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Mechanisms of Antibody-Mediated Protection for a Protein-Based Smallpox Vaccine

Abstract

Protein-based smallpox vaccines have shown to be effective alternatives to live virus vaccines in animal model challenge studies. It is believed that subunit vaccine protection is mainly mediated through the generation of antibodies. We, and others, have shown that Th1-type antibody responses are important for protection against poxvirus infections. This finding suggests that antibody-mediated protection by a protein-based smallpox vaccine may involve antibody that can fix complement and/or activate Fc receptors. Vaccinia virus, the prototype member of the poxvirus family, produces two infectious forms of virus: mature virus (MV) and extracellular virus (EV). EV is relatively resistant to neutralization by antibody, yet antibody against EV has been shown to be protective *in vivo*. Therefore, we examined the importance of complement and Fc receptors in the protection afforded by antibody against EV. In the first part of this thesis, we found that polyclonal antibody against the EV proteins A33 and B5 can fix complement to efficiently neutralize EV *in vitro*. Additionally, we found that the complement activation requirements necessary for neutralization differ depending on the EV protein target used. We attribute these differences to the amount of A33 and B5 protein found on the EV outer membrane. We then show that in mice, both complement and Fc receptors are important for protection mediated by polyclonal antibody against the EV protein B5. In the last section, we found that non-human primates are better protected from monkeypox virus challenge by protein vaccination when the vaccine formulation generates more Th1-like antibody responses. Individual non-human primates receiving this vaccine had more homogeneous antibody responses that could neutralize EV in the presence of complement. Together, these studies implicate an important role for complement and Fc receptors in the protection by antibody against the EV form of poxviruses. This work highlights the importance of analyzing the mechanisms by which antibody provides protection from viral infection so that antibody-based vaccines and therapeutics can be more efficiently designed.

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PROTEIN-BASED SMALLPOX VACCINE

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Abstract

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MATTHEW E. COHEN

STUART N. ISAACS

Protein-based smallpox vaccines have shown to be effective alternatives to live virus vaccines in animal model challenge studies. It is believed that subunit vaccine protection is mainly mediated through the generation of antibodies. We, and others, have shown that Th1-type antibody responses are important for protection against poxvirus infections. This finding suggests that antibody-mediated protection by a protein-based smallpox vaccine may involve antibody that can fix complement and/or activate Fc receptors. Vaccinia virus, the prototype member of the poxvirus family, produces two infectious forms of virus: mature virus (MV) and extracellular virus (EV). EV is relatively resistant to neutralization by antibody, yet antibody against EV has been shown to be protective *in vivo*. Therefore, we examined the importance of complement and Fc receptors in the protection afforded by antibody against EV. In the first part of this thesis, we found that polyclonal antibody against the EV proteins A33 and B5 can fix complement to efficiently neutralize EV *in vitro*. Additionally, we found that the complement activation requirements necessary for neutralization differ depending on the EV protein target used. We attribute these differences to the amount of A33 and B5

protein found on the EV outer membrane. We then show that in mice, both complement and Fc receptors are important for protection mediated by polyclonal antibody against the EV protein B5. In the last section, we found that non-human primates are better protected from monkeypox virus challenge by protein vaccination when the vaccine formulation generates more Th1-like antibody responses. Individual non-human primates receiving this vaccine had more homogeneous antibody responses that could neutralize EV in the presence of complement. Together, these studies implicate an important role for complement and Fc receptors in the protection by antibody against the EV form of poxviruses. This work highlights the importance of analyzing the mechanisms by which antibody provides protection from viral infection so that antibody-based vaccines and therapeutics can be more efficiently designed.

Table of Contents

Acknowledgements	ii
Abstract.....	iii
Table of Contents	v
List of Tables	viii
List of Figures.....	ix
Chapter One: Poxviruses and the Contribution of Antibody to Protection In Vitro and In Vivo	1
I. Introduction	2
II. Virus structure and lifecycle.....	5
A. Virus structure	5
B. Virus lifecycle.....	7
III. Poxvirus Immunity	10
A. Protective immune responses from primary infection	10
B. Vaccination induced protective immune responses.....	12
IV. Antibody Neutralization of Poxviruses	14
A. Overview	14
B. Antibody targets	15
Neutralizing targets on mature virus (MV).....	16
Neutralizing targets on extracellular virus (EV).....	18
V. Antibody Effector Mechanisms	21
A. Overview	21
B. Complement.....	22
The complement cascade	22
Complement and defense against viral infection	23
C. Fc receptors.....	25
Overview	25
Fc receptor activation.....	25
Fc receptors and defense against viral infection	27
Chapter Two: Improved Smallpox Vaccines	28
Introduction	29
Live vaccinia virus-based vaccines.....	29
Development of a vaccine propagated in cell culture	29
Development of more attenuated live virus vaccines.....	31
Modified vaccinia Ankara (MVA).....	32
Subunit-based vaccines	33
Protein-based subunit vaccines	35
DNA-based subunit vaccines	46
Vector-based subunit vaccines	49
Conclusions.....	50
Chapter Three: Antibody Against Extracellular Vaccinia Virus (EV) Protects Mice Through Complement and Fc Receptors	52
Abstract	53
Introduction	54

Materials and Methods	56
Proteins and vaccine formulations	56
Mice, immunizations, and challenge	57
Antibody ELISA	58
In vivo complement depletion	59
EV production	59
EV neutralization in the presence of complement	60
Statistics	64
Results	64
Protection from lethal VACV challenge requires the inclusion of CpG adjuvant and correlates with the induction of IgG2a antibody	64
Sera from mice vaccinated with A33 or B5 /CpG/alum can neutralize large numbers of EV particles in the presence of complement	66
Anti-A33 C ³ -mediated antibody neutralization requires steps that could lead to EV outer envelope lysis, while anti-B5 C ³ -mediated antibody neutralization can occur by opsonization.	68
Complement is partially responsible for the protection seen in mice after passive or active immunization.	76
Fc receptors protect mice passively transferred with anti-B5 antibody in the absence of complement.	78
Discussion	81
Acknowledgements	89
Author Contributions	90
Financial Disclosure	90
Chapter Four: A Protein-based Smallpox Vaccine Generates a Protective Th1-biased Antibody Response in Non-human Primates	91
Abstract	92
Introduction	92
Materials and Methods	93
Non-human primates	93
VACV proteins	94
Vaccinations	94
MPXV challenge	95
Antibody ELISA	98
Depletion of sera of anti-L1 antibodies	99
Virus neutralization	99
Statistical analysis	100
Results	101
Vaccine safety	101
Challenge with MPXV	101
Negative and positive vaccine controls	101
Subunit vaccine provides protection	102
Benefit of CpG	102
Trivalent versus tetraivalent protein vaccines and three versus two-dose regimen.	105
Vaccine immunogenicity and correlation with protection.	105
Neutralization activity correlates with protection.	106
Antibody isotype contributes to protection.	106
Anti-A27 antibodies do not neutralize virus.	107
Discussion	110

Acknowledgements	114
Chapter Five: Concluding Remarks	115
Introduction	116
Complement-dependent EV neutralization.....	119
Fc-dependent protection by antibody in mice.....	122
Antibody isotype and function in NHPs	124
Appendix: Preliminary results on the production and characterization of a recombinant VACV with β-lactamase fused to the core protein A4	127
Introduction	128
Methods, Results, and Discussion	129
Plasmid Construction.	129
Detection of β -lactamase-A4 fusion protein expression and activity from plasmid pMC243.....	133
Recombinant virus isolation.....	135
Detection of β -lactamase-A4 fusion protein in purified recombinant virions.	138
Bibliography	140

List of Tables

Table 2-1. Individual gene targets	36
Table 2-2. Combination Gene Targets.....	40
Table 3-1. Summary of survival and maximum weight loss after challenge with 4x10 ⁶ pfu VACV	65
Table 4-1. Three-dose vaccination study (5 monkeys/group)	96
Table 4-2. Two-dose vaccination study (3 monkeys/group)	97
Table 6-1. Primers used in β -lactamase-A4 plasmid pMC243 and pMC245 construction.....	132

List of Figures

Figure 1-1. The poxvirus lifecycle.....	8
Figure 3-1. Cobra Venom Factor (CVF) treatment of BALB/c mice transiently depletes complement.....	61
Figure 3-2. CD55 and CD59 are detected in stably transfected SVAREC cell lines.	62
Figure 3-3. Antibody isotypes produced in BALB/c mice after vaccination.	67
Figure 3-4. Neutralization of large numbers of EV particles using anti-B5 mAbs and complement.....	69
Figure 3-5. Neutralization of large numbers of EV particles using sera from mice vaccinated with A33 or B5 in CpG/alum or alum only.	70
Figure 3-6. Contribution of steps leading to the formation of the membrane attack complex in complement-mediated neutralization of EV.	73
Figure 3-7. Contribution of complement component C8 in complement-mediated neutralization of EV.....	75
Figure 3-8. Protection of EV by human regulators of complement from complement-mediated neutralization at high and low amounts of antibody.	77
Figure 3-9. Protection from vaccinia virus challenge by anti-B5 rabbit pAb in C57BL/6 and C3KO mice.....	79
Figure 3-10. Protection from vaccinia virus challenge by B5/CpG/alum vaccination in C57BL/6 and C3KO mice.....	80
Figure 3-11. Protection from vaccinia virus challenge by anti-B5 rabbit pAb in FcRKO mice.	82
Figure 3-12. Protection from vaccinia virus challenge by anti-B5 mAb B126 is FcR dependent.	83
Figure 4-1. Kaplan-Meier survival analyses and pock lesion summary.....	103
Figure 4-2. Viral load on days 0-27 post-challenge.....	104
Figure 4-3. Relationship between PRNT50, lesion number, and viral load.	108
Figure 4-4. Protein vaccines adjuvanted with CpG/alum provide higher IgG1 to IgG2 ratios that result in more consistent complement-enhanced neutralization of EV.	109
Figure 4-5. Antibody against A27 does not neutralize MV.....	111
Figure 5-1. Model of complement-dependent EV neutralization by anti-B5 or anti-A33 antibody.....	118
Figure 6-1. Diagram of pMC245.	131
Figure 6-2. Protein expression of β -lactamase-A4 fusion protein from pMC243 transfected cells infected with VACV.	134
Figure 6-3. β -lactamase activity in pMC243 transfected and VACV infected cell lysates.....	136
Figure 6-4. β -lactamase-A4 fusion protein is incorporated into recombinant virions.	139

**Chapter One: Poxviruses and the Contribution of Antibody to Protection In Vitro
and In Vivo**

Introduction, Part I

Matthew E. Cohen and Stuart N. Isaacs

I. Introduction

Poxviruses are large, double-stranded DNA viruses. They include many species that infect humans and can cause disease such as monkeypox virus (MPXV), cowpox virus (CPXV), and variola virus (VARV), the highly transmissible causative agent of smallpox. Smallpox plagued humankind for thousands of years with the earliest evidence of telltale pockmark disease found on the mummified remains of ancient Egyptian Pharaoh Ramses V, who died in 1157 B.C. (1). Fatality rates from smallpox infections can reach 50% in the unvaccinated (2); therefore, it's no surprise that billions suffered from this terrible disease (3). In addition to smallpox, both MPXV and CPXV are zoonotic infections that can be transmitted to humans from their rodent reservoirs. Most human MPXV infections occur in Central and West Africa and have mortality rates between 1-15% (4,5). In 2003, a MPXV outbreak in the Midwestern United States highlighted the need to monitor this virus as a potential deadly emerging human pathogen (6). CPXV was the source of the original smallpox vaccine used by Edward Jenner in 1796 (3) and is currently endemic in Western Europe (7). Although disease is typically mild, severe and deadly infections can occur in people who are immunocompromised or atopic (8).

