a Th2 response following VACV infection, a predominantly Th1 response is associated with smaller lesion size and decreased viral replication (150, 179). Furthermore, following VACV infection, persons with atopic dermatitis produce higher levels of cytokines and antibodies associated with the less protective Th2 response (11), resulting in uncontrolled replication of virus in the skin. We found no evidence that A35 was mediating immune deviation by skewing the immune response towards the less protective Th2 response since IgG1 production was not increased in the presence of A35. We also measured the levels of VACV-specific IgM, the first antibody to be produced during an infection, and found that A35 had no effect on the production of this antibody on days 7 and 10 pi. On these same days, there were significantly higher levels of the other antibody isotypes measured in the MVA35Δ-infected mice compared to the MVA-infected mice. These data indicate that mice infected with the MVA35Δ virus had enhanced switching of antibody to the more protective isotypes, such as IgG and its various subclasses.

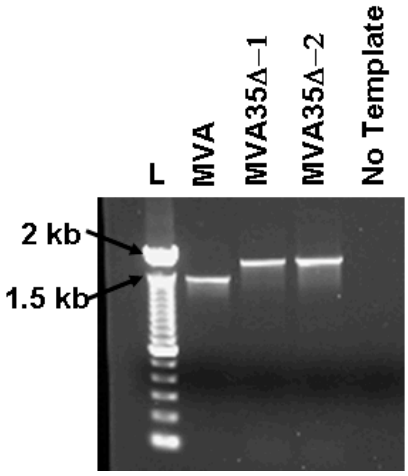
Our data with A35 indicate that the protein regulates the host adaptive immune response. The presence of A35 in both WR and MVA correlated with decreased T and B lymphocyte responses in mice. Our data using the WR virus model showed that the removal of the A35 gene

caused elevated antibody levels at least a month after vaccination (164), suggesting that A35 deletion mutant viruses may give effective protection for longer time periods. Since MHC class II antigen presentation to CD4+ T cells is necessary for the generation of optimal T and B cell immune responses in mice (224), it would be interesting to see if splenocytes from MVA- or WR-infected mice would be deficient in presenting antigen to CD4+ T cells *ex vivo*, as compared to MVA35Δ- and A35Δ-infected mice, similar to what we have observed following *in vitro* infection with VACV (162). It has been reported that splenic DCs from VACV-infected mice were deficient in presenting a model antigen to specific CD4+ T cells, but no VACV gene was identified as contributing to this effect in this model (227), so this would indeed be an exciting find. Nevertheless, the data presented further support that the removal of A35 from any poxvirus-based vaccine would result in increased antibody and T cell responses.

Figures

Figure 30: Molecular characterization of MVA35Δ. a) PCR. MVA-infected cells were transfected with a recombinant PCR fragment containing the *E. coli gpt* gene inserted between the A35 flanking regions and recombinant viruses were selected in mycophenolic acid-containing media. Virus crude stocks were PCR analyzed using primers in the A35 flanking regions. Wild type A35 locus yields a product of 1400 kb size and the mutants with gpt inserted yield a size of approx 1900 kb. b) Western blot showing that A35 is not expressed in MVA35Δ-infected cells. BHK-21 cells were infected with listed viruses at an MOI of 20 for 2 h and analyzed by SDS-PAGE. Blots were incubated with rabbit anti-A35 antibody at a 1:1000 dilution and developed with an anti-rabbit alkaline phosphatase-conjugated secondary antibody followed by incubation with the substrate Western Blue (Promega).

A.


L
MVA
MVA35Δ–1
MVA35Δ–2
No Template
2 kb
1.5 kb

B.

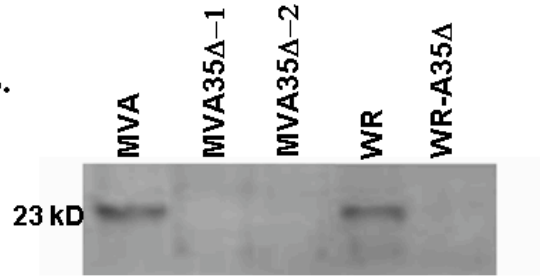

MVA
MVA35Δ–1
MVA35Δ–2
WR
WR-A35Δ
23 kD

Figure 31: A35 is not required for replication of MVA. a) MVA and MVA35Δ mutant viruses form similarly sized foci on BHK-21 monolayers. Virus-infected cells were visualized by immunostaining. b) One step growth curve. BHK-21 cells were infected (MOI=10) with MVA or MVA35Δ, and the amount of virus associated with cells and in the supernatants was titered on BHK-21 cells at various times post infection.

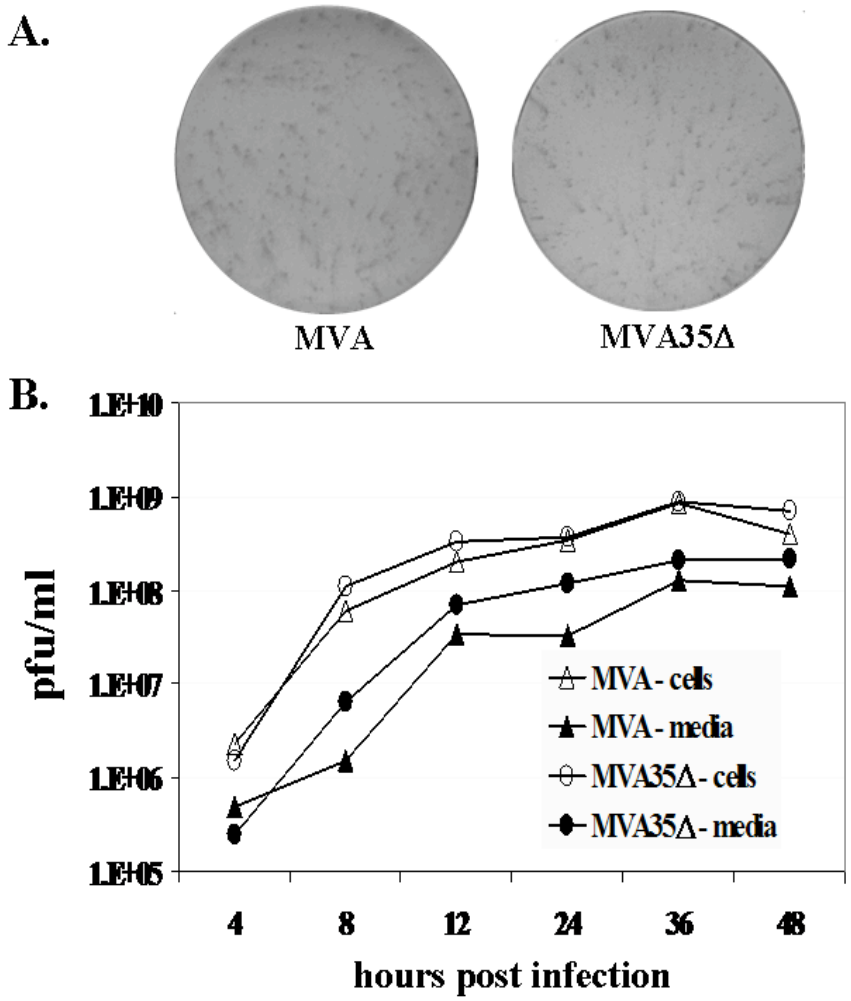

A.
MVA
MVA35Δ
B.
1.E+10
1.E+09
1.E+08
1.E+07
1.E+06
1.E+05
pfu/ml
MVA - cells
MVA - media
MVA35Δ - cells
MVA35Δ - media
4
8
12
24
36
48
hours post infection

Figure 32: Infection of mice with MVA and MVA35Δ. Groups of mice (n=5) were infected i.m. with 10^7 pfu/mouse of MVA or MVA35Δ virus, or mock-vaccinated with PBS and weighed (g $\pm$SD) for 4 weeks. No mice lost weight or showed signs of illness.

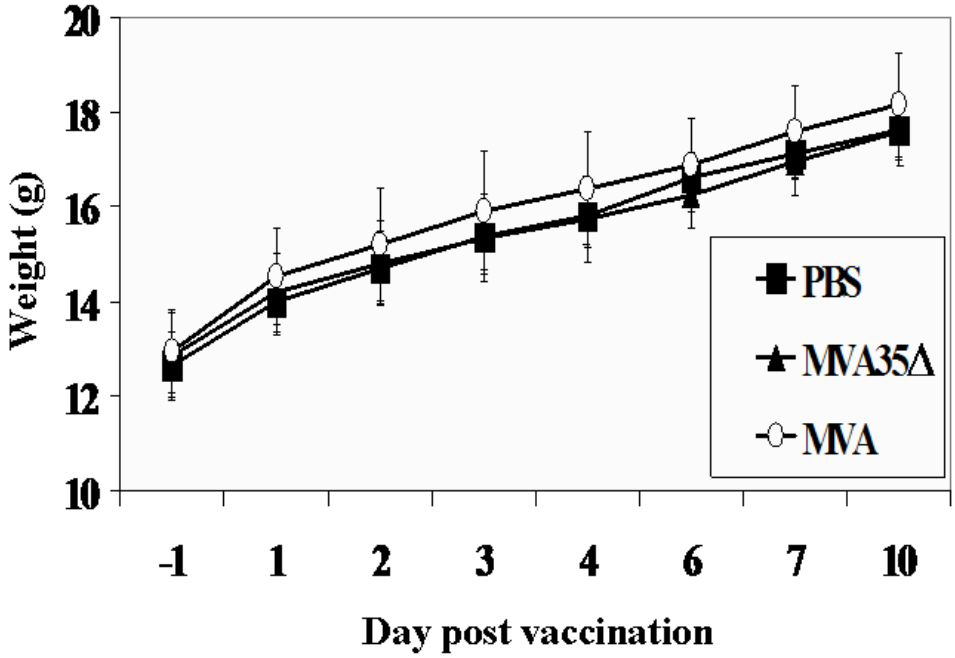

20
18
16
14
12
10
Weight (g)
-1
1
2
3
4
6
7
10
Day post vaccination
PBS
MVA35Δ
MVA

Figure 33: VACV-specific antibody response. Mice (PBS n=3 day 7, n=4 day 10; MVA35Δ n=4; MVA n=4) were infected i.m. with MVA or MVA35Δ virus, and blood was collected on various days pi. Total VACV-Ig was measured by ELISA on VACV coated plates for day 7 and 10. Data show the average absorbance at 405 nm (+/- SEM).