Fortunately, VARV was declared eradicated in 1980, thanks in large part to heroic effort by the World Health Organization, and vaccination with an antigenically cross-reactive virus called vaccinia virus (VACV). Inoculation with live VACV in the skin greatly reduces incidence of morbidity and mortality and severs the transmission chain of the virus. After eradication of smallpox was declared, vaccination rates greatly declined given the potential serious adverse events from live VACV infection. Historical data

suggests death occurs in approximately one in a million people that receive the live virus vaccine (9,10) though that may be higher now with recent increases in the numbers of immunocompromised individuals due to HIV/AIDS, cancer, and transplantation (11). Additionally, a rise in the numbers of those with atopic dermatitis and other underlying skin conditions increases the potential for serious adverse events following vaccination.

Vaccination with live VACV can result in four major types of serious complications (12,13). Eczema vaccinatum can occur in those suffering from or with history of eczema. Skin eruptions occur at sites of the body that were at that time or previously had been affected by eczema with eruptions sometimes spreading to healthy skin. Symptoms are severe with the outcome largely dependent on the amount of skin affected. Progressive vaccinia or vaccinia necrosum occurs in those suffering from immunodeficiency. In these cases, the primary vaccination site fails to heal and secondary lesions are found on other parts of the body. The infection continues to spread with a high rate of mortality occurring 2 to 5 months after vaccination. Another serious complication is postvaccinial encephalitis, which occurs in two main forms. The first occurs most often in infants less than two years old and has a violent onset, characterized by convulsions. Even after recovery, many patients are left with cerebral impairment and paralysis. The second form occurs most often in children older than two years and has an abrupt onset, with fever, vomiting, headache, and malaise, followed by loss of consciousness, amnesia, confusion, restlessness, convulsions and coma. The fatality rate is high with death occurring within a week. A fourth, recently rediscovered complication is myopericarditis (14,15). Recent smallpox vaccination programs have found an association between vaccination and patients experiencing chest pain, shortness of breath,

and heart attack. Currently, vaccination with live VACV is contraindicated in the following populations: pregnant women, those with immune disorders or therapeutically induced immunosuppression, those with HIV infection, those with a history of eczema, infants under 12 months of age, and those with heart disease.

Fortunately, routine smallpox vaccination is no longer needed, as VARV is not endemic in any part of the world. Presently, VARV stocks are kept under close guard at the CDC in Atlanta and in Russia at the State Research Center of Virology and Biotechnology, known as Vector. However, the Soviet Union had developed VARV as a bioweapon and after the collapse of the Soviet Union, it was unknown whether these stocks were properly destroyed and/or accounted for. Because of this, some speculate that VARV could be used as a biological weapon by terrorists or a rogue nation state (16). In addition, recent technical advances that could allow whole genome synthesis (17-19) has sparked concern that an intentional or unintentional release of VARV could occur and spark an outbreak of smallpox in the rapidly growing unvaccinated population. Also, the fear that MPXV could evolve to transmit from human to human more efficiently and have increased virulence has led to the stockpiling of smallpox vaccine in the United States and other developed countries around the world (20-22).

Given the serious risks associated with vaccination, a large effort has been made to invent and develop new, safer smallpox vaccines (23). These include attenuated strains of VACV as well as subunit DNA and protein vaccines and are discussed further in chapter two of this thesis. Protein vaccination is an attractive, safe option as it relies on the delivery of a small number of proteins and adjuvant to induce a protective antibody immune response. The work presented here examines both *in vitro* and *in vivo* the

mechanisms through which this antibody response can provide protection and highlights the importance of evaluating the immune requirements for protection from disease to aid in the rational design of new vaccines and therapeutics.

II. Virus structure and lifecycle

A. Virus structure

Infection with poxviruses results in the formation of two, morphologically distinct infectious forms: mature virus (MV) and extracellular virus (EV) (24). The MV form is the predominant form of virus produced during infection. MV is released in large number from killed infected cells, is environmentally stable, and thought to be responsible for host-to-host transmission. MV particles have a brick-shaped morphology and contain over 20 viral proteins in their lipid envelope. Some of these surface proteins are responsible for attachment to cells via cell surface proteoglycans (25-29). Other viral envelope proteins on MV are part of the indispensable multi-protein entry fusion complex (EFC) and mediate fusion of the virus with cellular lipid membranes (30,31). Protein L1 is found on the surface of MV and is an essential attachment and entry factor, though not directly part of the EFC (32). Within the MV envelope is a dense protein core that contains a double-stranded DNA genome and other proteins that initiate infection upon entry into the cell (33).

EV particles are MV wrapped with an additional lipid membrane containing six viral proteins and are released from infected cells using an active process (34-36). The six viral proteins found on the outer membrane of EV are A33, A34, A56, B5, F13, and F12. Of these, only A33, A34, A56, and B5 are exposed on the surface of EV and therefore are

potential targets of a neutralizing antibody immune response. Because none of these proteins are capable of fusing with cells, this presents a unique morphological problem for EV to enter cells. To solve this, the B5 protein is capable of interacting with cell surface proteoglycans to trigger the non-fusogenic dissolution of the outer membrane of EV, thereby exposing the EFC of MV and allowing entry (37,38).

EV is responsible for dissemination of virus within an infected host. Indeed, the deletion of viral proteins responsible for formation of EV leads to highly attenuated viruses that spread poorly *in vivo* (39-43). The outer lipid membrane of EV provides a number of unique biological advantages that allow successful spread within a host. Compared to MV, EV particles are relatively resistant to antibody neutralization (44,45). The EV particle is also relatively resistant to complement as it contains a number of host-cell derived complement regulatory proteins that down-modulate the deposition of complement proteins on the virus as well as preventing the lysis of viral particles (46). The small number of viral proteins on EV also makes it less likely to be detected by the host immune response.

Poxviruses have large genomes, consisting of ~200 kb of covalently-closed linear double-stranded DNA that encodes for >200 proteins (33). Genes responsible for replication and assembly of viral particles are highly conserved, while immune modulatory and host-range proteins are much less well conserved and give diversity to different poxvirus species (47). Notably, it's believed that pathogenesis in each specific host for each poxvirus species can at least be partially attributed to the adaptation of these variable proteins. Many of the proteins that are targeted by the antibody response to infection are partially conserved throughout the poxviruses, though can contain some

amino acid differences from species to species (48,49). This genetic similarity allows for cross-species protection and for VACV vaccination to protect against the related VARV and MPXV.

B. Virus lifecycle

The poxvirus lifecycle is depicted in Fig. 1-1 (modified from (50) and reprinted with permission). Poxviruses first bind to cell surface glycosaminoglycans (GAGs), which can increase the efficiency of cell penetration (29). The mechanism for entry of poxviruses into cells can occur via two different pathways: Fusion at the plasma membrane (29,51,52) or apoptotic mimicry and the hijacking of macropinosomes followed by low-pH induced fusion within the macropinosome (51,53-56). The cellular receptor(s) that triggers entry/fusion is unknown, as is the mechanism by which the EFC is used to fuse the viral and cellular membranes to deliver the viral core into the cytoplasm. After fusion of the MV membrane with the cellular membrane, the viral core is transported along microtubules to a perinuclear location in the cell (57). As the core begins to uncoat, early gene expression begins to occur as the virus starts to prepare the cell environment for optimal conditions to replicate and create more viral particles. At this time, the virus begins to create a “virus factory” adjacent to the nucleus where the ultimate assembly of new virus will take place (58). The “factory” is surrounded by membrane, likely derived from the endoplasmic reticulum (59), and it is where all viral transcription, translation, and DNA replication take place. Confining the space within which these processes occur likely enhances the efficiency of poxvirus replication and facilitates competition for cellular resources.

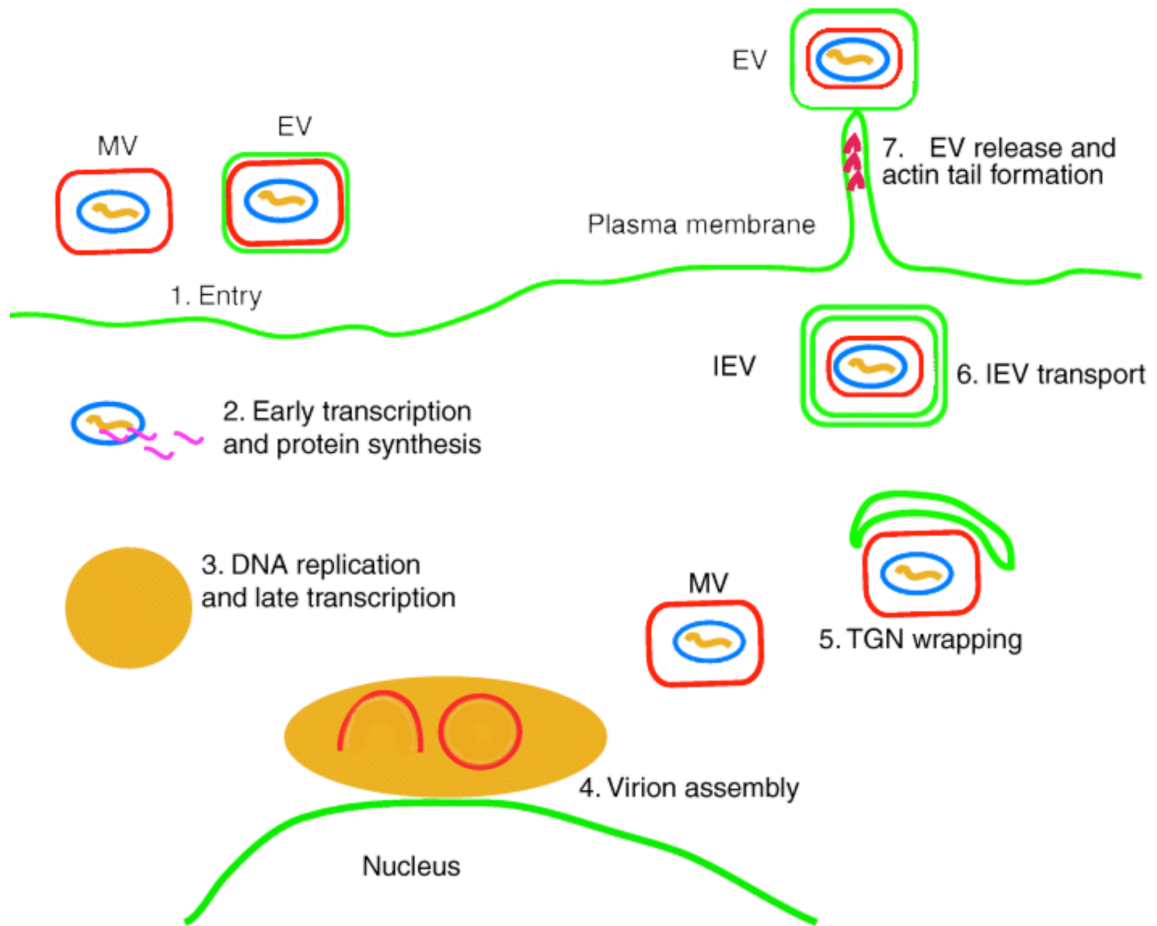


Figure 1-1. The poxvirus lifecycle.