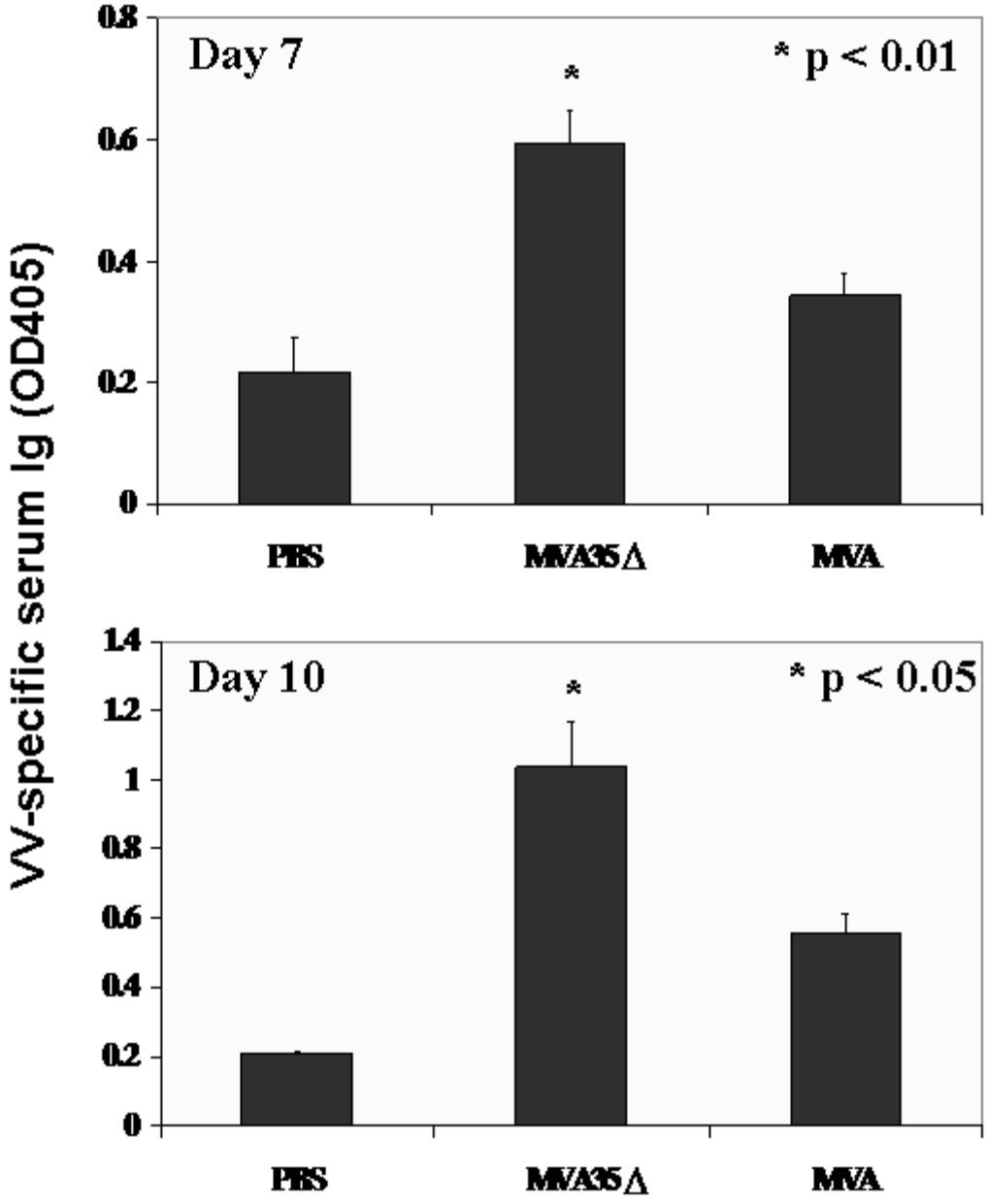

Day 7
* p < 0.01
*
0.8
0.6
0.4
0.2
0
PBS
MVA35Δ
MVA
Day 10
* p < 0.05
*
1.4
1.2
1
0.8
0.6
0.4
0.2
0
PBS
MVA35Δ
MVA
VV-specific serum Ig (OD405)

Figure 34: VACV-specific antibody isotype response. Mice (n=3-5) were infected i.m. with MVA or MVA35Δ virus, and blood was collected on various days pi. VACV-specific IgM, IgG, IgG1, and IgG2a were measured by ELISA on VACV coated plates for day 7 and 10. Data show the average absorbance at 405 nm (+/- SEM).

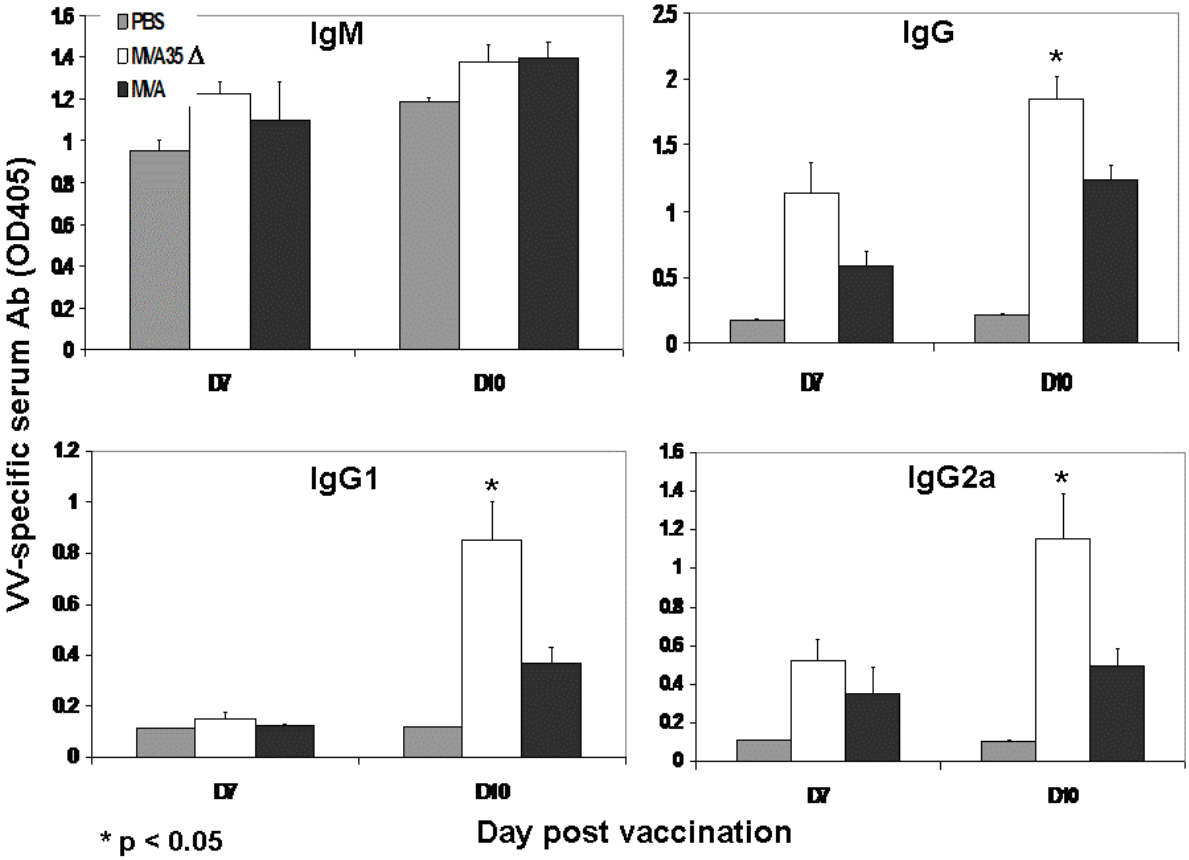
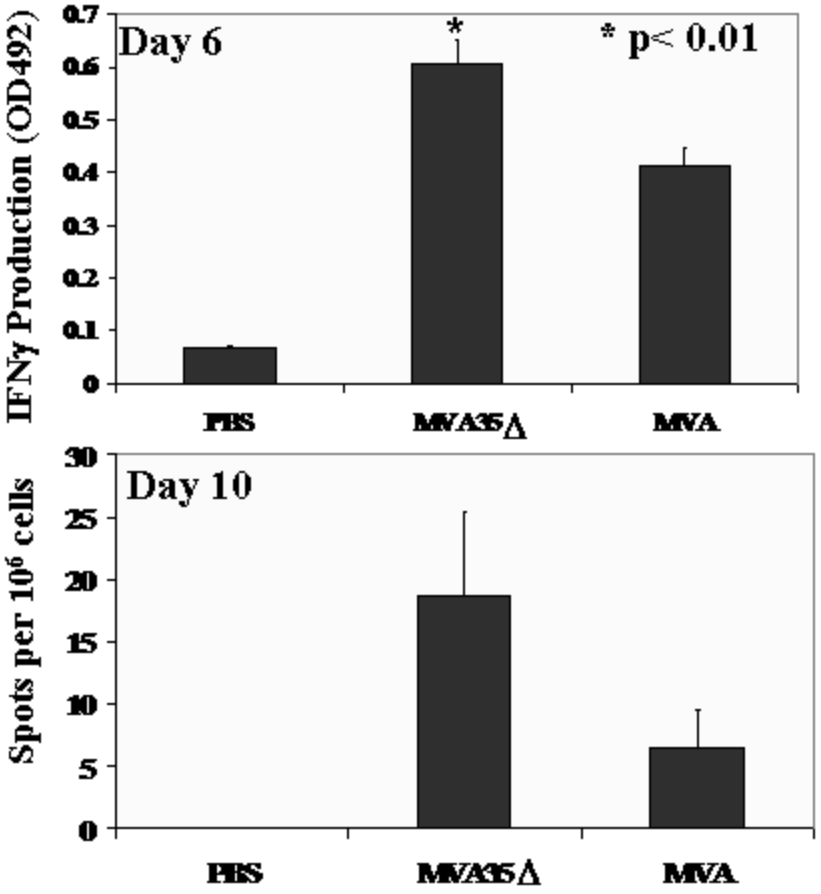

PBS
MVA35 Δ
MVA
IgM
IgG
IgG1
IgG2a
*
D7
D10
VV-specific serum Ab (OD405)
* p < 0.05
Day post vaccination

Figure 35: VACV-specific IFNγ-producing cells. a) IFNγ production. On days 6 and 10 pi (i.m.), the spleens from 5 mice/group were harvested and splenocytes were analyzed by ELISPOT for virus-specific IFNγ production 48 h after stimulation with VACV-WR virus. b) CD8+ cells. On days 7 and 10 pi, spleens from vaccinated mice were analyzed by flow cytometry for the percentage of CD8+ T cells. Data show the average (+/- SEM).

A.


Day 6
* p< 0.01
IFNγ Production (OD492)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
PBS
MVA35Δ
MVA
Day 10
Spots per 10^6 cells
30
25
20
15
10
5
0
PBS
MVA35Δ
MVA

B.

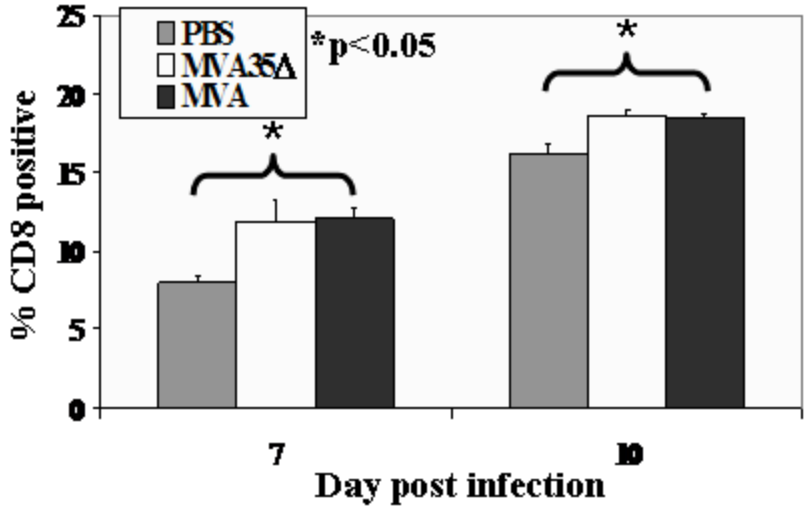

PBS
MVA35Δ
MVA
*p<0.05
% CD8 positive
25
20
15
10
5
0
7
10
Day post infection

Figure 36: Cellular subsets in spleens. On day 6 pi, spleens from MVA and MVA35Δ-infected mice (n=5) were stained for various cell surface markers to enumerate percentage of different cell types. Data show average percentage (+/- SEM). Gran, granulocytes; NK, natural killer; MO, macrophage; DC, dendritic cell.