Poxviruses have two infectious forms that are capable of infecting cells, the mature virus (MV) and extracellular virus (EV). After entry (1), the core begins to uncoat and early transcription/translation begins (2). DNA replication and virion assembly occur in the perinuclear viral factory (3 and 4). MV is the predominant form assembled; however, some of the MV is targeted to the trans-Golgi network (5) where it is wrapped with two additional membranes to become intracellular enveloped virions (IEV) (6). The outer membrane of IEV fuses with the plasma membrane leaving the EV form of the virus attached to the infected cell surface. EV can then polymerize actin to propel the virus away from the infected cell (7) or be released from the cell through an active cell signaling process. Modified figure reprinted from (50) with permission.

Poxvirus gene expression is temporally regulated (60) and is divided into three main categories: early, intermediate, and late. Early gene expression provides the virus a suitable environment to replicate and is required for succession to DNA replication, intermediate gene expression, and late gene expression (33). Early genes include proteins that shut off the host defense response which attempt to abort viral replication (61). Following early gene expression, DNA replication occurs and is a required precursor to intermediate and late gene expression (62,63). If DNA replication is prevented, late gene expression cannot occur. As DNA replication occurs, the transcription of intermediate genes begins. Intermediate gene products are few in number and mostly serve as transcription factors that turn on late gene expression (62,64,65). Lastly, late gene expression occurs and provides the proteins necessary for assembly of new virions (33), as well as proteins that are incorporated into new virions to allow early gene expression to occur in the next round of infected cells (66).

Viral assembly within the “virus factory” is a complicated process that includes non-infectious intermediate-step viral particles. Assembly starts with the formation of protein scaffolded membrane crescents (67,68) that mature into complete protein shells called immature virions (IV). Next, viral genomes and proteins required for early gene transcription are packaged into IV (69). Cleavage of core proteins occurs creating MV that leaves the factory (70,71). While this is the predominant form of virus produced, a small subset of MV leave the factory and acquire two additional membranes from the trans-Golgi network (TGN) or endosomes (72,73). These viral particles are known as intracellular enveloped virions (IEV) and contain a distinct subset of viral proteins, including those found on the EV particle (33). During infection the EV unique proteins

are targeted to the TGN and endosomes, as well as the cell surface. After wrapping, the IEV are transported to the perimeter of the cell on microtubules (74-76). When the plasma membrane of the cell is reached and the cortical layer of actin breached (77), the outer membrane of IEV fuses with the cellular plasma membrane leaving a double membrane cell-associated extracellular virus (CEV) particle attached to the cell surface (78). The CEV can infect an adjacent cell or can be pushed away from the cell it is attached to toward uninfected cells by activating actin filament polymerization underneath it in a Src-kinase dependent manner (79-81). Additionally, the CEV can be released from the cell in an active, Abl-kinase dependent manner to form EV (35). The release of CEV to EV is important for full virulence during a poxvirus infection as evidenced by a reduction in virulence *in vivo* by an Abl-kinase inhibitor drug that blocks release of EV from the host-cell surface (35).

III. Poxvirus Immunity

A. Protective immune responses from primary infection

The first line of host defense against poxvirus infection is the innate immune response. This response is important for controlling virus replication early during infection and allows sufficient time for, as well as helps activate, an adaptive immune response. Evidence for the importance of innate immunity during poxvirus infections is found in the numerous innate immune response modifier proteins encoded by poxvirus genomes (61). These poxvirus virulence proteins target key innate pathways including interferons, chemokines, complement, and the toll-like receptor (TLR) family of pattern recognition receptors.

The ectromelia virus (ECTV) model system has been frequently used to examine immune requirements for protection from primary infection in mice because it is a highly lethal, species-specific poxvirus infection similar to VARV in humans. The IFN α/β binding protein from ECTV has been found to be critical for virulence indicating that IFNs are important in mounting a host response against ECTV (82). Many poxviruses encode numerous chemokine-binding proteins that could act to modify the recruitment of cellular responses to infection. Indeed, NK cells have been shown to be critical for control of ECTV following challenge of ECTV resistant strains of mice (83-85). Also, mice lacking complement have been found to be more susceptible to a primary ECTV infection (86). TLR9 signaling to activate dendritic cells is critical in survival from a lethal ECTV infection in mice (87). While innate immune responses are important and help shape adaptive immune responses to infection, they are not sufficient to control and clear poxvirus infections.

In addition to innate immunity, adaptive immunity is also required to control and clear infection of poxviruses. Studies with ECTV infection in mice have shown that *both* B cell and T cell responses are critical for controlling a primary infection and ultimately protecting mice from lethal disease. B cell deficient mice (μ MT $^{-/-}$), despite mounting robust CD8 $^{+}$ T cells responses, succumb to ECTV infection within 3 weeks post infection (88). A similar study found that mice that are incapable of mounting antibody responses (μ MT $^{-/-}$; MHCII $^{-/-}$; or CD40 $^{-/-}$) are able to survive infection for greater than 1 month, but ultimately are unable to clear the virus and succumb to infection (89). T cell responses are also critical for clearing a primary ECTV challenge. The depletion of CD8 $^{+}$ T-cells from mice normally resistant to ECTV makes them highly susceptible to ECTV

and these mice succumb to infection by day 8 (88,89). Together, these studies illustrate that CD8⁺ T-cells, CD4⁺ T-cells, and antibody are critical in controlling primary ECTV in mice. These findings with ECTV also suggest that for a highly lethal, species-specific poxvirus infection, T-cells are required to control infection early and limit replication at the site of infection, while antibody is critical to clear the virus and prevent dissemination within the host.

B. Vaccination induced protective immune responses

The smallpox vaccine induces strong, long-lived adaptive immune responses. In humans, vaccinia-specific antibody levels and neutralizing titers have been shown to persist for decades and vaccinia-specific memory B-cell responses are functional and maintained for more than 50 years (90-92). CD4⁺ and CD8⁺ T-cell responses peak at 2-4 weeks post vaccination and contract to form a stable pool of memory T-cells that can last for decades (92,93).

While smallpox vaccine generates a fully protective immune response, the specific correlates of protection in humans are unknown. In order to develop new, safer vaccines against smallpox, researchers have turned to animal models to study the ability of antibody and T-cells to provide protection together or on their own. Passive immunization with anti-vaccinia sera or anti-vaccinia primed CD8⁺ T-cells in mice has been shown to be sufficient on their own to provide protection from VACV or ECTV challenge (89,94-101). Mice vaccinated with VACV and then depleted of CD8⁺ or CD4⁺ T cells were fully protected from challenge (100). Vaccinated mice depleted of B-cells prior to VACV challenge lost some protection normally seen in wild-type mice, though

mice still survived VACV challenge indicating that T-cells may be sufficient to prevent death (100). If these mice were depleted of both B-cells and T-cells, mice succumbed to infection. In a separate study, vaccinia-specific memory CD8⁺ T cells were transferred into naïve mice and had the ability to protect in the absence of other preexisting adaptive immunity (102).

While these results suggest a role for CD8⁺ T-cells in protection after vaccination, using the ECTV and MPXV models reveals an even greater dependence on antibody for protection after live virus vaccination. Mice vaccinated with an avirulent strain of ECTV and subsequently depleted of CD8⁺, CD4⁺, or CD8⁺/CD4⁺ T-cells are protected from a lethal ECTV challenge (103). The engagement of B-cells and the production of an antibody response are critical for protection after vaccination with an avirulent ECTV. Mice lacking B-cells (μ MT^{-/-}) or the ability to mount an antibody response (MHCII^{-/-} or CD40^{-/-}) vaccinated with avirulent ECTV remained susceptible to ECTV infection and had similar times to death as naïve mice (103). All protection correlated with the production of an antibody response and effective virus control.

In a model of MPXV challenge, nonhuman primates (NHPs) are protected from lethal infection if they are first vaccinated with VACV (104-106). Depletion of CD8⁺ or CD4⁺ T cells at the time of challenge had no effect on this protection and NHPs were free from signs of disease (107). However, depletion of B-cells at the time of challenge abrogated protection and led to the death of 3 of 4 NHPs with the surviving NHP showing signs of morbidity after challenge (107). These MPXV data are in agreement with the ECTV data and reflect the requirement for generating an antibody response to induce protection against poxviruses. The finding that antibody is necessary and

sufficient for protection against challenge with lethal, systemic infection suggests that new vaccine strategies that focus on inducing an antibody response can be sufficient for protection. Indeed, subunit protein vaccination that induces primarily a strong antibody response has been promising in mouse and NHP models of infection (98,105,108-110). These vaccines are discussed in greater detail in chapter two.

IV. Antibody Neutralization of Poxviruses

A. Overview

Antibody neutralization of poxvirus plays a critical role in protection from disease. Many studies have used VACV as a model to ascertain the protein targets of neutralization. Both the MV and EV forms of the virus are targets of the neutralizing antibody response (44,111-116). While MV is much more sensitive to antibody neutralization, EV neutralization can be achieved with higher concentrations of immune serum or purified specific antibody (44,109). Importantly, antibody against MV is incapable of neutralizing EV, and vice versa. Not surprisingly, it has been shown that inducing or transferring antibody against both forms is more protective than antibody against either form alone (96,98). Antibody targeting the MV form is thought to reduce the infecting inoculum while antibody that targets EV could alter the spread and dissemination of the virus within the host. Therefore, it has been critical to identify proteins on both the MV and EV forms that can be targeted by antibody and provide protection from challenge. Because poxviruses are so complex and encode for more than 200 proteins, considerable effort has been made to identify targets of the natural antibody response to VACV as well as targets suitable for vaccination.