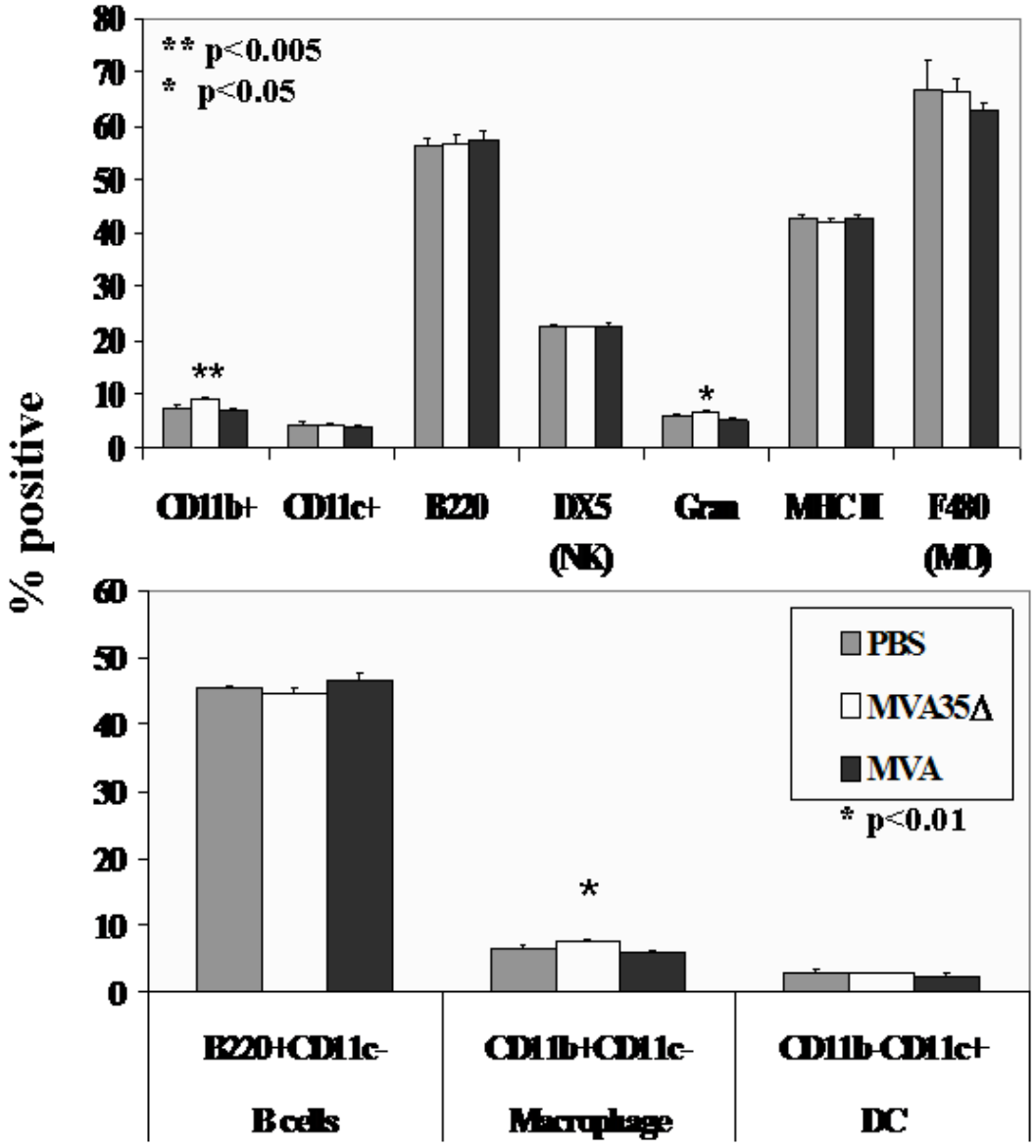
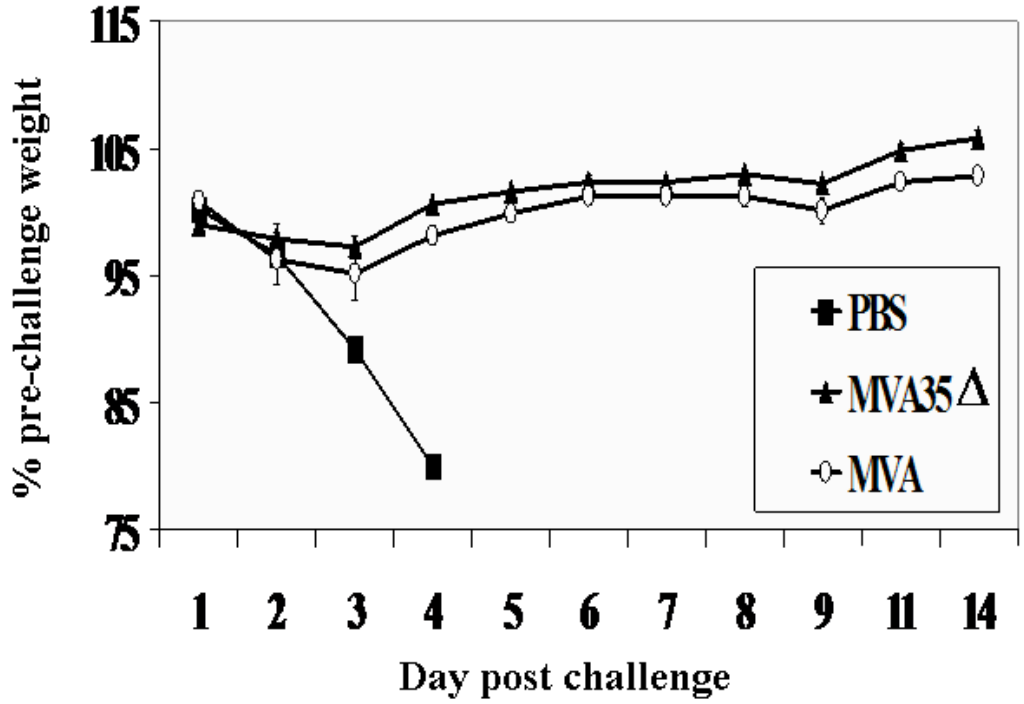

% positive
** p<0.005
* p<0.05
CD11b+
CD11c+
B220
DX5 (NK)
Gran
MHC II
F480 (MO)
PBS
MVA35Δ
MVA
* p<0.01
B220+CD11c- Bcells
CD11b+CD11c- Macrophage
CD11b-CD11c+ DC

Figure 37: Protection from lethal challenge. Mice (n=5) were vaccinated i.m. on day with 10^7 pfu of MVA or MVA35Δ virus, or mock-vaccinated with PBS. Four weeks later, the mice were challenged i.n. with LD_{50} x 500 virulent WR virus. Data show average percent change in pre-challenge weight (+/- SEM). All mice died in the PBS mock vaccinated group.


115
105
95
85
75
% pre-challenge weight
PBS
MVA35Δ
MVA
1
2
3
4
5
6
7
8
9
11
14
Day post challenge

CHAPTER 6: DISCUSSION

VACV encodes numerous proteins that function to evade the immune system, including inhibitors of apoptosis and soluble homologs of various cytokine receptors. We expand the repertoire of known poxviral immune evasion tactics and show that infection of APC with VACV significantly reduced MHC class II antigen presentation (163), resulting in the decreased synthesis of 13 chemokines and cytokines. We show that VACV specifically exerts its effects on the APC, rather than on the responding T cell (163). VACV significantly decreased antigen-independent NO production from PEC and RAW macrophages, induced apoptosis of the PEC, significantly decreased the amount of a model peptide displayed in the cleft of the MHC class II molecule and mildly reduced MHC class II surface expression on PEC and 1153 B cells (163). Interestingly, VACV decreased MHC class II expression on the 1153 B cells, but did not induce apoptosis of these cells (163). This indicates that VACV affects B lymphocytes differently than other APCs and that the reduction in MHC class II expression and antigen presentation is **not** always apoptosis dependent, as had been previously suggested (95). Our data suggests that the key mechanism of VACV inhibition of antigen presentation is its reduction of antigenic peptide loaded into the cleft of MHC class II molecules (163), which is consistent with data from the Blum laboratory showing that VACV decreases the amount of GAD peptide associated with MHC class II molecules in a human B cell line (116).