In addition to targets, the type of antibody response must be considered. Vaccination with live VACV induces a potent Th1-biased immune response that generates antibody isotypes of IgG2a, IgG2b, and/or IgG2c in mice (105,117,118) or IgG1 in non-human primates (Xiao, Isaacs; unpublished results). While direct neutralization of virus is dependent on the variable region of the antibody, the ability of the Fc region to fix complement can greatly enhance the potency with which an antibody can neutralize virus. A number of viruses have been shown to be more easily neutralized in the presence of antibody and complement, including West Nile (119), influenza (120,121), herpes simplex (122,123), and poxviruses (97,108,124-126). While VACV MV is efficiently neutralized by antibody alone, studies have shown that EV is much more effectively neutralized by antibody in the presence of complement and protective effects *in vivo* are partially dependent on complement (125). Designing vaccines and therapeutic antibodies must take into account not only the antibody targets, but also the isotype and Fc functional activity of antibody being generated.

B. Antibody targets

The majority of research to identify targets of the antibody response to VACV has focused on the surface proteins of MV and EV. Protein targets were identified based on the ability of antibody against them to neutralize the virus *in vitro* or provide protection *in vivo* after passive transfer. Vaccinia Immune Globulin (VIG), a preparation of serum from recently vaccinated individuals used to treat vaccine-related complications, has been studied to identify what antibodies contained therein are able to neutralize VACV (113-115,127-129). More recently, a number of labs have used protein microarrays to identify

protein targets of the antibody response after vaccination with VACV (114,130-133). These studies have shown that the humoral response in humans has a large degree of interindividual variability and many different protein targets have antibody generated against them. No specific immunodominant targets of the antibody response to MV were identified. These studies revealed that antibody is generated against not only surface membrane proteins of VACV, but also core proteins and proteins only expressed in infected cells. The importance of these responses is currently unknown. The following section will discuss the protein targets of neutralizing antibody that have been identified.

Neutralizing targets on mature virus (MV)

Neutralization of the MV form of VACV has been the classical standard for determining the ability of antibody to provide protection *in vivo*. MV is typically seen by a naïve immune system upon initial infection and this perhaps allows for its enhanced sensitivity to neutralization compared to EV (44). To date, the proteins recognized as neutralizing targets of antibody on MV are A27, A28, L1, D8, and H3.

A27 is a 14 kDa trimeric protein found on the surface of MV and is involved in virus attachment to GAGs on cells, virus–cell fusion, and virus release from cells (26,134,135). Monoclonal antibody against A27 generated from VACV immunized mice neutralizes MV *in vitro* and protects mice after passive transfer *in vivo* (136). Immunization with smallpox vaccine induces A27 neutralizing antibody, but represents only a small fraction of the total neutralizing activity of VIG (128). A27 vaccinations with recombinant protein or DNA have had varying degrees of success with providing protection from challenge (110,137-139).

A28 is an 18 kDa membrane protein found on the surface of MV and is highly conserved between poxvirus species. It was the first identified member of the poxvirus EFC (30,140) and has been shown to interact with another protein member of the complex called H2 (30,141,142). Vaccination of rabbits with recombinant A28 protein induced neutralizing antibody and passive transfer of this antibody protected mice from lethal challenge (143). Interestingly, vaccinating mice with A28 and H2 at the same time, in the same location, induced a more potent neutralizing antibody response than vaccinating mice with A28 alone or A28 and H2 at different sites (144). Because these proteins interact on the virus, it is likely that presenting recombinant A28 associated with H2 provides a more natural epitope for B-cells to recognize and produces antibody that can recognize A28 in its complex form on the virion. This concept is important and likely could be extended to a number of poxvirus protein targets. To date, A28 is the only member of the EFC that has generated neutralizing antibody, but perhaps the right complex of proteins could generate neutralizing responses to other members.

L1 is a 29 kDa myristylated protein found on the surface of MV particles and has been shown to be involved in virus penetration into cells, though hypothesized to only peripherally associate with the EFC (32). Some of the first identified neutralizing monoclonal antibodies to VACV were found to target the L1 protein (145-147). Recently, it was described that L1 can bind to cells independently of GAGs, suggesting that it may be responsible for interacting with an unknown cellular receptor responsible for initiating membrane fusion (148). While both A27 and L1 have been studied extensively as targets for vaccination, L1 has outperformed A27 and consistently has produced protective neutralizing antibody (98,106,108,110,137,149).

D8 is a 32 kDa protein found on the surface of MV and has been shown to bind to the GAG chondroitin sulfate and mediate adsorption of MV to cells (28,150). Deletion of D8 leads to a reduction in MV titer when growing virus in cell culture (28) and replication of a recombinant VACV that has D8 deleted is attenuated in brain tissue (151); therefore, blocking interaction of the cell and D8 hampers the ability of poxvirus to efficiently enter cells. Antibodies to D8 have been detected in mice immunized with VACV (152). Additionally, immunization of mice with D8 in the form of an optimized DNA vaccine induced neutralizing antibody (153).

H3 is a 35 kDa integral membrane protein of MV and mediates the binding of virus to cells through heparin sulfate (154). H3 deleted recombinant virus has a small plaque phenotype, though this is partially attributed to defects in morphogenesis. Mice passively given rabbit polyclonal anti-sera to H3 are partially protected from challenge with VACV (154). Additionally, H3 is a major target of the antibody response to VACV vaccination in humans and mice vaccinated with recombinant H3 protein are partially protected from challenge (99).

Neutralizing targets on extracellular virus (EV)

Compared to MV, EV requires higher concentrations of antibody or serum to achieve even modest levels of neutralization in plaque reduction assays (44,45,97,108,109,113,125,129,155). Another mechanism by which antibody “neutralizes” EV is thought to be through the inhibition of release of EV from infected cells (109,110,155). Additionally, recent studies have elucidated a mechanism of complement-enhanced antibody neutralization whereby the outer envelope of EV could

either be opsonized with complement (125) or lysed by the membrane attack complex (MAC) (97). The two identified targets on EV of neutralizing antibody and a protective antibody response are A33 and B5.

A33 is a 23 kDa type II transmembrane glycoprotein found as a dimer on the surface of EV as well as the surface of infected cells both early and late during infection (34,156). A33 plays a role in chaperoning A36, a protein important for actin filament formation underneath CEV, to the cell surface (157). A33 also plays a role in antibody resistant cell-to-cell spread of EV, though the exact mechanism is unknown (158).

Antibody against A33 is found in the sera of VACV vaccinated humans (112).

Additionally, a number of studies have shown that passive transfer of A33 antibody is protective *in vivo*, although antibody against A33 does not directly neutralize virions (96,109,159). While anti-A33 antibody has not been shown to neutralize EV in a plaque reduction assay, anti-A33 antibody has been shown to alter “comet-tail” formation in cell culture, a phenomenon mediated by the release of EV from infected cells (97,110). This prevention of “comet-tail” formation is attributed to the ability of A33 antibody to prevent the release of CEV (34). A33 antibody has also been shown to lyse the outer membrane of EV in the presence of complement (97). Neutralizing MV antibody was then shown to be able to neutralize the MV particle that was released. Finally, A33 antibody and complement has been reported to be able to lyse infected cells (160), indicating that A33 on the infected cell surface can serve as another target of A33 antibody. While these mechanisms have all been reported *in vitro*, it’s unclear which ones contribute to protection *in vivo*. Nevertheless, numerous groups have successfully used A33 as a vaccination target (98,105,106,108-110,138,149,153,160-162).

B5 is a 42 kDa type I transmembrane glycoprotein and like A33, it is found on the surface of EV and on the surface of infected cells both early and late in infection (34,163,164). B5 is required for the formation of EV and deletion of B5 leads to a reduction in the amount of EV released due to a reduction in the amount of MV that gets wrapped in the golgi/endosome (39,165). Because of a reduction in the amount of EV released, B5 deleted VACV is attenuated *in vivo* (39,165). Additionally, B5 is required for non-fusogenic dissolution of the outer membrane of EV (37) and deletion of the key residues of B5 protein decreases EV infectivity by blocking EV unwrapping to expose the EFC on MV (38). Currently, B5 is the only identified target of antibody that can directly neutralize EV (44,109) and passive transfer of polyclonal or neutralizing monoclonal antibody against B5 can decrease post-challenge morbidity *in vivo* (96,109,124,125,159,166,167). The EV neutralizing activity of VIG is mainly attributed to antibody against B5 (113,129). Recently, a monoclonal antibody against B5 was shown to neutralize EV, but only in the presence of complement (125). This ability has been attributed to the complement fixing IgG2a isotype of this monoclonal antibody and protection *in vivo* was dependent on complement. Unlike A33, opsonization (coating) of the EV outer membrane with antibody and complement was shown to be a mechanism for complement-mediated neutralization of EV when this B5 monoclonal was used (125). Similar to A33, antibody against B5 can lyse infected cells in the presence of complement (125). Including B5 as a target of vaccination has been widely successful in protecting mice and NHPs from lethal challenge (98,105,106,108-110,138,149,162,168,169).

V. Antibody Effector Mechanisms

A. Overview

Antibodies possess two functional domains: Fab and Fc. The Fab portion of antibody is highly variable and allows antibody to recognize and bind widely diverse molecular structures. The Fc portion of the antibody is more highly conserved and performs two basic functions (170). First, it interacts with the rest of the immune system after an antibody has bound its target. This interaction can lead to the activation of a variety of effector mechanisms: antibody dependent cellular cytotoxicity (ADCC), antibody dependent cellular phagocytosis (ADCP), complement dependent cytotoxicity (CDC), and complement-mediated neutralization. ADCC and ADCP are mediated by the interaction of the Fc portion with Fc receptors (FcRs) expressed on a variety of immune cells including natural killer (NK) cells, macrophages, neutrophils, and monocytes (171). CDC and complement-mediated neutralization are mediated by the interaction of the Fc with complement component C1 and the resulting activation of the complement system (172). The second function of the Fc portion of the antibody is the interaction with the neonatal Fc receptor (FcRn) to regulate the half-life of antibody in circulation (170).

The classical definition of antibody neutralization has often been defined as the interaction between the Fab portion of the antibody and a pathogen target structure such that the ability of the pathogen to interact with a cellular receptor or enter cells is prevented. However, *in vitro* non-neutralizing antibodies can actually be neutralizing if the assay is performed under conditions that allow Fc domain to function. Antibody neutralization can be aided by complement and Fc receptors. After the Fab portion recognizes the pathogen, the Fc portion can activate the complement cascade to

neutralize the pathogen or it can interact with Fc receptors to form an immune complex that can lead to degradation of the pathogen by opsonophagocytosis. For antibody against pathogens, the ability to activate these Fc-dependent mechanisms can be crucial for protection from disease. Recent studies have encouraged a deeper appreciation for the Fc requirements of antibody to provide disease protection from a number of virus pathogens including HSV, West Nile, yellow fever, influenza, HIV, and VACV (124,125,173-183).