While many VACV immunomodulatory proteins have been identified and characterized, nearly 25 highly conserved genes remain uncharacterized (213). The poxvirus A35 protein was initially characterized by our laboratory in 2006 (170). This protein is highly conserved in mammalian-tropic poxviruses, but has no sequence similarity to any other known protein, suggesting an important and novel function. The initial characterization indicated that A35 was

not required for replication in eight cell lines, but was required for virulence when delivered i.n. in the mouse model (170). Here we demonstrate that the A35 protein is also important for virulence when delivered i.p., bypassing the respiratory system (164). In the i.n. mouse model, our data here, together with our previous data (170), show that 10^6 pfu of A35Δ-mutant virus is required to reach the same level of weight loss caused by 10^3 pfu of WR virus, a 1000-fold difference (164). It was therefore important to determine the function of A35. We hypothesized that the protein promoted virulence by either affecting viral replication in certain cell types/tissues or by regulating the host immune response. Thus far, A35 has been found to not be necessary for replication in various cell lines and primary cells from six different mammalian hosts (162). Furthermore, A35 is not required for the replication of the virus in the murine host organs and tissues early after infection (days 1-3) before the development of specific immunity (164). Therefore we explored the effects of A35 on specific immunity.

To study the effects of A35 on the adaptive immune response, we used a model *in vitro* MHC class II-restricted antigen presentation assay and report that A35 significantly decreased the amount of IL-2 and NO that was produced by the T cells and the macrophages, respectively, as well as 13 other cytokines/chemokines (162). Further data showed that A35 did not have any significant effect on the T cells used in the assays, implicating the APC as the target of the protein (162). A35Δ virus was able to infect the PEC as efficiently as WR, and A35 did not induce apoptosis of these cells (162). A35 did not reduce antigen-independent NO production, indicating that A35 regulated aspects of the APC-T cell interaction. A35 localized to the endosomes of the APC, modestly reduced MHC class II and B7.2 surface expression on the APC and significantly decreased the amount of peptide that was associated with MHC class II molecules (162) (Figure 38). Whether these minor decreases in MHC class II antigen

presentation will have *in vivo* significance is unknown, but it is known that both CD4 and MHC class II are needed for full protection from disease and for the generation of protective antibody (224), while an absence of CD8 or MHC class I did not reduce protection (221, 224).

VACV has been reported to decrease MHC class II antigen presentation in several model systems. Deng et al reported a decrease in the presentation of KLH peptide by primary Langerhans cells to cognate CD4+ T cells following VACV infection (51). Mann et al found that VACV infection decreased MHC class II mediated antigen presentation using primary macrophages as APC (126), and the Blum laboratory has reported a decrease in MHC class II antigen presentation using both professional and non-professional APC presenting numerous peptides (116). Decreases in MHC class II antigen presentation, concomitant with a decrease in MHC class II surface expression, has also been observed in *in vivo* infected murine splenic dendritic cells (227). Collectively, there is a growing amount of evidence to indicate that VACV perturbs peptide antigen presentation; however, **no gene** had been previously identified as contributing to this effect. We report here that A35 decreases the association of peptide with MHC class II molecules, while increasing the number of CLIP-MHC class II complexes on the cell surface (162) (Figure 38). This suggests that A35 may interfere with the processing and/or presentation of a wide variety MHC class II peptides. This is the first report to identify a specific VACV gene product, A35, as contributing to the inhibition of peptide antigen presentation (162). Since A35 is highly conserved in all sequenced mammalian tropic poxviruses (170), it may have a similar function in many different poxviruses that infect different host species.

Since MHC class II antigen presentation to CD4+ T cells is the seminal initiating event of the specific immune response, and both CD4 and MHC class II are important in the generation of a protective immune response *in vivo* (221, 224), we hypothesized that A35 would also decrease

the downstream effectors resulting from this event, including the generation of antigen-specific antibody-producing B cells, IFNγ-secreting T cells, and cytolytic T cells. After infection of mice with a low dose of virus, we have shown that, compared to WR infected mice, A35Δ infected mice had larger spleens (suggesting a robust immune response), increased antigen-specific antibody production, increased IFNγ secreting T cells, and increased CTL killing (164). The enhanced immunogenicity of the A35Δ virus correlated with decreased viral replication in target organs (days 6-9 post infection) (164). Importantly, both A35Δ mutant virus and WR could replicate similarly in the organs of the mouse on days 1-3 post infection (164), which is before an adaptive immune response has been generated (36). Thus, the decrease in viral replication seen in the A35Δ infected mice correlated with the development of the specific immune response. The increased immunogenicity of the A35Δ virus compared to WR was evident up to 34 days post infection, despite the presence of an increased antigen load following WR infection (164). Together, our data indicate that A35 is decreasing MHC class II antigen presentation to CD4+ T cells, which would result in smaller spleen size as a result of decreased lymphocyte proliferation or migration of immune cells to this organ, leading to a decrease in specific immunity including antibody production, IFNγ secretion, and CTL killing, all of which are important for protection from poxviruses (14, 32, 151, 224).

Since the A35Δ virus is 1000 times safer (164, 170) and is more immunogenic than WR virus (164), we hypothesized that it would be a superior vaccine choice. Impressively, the A35Δ virus was able to protect mice as well as WR from a lethal challenge of up to 1000 x LD_{50}, even when given at a low dose (500 pfu/mouse) (164). To be effective, vaccine strains need to retain the genes that encode proteins with protective B and T cell epitopes. Several studies have not detected A35 as having B or T cells epitopes (48, 88), but a recent study has shown that epitopes

derived from A35 can bind certain MHC class I alleles (89). Our data indicate that A35 deletion mutant vaccines would be superior to wild type strains, providing vastly increased safety coupled with improved immunogenicity and effective protection.

We performed similar replication and immune response experiments using the attenuated vaccine strain MVA. MVA was created by serial passage of wild-type VACV in CEFs. This resulted in the deletion of nearly 25 kb of genetic material (136) and a highly attenuated virus. While A35 was not found to be required for replication in WR, it was possible that its loss was being compensated for by another redundant VACV gene. Since MVA is missing a significant portion of the genome (136), we hypothesized that a replication function for A35 might be uncovered using this virus strain. However A35 was not required for MVA replication in BHK-21 cells, although it remains a possibility that A35 may be required for the replication of the virus in CEFs, the cells used during its attenuation.