B. Complement

The complement cascade

The complement cascade is part of the innate immune response and is one of the earliest defense systems to recognize invading pathogens (184,185). Complement is activated by three different pathways that use distinct mechanisms to recognize non-self from self: classical, lectin, and alternative. The classical pathway is activated by IgM or IgG antibody bound to antigen. The C1q complement protein recognizes the Fc portion of the bound antibody and initiates the cascade. In mice, IgG2a, IgG2b, and IgG2c are much more efficient at activating complement via the classical pathway than IgG1 (186-188) and in humans and non-human primates, IgG1 is more efficient at activating complement than IgG2 (189,190). The lectin pathway is activated by the mannose-containing polysaccharides found on pathogens. The alternative pathway has no specific activator, but simply results from low-level cleavage of the C3 protein of complement. If complement regulatory proteins are missing from the surface of a pathogen or cell, the alternative pathway is amplified in a feedback loop.

All three pathways converge on the formation of a C3 convertase, responsible for the continued cleavage of C3 to its active form, C3b, which can bind covalently to nearby surfaces. As C3b continues to form, some of it binds back to the C3 convertase to form the C5 convertase. The C5 convertase cleaves C5 into C5a and C5b. C5b recruits C6 through C9 to form the membrane attack complex (MAC), a lytic pore that can lyse pathogens and infected cells.

Complement and defense against viral infection

Complement is an important first line of defense against virus infections (191,192). Complement can opsonize viral particles by the covalent attachment of C3b. This can block viral proteins from interacting with the host cell and effectively neutralizes the virus. Additionally, viral particles bound with C1q and/or C3b can be taken up and degraded by phagocytic cells such as macrophages and neutrophils. The formation of the MAC on enveloped viral particles can lyse the membrane and prevent the virus from being able to infect the cell. Infected cells that express viral proteins on the cell surface can be recognized by antibody and activation of the classical pathway on those cells can occur. Formation of the MAC on infected cells is called complement dependent cytotoxicity (CDC) and prevents the continued replication of virus in those cells. Complement-mediated neutralization and CDC have been shown to be important in many viral infections, including influenza, HSV, and poxviruses (125,173,183,193-195). In addition to opsonization and the formation of the MAC, complement has been shown to enhance adaptive immune responses through its various cleavage products (196).

Because of the anti-viral effects of complement, many viruses have evolved ways to evade complement attack. Poxviruses, paramyxoviruses, and several retroviruses recruit host regulators of complement and incorporate them into their outer envelope (46,197,198). For example, VACV EV is relatively resistant to complement because it incorporates the host regulators of complement CD46 (membrane cofactor protein, MCP), CD55 (decay accelerating factor, DAF), and CD59 (an inhibitor of MAC formation) into its outermost envelope (46). CD46 acts as a cofactor with host regulator factor I to degrade C3b into an inactive form effectively preventing the further formation of C3 convertases. CD55 is capable of destabilizing the C3 convertase complex, rendering it inactive. CD59 can prevent the incorporation of the MAC into membranes (199).

Poxviruses, HSV, and flaviviruses encode their own proteins that regulate the complement cascade (126,183,200-203). Poxviruses encode complement control proteins (CCPs) that contain structural homology to host regulators of complement. VACV expresses the vaccinia virus complement control protein (VCP) that protects infected cells and free virions from complement mediated attack (126,193). HSV and flaviviruses encode their own protein regulators of complement, but these contain no structural similarity to host regulators of complement. However, both are effective at allowing the virus to evade complement and replicate more efficiently (183,201-203).

C. Fc receptors

Overview

The Fc portion of antibody engages FcRs after it has bound its antigen (171). In the case of ADCC, the result of the three-way interaction between target cell, antibody, and an FcR expressing cell (such as an NK cell or macrophage) is cell death. It has long been recognized that in the presence of antibody and peripheral lymphocytes, VACV infected cells are lysed and killed (ADCC) (204). Additionally, viral pathogens can be directly linked to FcR expressing phagocytic cells through antibody and taken up to be degraded. Lastly, engagement of FcRs can lead to the expression of cytokines and the release of inflammatory mediators, bolstering the immune response in the vicinity of bound antibody.

Fc receptor activation

There are two main classes of Fc receptors recognized: activation receptors and an inhibition receptor (171). Both classes are often found on the same cell and function in concert (205). Almost always, co-engagement of these receptors occurs, setting the threshold and magnitude of the responses generated. Levels of each type of receptor on FcR expressing cells are carefully regulated during the development and differentiation of effector cells so that FcR activation receptors are more highly expressed when a strong cell-mediated response is needed and FcR inhibition receptors are more highly expressed when the response needs to be turned off. In mice, the high-affinity Fc γ RI, low-affinity Fc γ RIII, and intermediate affinity Fc γ RIV are activating receptors and share a common immune activating tyrosine motif (ITAM) subunit called the γ -chain (171). High-affinity

Fc γ RI can bind monomeric IgG and is found exclusively on macrophages and neutrophils. Low-affinity Fc γ RIII is expressed on NK cells, macrophages, neutrophils, and mast cells. The intermediate-affinity Fc γ RIV was more recently discovered and is exclusively expressed on neutrophils, monocytes, macrophages, and dendritic cells (206). If the common γ -chain is knocked out, as is the case with FcRKO mice, the activating FcRs are still expressed on the cell surface but fail to provide signals to the cells, rendering the activation pathways of the FcR system inert. Indeed, activated macrophages from FcRKO mice fail to phagocytose antibody-coated beads despite normal binding (207). Additionally, NK cells from these mice are defective at performing ADCC (207).

The ability of IgG to bind and stimulate activation FcRs is heavily dependent on the isotype of the antibody (208). Generally, Th1-type IgG is better at activating Fc receptor dependent processes. This is not surprising, as Th1-type cytokines upregulate expression of activating FcRs (171). In mice, the high-affinity Fc γ RI exclusively binds IgG2a, while the low-affinity Fc γ RIII binds IgG1, IgG2a, and IgG2b (171). Fc γ RIV exclusively binds IgG2a and IgG2b with intermediate affinity and does not interact with IgG1 or IgG3 antibody isotypes (206). Furthermore, recent findings have suggested that immune complexed IgG2a and IgG2b are functionally dependent on Fc γ RIV, despite having an affinity for Fc γ RI (in the case of IgG2a) and Fc γ RIII (in the case of IgG2a and 2b) due to Fc γ RI being occupied by monomeric IgG2a and Fc γ RIII having too low an affinity to have productive engagement at typical serum levels of antibody (171).

Fc receptors and defense against viral infection

In support of FcγRIV driving Fc-dependent Th-1 type IgG mechanisms, a protective IgG2a West Nile virus monoclonal antibody is rendered ineffective in FcRKO mice, but not FcγRIII or NK cell depleted mice, indicating that ADCC by NK cells does not mediate protection from West Nile virus infection by IgG2a monoclonal antibody (180). Protective antibody to influenza infection has also been shown to be dependent on macrophages and not NK cells by an FcR dependent mechanism (178). While NK cells and macrophages play a role early during an initial poxvirus infection in a naïve host (83,84,209-211), it is less clear what role they might play in a protective Fc-dependent antibody mechanisms. Recently, FcRKO mice were shown to be protected from lethal ECTV challenge if given a post-exposure immunization with Modified Vaccinia Ankara (MVA), an attenuated smallpox vaccine candidate (212). However, in this model, the lack of FcRs may be compensated by other immune mechanisms. This thesis begins to explore the *in vivo* role of Fc receptors in protection from VACV challenge in the presence of a protective antibody response.

In the next chapters, we examine the Fc effector mechanism requirements for *in vitro* neutralization of VACV EV with polyclonal antibody responses against the A33 and B5 proteins of EV and the Fc effector mechanism requirements for *in vivo* protection from poxvirus challenge after passive and active immunization against B5.

Chapter Two: Improved Smallpox Vaccines

Introduction, Part II

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Introduction

The first preventative vaccines against an infectious agent were orthopoxviruses, which were used to prevent smallpox (3). Vaccination with live vaccinia virus resulted in cross-protection against infection by variola virus, the causative agent of the deadly disease smallpox. Vaccination with vaccinia virus was part of the World Health Organization's (W.H.O.) successful strategy to eradicate smallpox, which made smallpox the first, and currently only, disease to be eradicated from plaguing humankind (3). However, this success has now led to concern about the accidental or intentional release of variola virus (213,214). Because routine vaccination of civilians with vaccinia virus vaccines ended with the eradication of smallpox, a large portion of the world population is susceptible and for those previously vaccinated, full protection from smallpox may be incomplete. In addition to concerns about variola virus, ongoing outbreaks of monkeypox in Africa (4,215) and the potential spread of monkeypox to non-endemic areas (6), has generated the necessity to have smallpox vaccine available. However, concerns about the safety of the smallpox vaccines used in the smallpox eradication program have led to the development of new orthopoxvirus vaccines (216-219).

Live vaccinia virus-based vaccines

Development of a vaccine propagated in cell culture

The smallpox vaccines that were used around the world to combat smallpox were developed in a time well before modern methods of growing and passaging live virus vaccines. In the United States, the smallpox vaccine used was Dryvax (manufactured by Wyeth Laboratories) and was grown and passaged in the skin of animals. Other parts of

the world used other strains of vaccinia virus, and depending upon the strain, different rates of vaccine-related severe complications (e.g., death or post-vaccination encephalitis) were observed (9). With the eradication of naturally occurring infections from variola virus, routine smallpox vaccination ended because the risks (both major and minor) of the vaccine were deemed to be too high in the setting of no disease. Without widespread use of the vaccine, production ended. While the Centers for Disease Control & Prevention (CDC) had access to old stocks of Dryvax, there were not enough doses available to combat a large-scale outbreak. Thus, there was great urgency to produce and stockpile more vaccine using modern vaccine manufacturing procedures. However, a major dilemma was that the virus contained in the Dryvax vaccine represented a swarm of virus with unlimited diversity (Russell Regnery, personal communication). A number of individual virus plaques from stocks of Dryvax were picked and characterized. The virus propagated from a single plaque that most closely resembled the *in vitro* and *in vivo* characteristics of the Dryvax swarm (but resulted in lower neurotoxicity in mice) was selected (220). This virus, grown in cell culture, was shown to protect animals against orthopoxvirus infections (220-222). Ultimately, this virus, called ACAM2000, became the FDA licensed smallpox vaccine in the U.S (223,224). However, like Dryvax, this vaccine generates infectious progeny that can spread within the host, and thus it is expected to result in the same minor and major complications of the parent virus (225,226). Therefore, the prescribing package insert for ACAM2000 contains the same warnings as Dryvax indicating that people with immunodeficiencies, common skin disorders like eczema and atopic dermatitis, cardiac disease, age less than 12 months, and pregnancy are still at risk for developing a severe complication from the vaccine and

should not get vaccinated in the setting where there is no active smallpox (227). Because of the concerns about complications from the smallpox vaccine, there has been a continued interest in developing safer smallpox vaccines that can be used in a diverse population, including people at risk for the severe complications from current live vaccinia virus vaccines.

Development of more attenuated live virus vaccines

Prior to the eradication of smallpox, some countries began testing more attenuated strains of vaccinia virus as potential smallpox vaccines. These vaccines were isolated by continual passage of a parental vaccine strain in cell culture, which resulted in random mutations that attenuated the virus. Many of these vaccines were given to large populations and appeared to have less side effects, but were used in countries where smallpox disease no longer existed. Examples of this strategy are LC16m8 and MVA strains of vaccinia virus.

LC16m8

In Japan, LC16m8 was developed as an attenuated vaccinia virus vaccine (228-230). This virus was isolated after passage of the parental Lister strain of vaccinia virus in rabbit cells at low temperature. The resulting virus made very small plaques in cell culture and showed less neurovirulence in animal models. Much of the attenuated phenotype of this virus is due to a mutation in the B5R gene (231), which encodes for an important glycoprotein needed for the optimal production of an infectious form of virus critical for virus spread. Thus, while LC16m8 can grow and make infectious particles, it

spreads poorly in cell culture. LC16m8 has been shown to generate protective immunity in mice (232), rabbits (232), and non-human primates (233). However, there are two important concerns about this vaccine. Since the key attenuating mutation in B5R is a one base deletion that results in a frame-shift and early truncation of the B5 protein (231), there is evidence that virus can revert back to wild type during growth (234). An additional concern is that the B5 protein is an important protective target of the humoral immune response to live vaccinia virus vaccination (113,129) and this aspect of protection may be lost in an LC16m8 vaccinated individual.

Modified vaccinia Ankara (MVA)

In Germany, modified vaccinia Ankara (MVA) was developed as a highly attenuated potential smallpox vaccine (235,236). This vaccine was obtained after 572 serial passages of the parental vaccinia strain on chick embryo fibroblasts. This resulted in about 25 kilobases deleted and a virus that no longer produced infectious progeny virus in almost all mammalian cell lines. That is, the virus could infect, replicate its DNA, and generate abundant amounts of key viral proteins, but could not assemble into infectious virions in most mammalian cells. Because of the inability to generate infectious virions in human cells, this type of virus would likely be safe to give to many people who have conditions that would not allow routine smallpox vaccination. Therefore, this virus has been intensively studied as a next generation smallpox vaccine that may ultimately gain FDA approval in the U.S.A. The virus has been widely studied and shown to generate antibody responses similar to Dryvax (130), as well as protection in mouse (237,238) and non-human primate challenge models (104,239,240). There is evidence that MVA

vaccination results in more rapid protection when compared to a fully replication competent vaccine, like Dryvax (241). While the mechanism for this enhanced early protection by MVA is not entirely known, part of the explanation may be that it induces more rapid immunity (241) because it is given at about a 1000-times higher dose than current replication competent vaccinia vaccines. It also appears that MVA can activate innate immune responses because it is missing genes present in replication competent vaccinia virus that encode proteins that may initially dampen the immune response (87,242).

Subunit-based vaccines

Until recently, it was believed that protection conferred by live vaccinia virus vaccination was predominantly due to anti-vaccinia T cell responses. This was mainly based on the fact that inactivated smallpox vaccines did not protect against smallpox (3). Thus, it was assumed that live vaccinia virus vaccination protected by potent anti-viral T cell responses. However, the inability of experimental inactivated vaccines to protect may have been due to denaturing of key targets (243), as well as the fact that the vaccine preparations did not contain some critical antigens that are present on a minor population of infectious virus (116,244). Furthermore, in recent years protection via vaccination with live vaccinia virus has been shown to be dependent on vaccinia specific, CD4⁺ dependent B-cell responses (94,100,107,245). Thus, future-generation smallpox vaccines that are capable of inducing protective antibody responses are viable alternatives to the current live-virus vaccines. One way to induce such antibody responses is to provide protein(s) directly to the immune system to which neutralizing and protective antibodies can be

generated. Strategies to present these critical proteins include direct injection of soluble proteins with adjuvants, introduction of recombinant DNA that host cells transcribe and translate, and live or attenuated vectors that deliver poxvirus proteins to the host immune system.

Since poxviruses are large DNA viruses that encode over 200 proteins, the identification of suitable proteins that would generate a protective immune response is complex. Most research has focused on different surface membrane proteins of the two infectious forms of virus, the mature virus (MV) and the extracellular virus (EV) (31,34). Furthermore, including targets against both MV and EV appears to provide the best protection from morbidity and mortality (98,110,138,160,246). Including targets against both forms of infectious virus is believed to provide a way to decrease the infecting inoculum (believed to be mainly MV) and then alter the spread and dissemination of the virus within an infected host (thought to be mainly EV) (73,247-250). Initial insights into appropriate targets against MV and/or EV proteins were based on the production of antibodies that could neutralize virus *in vitro* or provide passive protection against vaccinia virus challenge *in vivo* (28,96,109,145-147,154,160,166,251-254). Relevant protein targets were also identified by examining what proteins were recognized by vaccinia immunoglobulin (VIG) (113,115,127-129), serum from vaccinia virus vaccinated individuals that was used clinically to treat complications from live vaccinia virus vaccination. Many of the protein targets identified by these approaches are targets of potent neutralizing antibodies. The following sections and tables will cover the most widely studied viral targets and the effort that is being made to combine these targets into effective subunit vaccines.

Protein-based subunit vaccines

The first successful attempt at a subunit vaccine to protect against lethal vaccinia virus challenge was by Lai, et al. in 1991 (139). They intraperitoneally injected purified vaccinia virus A27 protein (an MV protein) generated in *Escherichia coli* and found that the antibody response generated was both MV neutralizing *in vitro*, and 100% protective against a lethal intraperitoneal challenge with vaccinia virus. The EV proteins, A33 and B5, produced in baculovirus were first shown to generate protective immune responses by Galmiche (109). They found that injection of A33 or B5 protein provided 100% protection from lethal intranasal challenge with vaccinia virus. While only B5 vaccination elicited *in vitro* EV neutralizing activity, the antibodies produced against A33 resulted in “comet inhibition”, indicating that they altered the way EV spread in cell culture. Antibody to A33 may also provide protection through the activation of complement (97). Table 2-1 summarizes the individual orthopoxvirus genes that have been examined as a subunit vaccine. Proteins have been expressed in bacteria (94,99,101,109,139,162,255), baculovirus (98,105,109,110), and even recombinant plants (168).

While work with individual proteins has helped identify appropriate targets to include in a subunit vaccine, the combination of multiple proteins is believed to provide the optimum protection (Table 2-2). For example, A33, B5, and L1 proteins have been used in combination to generate a mouse antibody response to both the MV and EV infectious forms of vaccinia virus (98,110). These trivalent subunit vaccines provide

Table 2-1. Individual gene targets

Gene(s) (target)	Origin	Delivery	Adjuvant	Animal Model	Challenge Virus/Route/Dose	Survival/Morbidity	Correlates of Protection Investigated	Reference
A13L (MV)	VACV, IHD-J strain	DNA, i.m., 4 doses	none	BALB/c mice	VACV, IHD-J strain/ i.n./1E7 pfu/mo	0%/severe	No antibody detected	(169)
A27L (MV)	VACV, WR strain	Protein (expressed in bacteria), i.p., 2 doses	Prime: Freund's complete, Boost: Freund's incomplete	BALB/c mice	VACV, WR strain/i.p./1.5E8 pfu/mo	100%/ND	MV NAb	(139)
	VACV, NYBH-CONN strain	DNA, gene gun (abdominal epidermis), 3 doses	DNA precipitated on gold	BALB/c mice	VACV, WR/i.p./5E8 pfu/mo	10%/severe	MV NAb	(138)
	VACV, IHD-J strain	DNA, i.m., 4 doses	none	BALB/c mice	VACV, IHD-J strain/ i.n./1E7 pfu/mo	80%/significant	No antibody detected	(169)
	CPXV	VEE virus replicon (VRP), 2 doses of 1E6 IU VRP	none	BALB/c mice	CPXV, Brighton red/i.n./1E6 pfu/mo (sublethal dose)	100%/significant	Anti-VACV antibody, Th1 response (IgG2a dominant)	(256)
	VARV	DNA, gene gun (abdominal epidermis), 4 doses	none	BALB/c	VACV, WR strain/i.p./5E7 pfu/mo	100%/moderate	MV NAb	(162)
	VACV, WR strain	Replication incompetent rAd35 vector, 1 dose	none	BALB/c mice	VACV, WR strain/i.p./2E8 pfu/mo	i.p.: 50%/severe	Anti-VACV antibody, not comet inhibitory, Th1/Th2 balanced response (IgG2a and IgG1 both produced), IFN γ producers detected	(246)
A33R (EV)	VACV, IHD-J strain	Protein (expressed in baculovirus system), s.c., 4 doses	Primary: Freund's complete / Boosts: Freund's incomplete	BALB/c mice	VACV, IHD-J strain/i.n./1E7 pfu/mo	100%/significant	Anti-VACV antibody	(109)
	VACV, IHD-J strain	DNA, i.m., 4 doses	None	BALB/c mice	VACV, IHD-J strain/i.n./1E6 pfu/mo	100%/significant	Anti-VACV antibody, protection not correlated with titers	(109)
	VACV, NYBH-CONN strain	DNA, gene gun (abdominal epidermis), 3 doses	DNA precipitated on gold	BALB/c mice	VACV, WR/i.p./5E8 pfu/mo	0%/severe	Anti-VACV antibody	(160)
	VACV, WR strain	Protein (expressed in baculovirus system), s.c., 4 doses	Ribi or QS21	BALB/c mice	VACV, WR strain/i.n./1E6 pfu/mo or 2E7 pfu/mo	1E6: 100%/mild 2E7: 70%/severe	Anti-VACV antibody, Th2 response (IgG1 dominant), comet inhibitory	(98)
	VACV, IHD-J strain	DNA, i.m., 4 doses	none	BALB/c mice	VACV, IHD-J strain/ i.n./1E7 pfu/mo	67%/significant	Anti-VACV antibody	(169)