MVA is being used as a safer alternative to the traditional replicating vaccine and also as a platform vaccine for other infectious diseases and cancer, although its immunogenicity and efficacy are of concern. MVA is unable to elicit the same levels of antibody and IFNγ secreting cells as the traditional replicating vaccine, even when given at 10-100 times the dose (14, 67, 221). The immunogenicity of MVA is of concern especially in persons with a weakened immune system due to HIV infection, organ transplantation, or cancer. It is therefore important to find strategies that can increase the immunogenicity of MVA. One way to do this is to create an MVA vaccine strain that is missing immunosuppressive genes. Indeed, infection of mice with a mutant MVA virus lacking the gene for the soluble IL-1β receptor resulted in improved immunogenicity compared to the parental MVA (196). Since removal of A35 from WR enhanced the immune response to infection (164), we hypothesized that its removal from MVA

may have a similar effect. In WR, it was possible that A35 required an interacting viral protein to be functional. Since MVA is missing nearly 25 kb of the VACV genome (136), it was possible that a protein that interacted with A35 had been deleted, rendering A35 ineffective. However, our data show that A35 still functions as an immunosuppressive gene in MVA, decreasing virus-specific antibody production, isotype switching, and IFNγ-secreting T cells. Thus, the removal of A35 from current vaccine strains will be beneficial by increasing their immunogenicity.

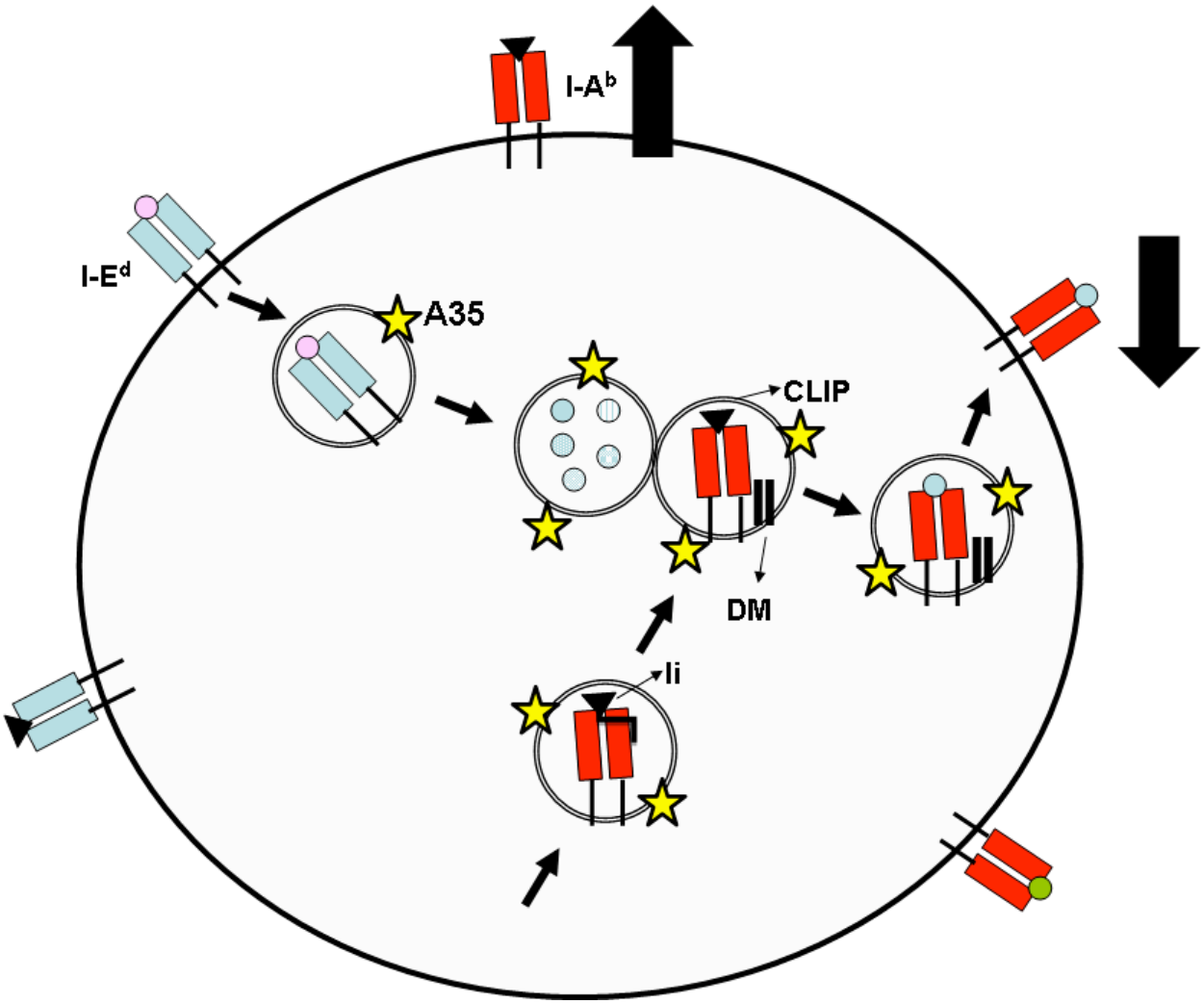
We have identified a new poxvirus virulence mechanism, the evolution of the A35 gene to block MHC class II antigen presentation. This is an important contribution to the repertoire of viral immune evasion mechanisms. This finding also has several important implications in human medicine. VACV is an effective vaccine against poxvirus infection, but its virulence results in a high rate of vaccine-related complications. It is therefore of importance to identify poxvirus virulence mechanisms in an effort to develop safer vaccines. The data presented herein strongly support the removal of A35 from poxvirus and poxvirus-based vaccines, such as MVA. A35 deletion mutant vaccines are 1000 times safer, more immunogenic than wild type strains, and offer protection from disease (164).

Besides contributing to the development of safer, more immunogenic poxvirus-based vaccines, our data are also applicable to the development of anti-poxvirus therapies. Post-exposure vaccination has been shown to be effective at preventing disease, with effectiveness decreasing as time between exposure and vaccination increases (130). Since the immune response to the A35Δ virus has improved kinetics compared to WR virus, we predict that A35Δ poxvirus vaccines would provide better protection from disease if given after exposure to the virus, even after the time when vaccination with the wild-type vaccine would be ineffective.