	ECTV (EVM135)	Protein (expressed in bacteria), s.c., 2 doses	Freund's incomplete	BALB/c mice	ECTV/footpad/3000pfu/mo	70%/moderate	Anti-VACV antibody, comet inhibitory, antigen specific CD8 ⁺ T cells	(101)
	CPXV	VRP, 2 doses of 1E6 IU	none	BALB/c mice	CPXV, Brighton red/i.n./1E6 pfu/mo (sublethal dose)	100%/moderate	Anti-VACV antibody, Th1 response (IgG2a dominant)	(256)
	VACV, WR strain	Replication incompetent rAd35 vector, 1 dose	none	BALB/c mice	VACV, WR strain/i.p./2E8 pfu/mo	i.p.: 75%/moderate to severe	Anti-VACV antibody, not comet inhibitory, Th1/Th2 balanced response (IgG2a and IgG1 both produced), IFN γ producers detected	(246)
A34R (EV)	VACV, IHD-J strain	Protein (expressed in bacteria), s.c., 4 doses	Primary: Friend's complete / Boosts: Friend's incomplete	BALB/c mice	VACV, IHD-J strain/i.n./1E7 pfu/mo	0%/severe	NR	(109)
	VACV, IHD-J strain	DNA, i.m., 4 doses	none	BALB/c mice	VACV, IHD-J strain/i.n./1E6 pfu/mo	~20%/severe	NR	(109)
	VACV, IHD-J strain	DNA, i.m., 4 doses	none	BALB/c mice	VACV, IHD-J strain/i.n./1E7 pfu/mo	0%/severe	No antibody detected	(169)
A36R (Cell*)	VACV, IHD-J strain	Protein (expressed in bacteria), s.c., 4 doses	Primary: Friend's complete / Boosts: Friend's incomplete	BALB/c mice	VACV, IHD-J strain/i.n./1E7 pfu/mo	0%/severe	NR	(109)
	VACV, IHD-J strain	DNA, i.m., 4 doses	none	BALB/c mice	VACV, IHD-J strain/i.n./1E6 pfu/mo	~50%/very severe	NR	(109)
	VACV, IHD-J strain	DNA, i.m., 4 doses	none	BALB/c mice	VACV, IHD-J strain/i.n./1E7 pfu/mo	60%/significant	No antibody detected	(169)
A56R (EV)	VACV, IHD-J strain	DNA, i.m., 4 doses	none	BALB/c mice	VACV, IHD-J strain/i.n./1E7 pfu/mo	50%/significant	Anti-VACV antibody	(169)
B5R (EV)	VACV, IHD-J strain	Protein (expressed in baculovirus system), s.c., 4 doses	Primary: Friend's complete / Boosts: Friend's incomplete	BALB/c mice	VACV, IHD-J strain/i.n./1E7 pfu/mo	~90%/severe	Anti-VACV antibody	(109)
	VACV, IHD-J strain	DNA, i.m., 4 doses	none	BALB/c mice	VACV, IHD-J strain/i.n./1E6 pfu/mo	~80%/significant	NR	(109)
	VACV, NYBH-CONN strain	DNA, gene gun (abdominal epidermis), 3 doses	DNA precipitated on gold	BALB/c mice	VACV, WR/i.p./5E8 pfu/mo	40%/severe	Anti-VACV antibody, non-MV NAb	(138)
	VACV, WR strain	Protein (expressed in baculovirus system), s.c., 4 doses	Ribi or QS21	BALB/c mice	VACV, WR strain/i.n./1E6 pfu/mo or 2E7 pfu/mo	1E6: 100%/moderate 2E7: 30%/severe	Anti-VACV antibody, Th2 response (IgG1 dominant)	(98)

	VACV, IHD-J strain	DNA, i.m., 3 or 4 doses	none	BALB/c mice	VACV, IHD-J strain/ i.n./1E7 pfu/mo	100%/moderate	Anti-VACV antibody, IFN γ - Type I response	(169)
	VACV, WR strain	Protein (expressed <i>in planta</i>), i.m., 3 doses	CpG, alum	BALB/c mice	VACV, WR strain/i.n./1.2E6 pfu/mo	100%/severe	Anti-VACV antibody, comet inhibitory	(168)
	CPXV	VRP, 2 doses of 1E6 IU	none	BALB/c mice	CPXV, Brighton red/i.n./1E6 pfu/mo (sublethal)	100%/significant	Anti-VACV antibody, Th1 response (IgG2a dominant)	(256)
	VARV	DNA, gene gun (abdominal epidermis), 4 doses	none	BALB/c mice	VACV, WR strain/i.p./5E7 pfu/mo	100%/significant	Anti-VACV antibody	(162)
	VACV, WR strain	Replication incompetent rAd35 vector, i.m., 1 dose	none	BALB/c mice	VACV, WR strain/i.p. or i.n./i.p.: 2E8 pfu/mo, i.n.: 2E7 pfu/mo	i.p.: ~90%/severe i.n.: 75%/severe	Anti-VACV antibody, comet inhibitory, Th1/Th2 balanced response (IgG2a and IgG1 both produced), minimal IFN γ producers detected	(246)
	CPXV, de novo synthesis	Replication incompetent rAd5 vector, i.m., 1 dose	none	BALB/c mice	3 months post vaccination: VACV, WR strain/i.n./1E5 pfu/mo (sublethal)	100%/moderate	Anti-VACV antibody, comet inhibitory	(257)
	CPXV, de novo synthesis	rVSV, i.m., 1 dose	none	BALB/c mice	3 months post vaccination: VACV, WR strain/i.n./1E5 pfu/mo (sublethal)	53%/significant	Anti-VACV antibody, slightly comet inhibitory	(257)
	CPXV, de novo synthesis	Replication incompetent rAd5 vector prime, rVSV boost, i.n., 1 dose each	none	BALB/c mice	3 months post vaccination: VACV, WR strain/i.n./1E5 pfu/mo (sublethal)	100%/mild	Anti-VACV antibody, comet inhibitory	(257)
B18R/ EVM1 66 (IRM)	ECTV	Protein (expressed in bacteria), i.m., 3 doses	none	BALB/c mice	ECTV/Footpad/300pfu/mo(60xLD)	100%/mild to moderate	Anti-ECTV antibody, neutralized protein activity	(82)
D8L (MV)	VACV, IHD-J strain	DNA, i.m., 4 doses	none	BALB/c mice	VACV, IHD-J strain/ i.n./1E7 pfu/mo	50%/severe	Anti-VACV antibody	(169)
	VACV, WR strain	DNA, gene gun (abdominal epidermis), 4 doses	none	BALB/c mice	VACV, WR strain/i.p./5E7	100%/moderate	MV Nab	(153)
	VARV	DNA, gene gun (abdominal epidermis), 4 doses	none	BALB/c mice	VACV, WR strain/i.p./5E7 pfu/mo	100%/moderate	MV Nab	(162)
H3L (MV)	VACV, IHD-J strain	DNA, i.m., 4 doses	none	BALB/c mice	VACV, IHD-J strain/ i.n./1E7 pfu/mo	33%/severe	No antibody detected	(169)
	VACV, WR strain	Protein (expressed in bacteria), i.m., 2 doses	Ribi	BALB/c mice	VACV, WR strain/i.n./~5E5 pfu/mo or ~2.5E7 pfu/mo	~5E5:100%/significant ant ~2.5E7:0%/severe	MV Nab	(99)

L1R (MV)	VACV, NYBH-CONN strain	DNA, gene gun (abdominal epidermis), 3 doses	DNA precipitated on gold	BALB/c mice	VACV, WR/i.p./5E8 pfu/mo	89%/significant	MV NAb	(160)
	VACV, WR strain	Protein (expressed in baculovirus system), s.c., 4 doses	Ribi or QS21	BALB/c mice	VACV, WR strain/i.n./1E6 pfu/mo or 2E7 pfu/mo	1E6: 100%/moderate 2E7: 30%/severe	MV NAb, Th2 response (IgG1 dominant)	(98)
	VACV, NYBH-CONN strain	DNA, gene gun (abdominal epidermis), 4 doses	DNA precipitated on gold	NHP	MPXV/ i.v./ 2E7 pfu per NHP	100%/severe	MV NAb	(106)
	VACV, IHD-J strain	DNA, i.m., 4 doses	none	BALB/c mice	VACV, IHD-J strain/ i.n./1E7 pfu/mo	0%/severe	No antibody detected	(169)
	VACV, WR strain	Replication incompetent rAd35 vector, 1 dose	none	BALB/c mice	VACV, WR strain/i.p. or i.n./i.p.: 2E8 pfu/mo, i.n.: 2E7 pfu/mo	i.p.: 100%/mild i.n.: 75%/severe	MV NAb, not comet inhibitory, Th1/Th2 balanced response (IgG2a and IgG1 both produced), IFN γ producers detected	(246)

Abbreviations used in Table:

alum, aluminum hydroxide

Cell*, A36 is a viral protein expressed on an infected cell that helps direct MV to become EV.

CPXV, cowpox virus (Brighton red strain)

ECTV, ectromelia virus (Moscow strain)

IHD-J, International Health Department strain J of VACV

i.d., intradermal

i.m., intramuscular

i.p., intraperitoneal

IRM, immune response modifiers

i.v., intravenous

MPXV, monkeypox (Zaire 79 strain)

MV, mature virus

NAb, neutralizing antibody

ND, not done

NHP, non-human primate

NR, not reported

NYBH-CONN, New York Board of Health-Connaught strain

pfu/mo, plaque-forming unit per mouse

QS21, non-toxic saponin derived from the soapbark tree, Quillaja saponaria

rAd, recombinant adenovirus

Ribi, MPL+TDM (monophosphoryl lipid A + trehalose dicorynomycolate) adjuvant

rVSV, recombinant Vesicular stomatitis virus

s.c., subcutaneous

VACV, vaccinia virus

VARV, variola virus (India 1967 strain)