A35 would also be a good anti-poxvirus drug target. Since it is not required for replication in tissue culture, it would be difficult for drug-resistant virus strains to be produced.

Collectively, the data presented describe a novel poxvirus immuomodulatory mechanism and expand our knowledge of the arsenal of VACV immune evasion tactics. An effective immune response will result in the clearance of virus from the infected host. Our data indicate that the presence of A35 impedes initiation of the adaptive immune response (164). This decrease allows the virus to establish itself throughout the host, resulting in sickness and death. Several questions regarding the mechanism of A35 remain. It will be important to determine whether splenocytes from A35Δ-infected mice are better at presenting antigen to cognate CD4+ T cells compared to splenocytes from WR-infected mice, and if this inhibition occurs, the mechanism by which it occurs. It has been reported that VACV can decrease MHC class II-restricted antigen presentation using *in vivo*-infected splenic dendritic cells as APC (227), but no protein was identified as contributing to the effect. It will also be important to determine with which cellular proteins A35 interacts. Several proteins are important in facilitating the exchange of the remnant chaperone protein CLIP with antigenic peptide, including DM (Figure 38). A35 could also interact with the CLIP peptide itself or with the invariant chain (Ii) (Figure 38). In preliminary microarray data, A35 did decrease the synthesis of both Ii and DM mRNA (data not shown). Furthermore, our data show that there is a population of cells in the spleen that is resistant to VACV and A35 (162). Indeed, other laboratories have shown that different cell types have different sensitivities to VACV infection (30, 175). It will be important to also determine how A35 affects immunity in other organs, such as the lungs. While some questions remain, it is clear that A35 is an important poxvirus immune evasion protein and its removal will increase both the immunogenicity and safety of poxvirus and poxvirus-based vaccines.

Figure 38: Model of A35 within the APC. A35 (shown by the yellow star) localizes to the endosomes within APC. It does not partition to a distinct endosomal compartment, but rather localizes throughout the range of endosomes. Data indicate that A35 reduces the surface expression of a model peptide within the cleft of MHC class II in a subset of cells within the splenocyte population, while increasing the expression of surface CLIP. The mechanism of how A35 does this is currently under investigation. It is not known whether A35 is outside of the endosome or inside. It is also not known with which cellular proteins A35 may interact. The chaperone protein DM, which is directly involved in the exchange of CLIP for antigenic peptide, is a likely candidate. A35 may also interact with MHC class II itself, or with the chaperone protein Ii.


I-A^b
I-E^d
A35
CLIP
DM
Ii

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APPENDIX A: ANIMAL AND BIOHAZARDOUS MATERIALS USE APPROVALS

EAST CAROLINA UNIVERSITY

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

August 25, 2006

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

Dear Dr. Roper:

Your Animal Use Protocol entitled, "Analysis of A35R Protein Interactions and Functions Using the Murine Challenge Model," (AUP #K139) was reviewed by this institution's Animal Care and Use Committee on 8/25/06. The following action was taken by the Committee:

"Approved as submitted"

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,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

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

June 10, 2009

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 A35R Protein Interactions and Functions Using the Murine Vaccination Challenge Model," (AUP #K139a) was reviewed by this institution's Animal Care and Use Committee on 6/10/09. The following action was taken by the Committee:

"Approved as submitted"

Please contact Dale Aycock at 744-2997 prior to biohazard 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,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

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

March 27, 2006

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

Dear Dr. Mannie:

Your Animal Use Protocol entitled, "Antigen-Specific Vaccines," (AUP #K144) was reviewed by this institution's Animal Care and Use Committee on 3/27/06. The following action was taken by the Committee:

"Approved as submitted"

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,

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

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

March 27, 2006

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

Dear Dr. Mannie:

Your research proposal entitled, "Antigen-Specific Inhibition of EAE," submitted to the National Multiple Sclerosis Society, was reviewed by this institution's Animal Care and Use Committee on 3/27/06. The following action was taken by the Committee:

"Approved as submitted"

Attached is an approval letter to be submitted by you to the National Multiple Sclerosis Society.

Please contact me if I can be of further assistance.

Sincerely yours,

Robert G Carroll, Ph.D

Robert G. Carroll, Ph.D.
Chairman, Animal Care and Use Committee

RGC/jd

enclosure

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

Occupational Medicine
Employee Health
252-744-2070

Radiation Safety
252-744-2236

Infection Control
252-744-3202

Biological Safety
252-744-3437

TO: Dr. Rachel Roper
Department of Microbiology & Immunology

FROM Benton Daw
Biological Safety

DATE: January 20, 2006

SUBJECT: Biohazardous Agent Update for RoperR01

The East Carolina University Institutional Biological Safety Committee approved your biohazardous agent update on January 19, 2006 for your research involving biosafety level 2 poxviruses infections of mice pending the following clarifications:

- Weighing Mice Section of Protocol #8
  - Add sanitized hands.
- Transport of Virus to animal care facilities
  - Add biohazardous label to outer container
  - Disinfect ice bucket after use
  - Use freight elevator
- Final lab inspection of animal facilities by the office of Biological Safety

After I have received a final copy of your protocol, I will notify comparative medicine and employee health.

Thank you for your continued cooperation, and please contact me with any further questions (744-2237).

The Brody School of Medicine
Office of Prospective Health
East Carolina University
188 Warren Life Science Building • Greenville, NC 27858-4354
252-816-2417 fax

Occupational Medicine
Employee Health
252-816-2070

Radiation Safety
252-816-2236

Infection Control
252-816-3202

Biological Safety
252-816-3437

8/17/06
Dear Dr. Roper:

Thank you for submitting your revised Registration for the Use of Biohazardous Agents. The Biosafety Committee reviewed your registration form and has approved the work of Project Title: Poxvirus virulence.

Thank you,

John Williams

John Williams
Associate Biological Safety Officer
Office of Prospective Health/ Biological Safety
Office: Brody GE-94
Telephone: 252-744-2237
Email: williamsjohna@ecu.edu

CC: Jeff Smith, PhD. Chair

APPENDIX B: COPYRIGHT PERMISSIONS

Chapter 2

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