VRP, Venezuelan equine encephalitis (VEE) virus replicon particle

WR, western reserve strain of vaccinia virus

Table 2-2. Combination Gene Targets

Genes	Origin	Delivery	Adjuvant	Animal Model	Challenge Virus/Route/Dose	Survival/Morbidity	Correlates of Protection Investigated	Reference
A27L, A33R	VACV, NYBH-CONN strain	DNA, gene gun (abdominal epidermis), 3 doses	DNA precipitated on gold	BALB/c mice	VACV, IHD-J strain/ i.p./5E8 pfu/mo	70%/severe	Anti-VACV antibody	(138)
A27L, B5R	VACV, NYBH-CONN strain	DNA, gene gun (abdominal epidermis), 3 doses	DNA precipitated on gold	BALB/c mice	VACV, IHD-J strain/ i.p./5E8 pfu/mo	95%/significant	MV NAb	(138)
	VACV, WR strain	Protein (expressed in bacteria), s.c., 3 doses	Ribi or TiterMax gold adjuvant	BALB/c mice	VACV, WR strain/i.n./~2.4E6 pfu/mo	100%/significant	MV NAb, comet inhibitory, Th1/Th2 balanced response in Ribi (equal IgG1 and IgG2a, Th2 response in TiterMax adjuvant (IgG1 dominant), Cd8 ⁺ and CD4 ⁺ responses	(255)
A27L, L1R	VACV, NYBH-CONN strain	DNA, gene gun (abdominal epidermis), 3 doses	DNA precipitated on gold	BALB/c mice	VACV, IHD-J strain/ i.p./5E8 pfu/mo	40%/severe	MV NAb	(138)
A33R, B5R	VACV, NYBH-CONN strain	DNA, gene gun (abdominal epidermis), 3 doses	DNA precipitated on gold	BALB/c mice	VACV, IHD-J strain/ i.p./5E8 pfu/mo	80%/severe	Anti-VACV antibody	(138)
	VACV, WR strain	Protein (expressed in baculovirus system), s.c., 4 doses	Ribi or QS21	BALB/c mice	VACV, WR strain/i.n./ 2E7 pfu/mo	90%/severe	Anti-VACV antibody, Th2 response (IgG1 dominant), comet inhibitory	(98)
A33R, L1R	VACV, NYBH-CONN strain	DNA, gene gun (abdominal epidermis), 3 doses	DNA precipitated on gold	BALB/c mice	VACV, WR/i.p./5E8 pfu/mo	100%/mild	MV NAb	(160)
	VACV, WR strain	Protein (expressed in baculovirus system), s.c., 4 doses	Ribi or QS21	BALB/c mice	VACV, WR strain/i.n./ 2E7 pfu/mo	90%/moderate	MV NAb, Th2 response (IgG1 dominant), comet inhibitory	(98)
	VACV, WR strain	Protein (expressed in baculovirus system), s.c., 2 doses	alum, or alum + CpG, or Ribi, or QS21	BALB/c mice	VACV, WR strain/i.n./2E7 pfu/mo	alum: 50%/severe, alum + CpG: 100%/moderate, Ribi: 80%/significant, QS21: 100%/mild	MV Nab (except alum – some anti-VACV antibody), comet inhibitory, Th1/Th2 balanced response in alum + CpG or Ribi (except for anti-L1) or QS21 (equal IgG1 and IgG2a), Th2 response in alum (IgG1 dominant)	(105)
B5R, L1R	VACV, NYBH-CONN strain	DNA, gene gun (abdominal epidermis), 3 doses	DNA precipitated on gold	BALB/c mice	VACV, IHD-J strain/ i.p./5E8 pfu/mo	100%/moderate	Weakly MV NAb	(138)
	VACV, WR strain	Protein (expressed in baculovirus system), s.c., 4 doses	Ribi or QS21	BALB/c mice	VACV, WR strain/i.n./ 2E7 pfu/mo	60%/severe	MV NAb, Th2 response (IgG1 dominant)	(98)
A27L, A33R, B5R	VACV, WR strain	Protein (expressed in baculovirus system), i.m., 2 doses	CpG, alum	BALB/c mice	VACV, WR strain/i.n./~6E6 pfu/mo	VACV: ~20%/severe	Weak MV NAb, comet inhibitory	(110)

	CPXV	VRP, 2 doses of 1E6 IU	none	BALB/c mice	CPXV, Brighton red/i.n./1E6 pfu/mo (sublethal dose)	100%/mild	Anti-VACV antibody, Th1 response (IgG2a dominant)	(256)
A27L, B5R, D8L	VACV, WR strain	Protein (expressed in bacteria), s.c., 3 doses	Ribi or TiterMax gold adjuvant	BALB/c mice	VACV, WR strain/i.n./~2.4E6 pfu/mo	100%/significant	MV NAb, comet inhibitory, Th1/Th2 balanced response in Ribi (equal IgG1 and IgG2a), Th2 response in TiterMax adjuvant (IgG1 dominant), Cd8 ⁺ and CD4 ⁺ responses	(255)
	VARV	Protein (expressed in bacteria), 2 doses	Freund's incomplete	BALB/c mice	VACV, WR strain/i.n./5E6 pfu/mo	100%/ND	Anti-VACV antibody, neutralization ND	(162)
	VARV	DNA, gene gun (abdominal epidermis), 4 doses	none	BALB/c mice	VACV, WR strain/i.p./5E7 pfu/mo	100%/mild	MV NAb	(162)
	VARV	DNA, gene gun (abdominal epidermis), 2 doses	none	BALB/c mice	VACV, WR strain/i.n./5E6 pfu/mo	80%/ND	MV NAb	(162)
A33L, B5R, L1R	VACV, WR strain	Protein (expressed in baculovirus system), s.c., 3 or 4 doses	Ribi or QS21	BALB/c mice	VACV, WR strain/i.n./ 2E7 pfu/mo	100%/mild	MV NAb, Th2 response (IgG1 dominant), comet inhibitory	(98)
	VACV, WR strain	Protein (expressed in baculovirus system), i.m., 4 doses	QS21	NHP	MPXV/i.v./5E7 (sublethal dose in this study)	100%/moderate	VACV MV Nab, MPXV MV Nab, VACV comet inhibitory, MPXV comet inhibitory	(105)
	VACV, WR strain	Protein (expressed in baculovirus system), i.m., 2 doses	CpG, alum	BALB/c mice	VACV, WR strain/i.n./~6E6 pfu/mo OR ECTV/i.n./1000 pfu/mo	VACV: 100%/mild ECTV: 100%/mild	MV NAb, comet inhibitory	(110)
A27L, A33R, B5R, L1R	VACV, NYBH-CONN strain	DNA, gene gun (abdominal epidermis), 3 doses	DNA precipitated on gold	BALB/c mice	VACV, IHD-J strain/ i.p./5E8 pfu/mo	100%/mild	Anti-VACV antibody	(138)
	VACV, NYBH-CONN strain	DNA, gene gun (abdominal epidermis), 4 doses	DNA precipitated on gold	NHP	MPXV/ i.v./ 2E7 per NHP	100%/mild	MV NAb	(106)
	VACV, NYBH-CONN strain	DNA, Skin Electroporation Device, 3 doses	none	BALB/c mice	VACV, IHD-J/ i.n./2E6 per muse	100%/mild	MV NAb, Th1/Th2 balanced response (IgG2a and IgG1 detected)	(161)
	VACV, WR strain	DNA, gene gun (abdominal epidermis), 4 doses	none	BALB/c mice	VACV, WR strain/i.n./5E6 per mouse	26%/significant	MV NAb	(153)
	MPXV	DNA, i.m. and i.d., 3 doses	none	NHP	MPXV/i.v./5E7 pfu	0%/severe	Weak Anti-VACV antibody, non-MV NAb, no CD8 ⁺ or CD4 ⁺ response	(149)

	MPXV	Protein, i.m., 3 doses	alum or CpG	NHP	MPXV/i.v./5E7 pfu	100%/mild to severe	VACV MV NAb, MPXV MV NAb, MPXV EV NAb, CpG adjuvant gave a CD8 ⁺ and CD4 ⁺ response	(149)
	MPXV	DNA, i.m. and i.d., 3 doses/ Boosted with protein, i.m., 3 doses	Protein in CpG	NHP	MPXV/i.v./5E7 pfu	100%/mild	VACV MV NAb, MPXV MV NAb, MPXV EV NAb, Detectable CD8 ⁺ and CD4 ⁺ response (IL-2 ⁺ and IFN γ ⁺ /TNF α ⁺)	(149)
	VACV, WR strain	Protein (expressed in baculovirus system), i.m., 2 doses	CpG, alum	BALB/c mice	VACV, WR strain/i.n./~6E6 pfu/mo OR ECTV/i.n./1000 pfu/mo	VACV: 100%/mild ECTV: 100%/mild	MV NAb, comet inhibitory	(110)
	VACV, WR strain	Replication incompetent rAd35 vector, 1 dose	none	BALB/c mice	VACV, WR strain/i.p. or i.n./i.p.: 2E8 pfu/mo, i.n.: 2E7 pfu/mo	i.p.: 100%/mild i.n.: 100%/mild to moderate	MV NAb, comet inhibitory, Th1/Th2 balanced response (IgG2a and IgG1 both produced), IFN γ producers detected	(246)
A27L, A33R, B5R, L1R, D8L	VACV, WR strain	DNA, gene gun (abdominal epidermis), 4 doses	none	BALB/c mice	VACV, WR strain/i.p./5E7 per mouse	100%/mild	MV NAb	(153)
	VACV, WR strain	DNA, gene gun (abdominal epidermis), 4 doses	none	BALB/c mice	VACV, WR strain/i.n./5E6 per mouse	66%/moderate	MV NAb	(153)

Abbreviations used in Table - see Table 2-1.

100% protection from a lethal intranasal challenge with vaccinia virus in BALB/c mice with only mild disease (as measured by weight loss). This protection was achieved with as little as 2 doses given only 2 weeks apart and challenge of the mice 3 weeks after the boost vaccination (110). Addition of the A27 protein to the other three proteins to form a tetravalent vaccine provided little additional benefit in mice (110). This is a remarkable achievement considering that an immune response to only 3 proteins can provide protection from a virus that encodes 200 proteins. In addition to these 4 proteins, other MV targets of neutralizing antibodies such as D8 and H3 have been explored, though they have not been shown to greatly enhance the ability of the trivalent protein vaccine (A33, B5, L1) to protect against disease symptoms (99,255).

The focus of protein subunit vaccination has mainly been on the envelope proteins of poxviruses that would target the infectious forms of the virus. However, poxviruses also encode a large assortment of non-structural proteins that encode immune response modifiers (IRMs) (258). These proteins allow poxviruses to dampen or alter the immune response of the host, in order to more efficiently spread throughout the host and ultimately infect the next host. Xu et al. (82) identified that the interferon (IFN) α/β binding protein encoded by the orthopoxvirus ectromelia virus (ECTV) EVM166 gene was critical for the efficient replication and spread of ECTV within its natural host, the mouse. With this in mind, they vaccinated mice with purified EVM166 protein to induce an antibody response that could neutralize the protein's biological activity. They found that vaccinated mice challenged with a lethal dose of ECTV (by a footpad infection) were protected against death with only mild to moderate disease symptoms (82). This was the first demonstration that a non-structural protein could be used in a subunit vaccine to

interfere with the ability of a virus to modulate the host immune response. This approach may be useful in future subunit smallpox vaccines, although it would be critical to determine which IRMs are most important for the replication and spread of smallpox.

While subunit vaccines have shown protection from vaccinia virus challenge, it is also important to show the ability of a vaccine to protect against a viral challenge in its natural host. Thus, the ectromelia virus (mousepox) challenge of mice has been a useful model since ECTV is a natural pathogen of the laboratory mouse (*Mus musculus*). Fang, et al. found that immunization with 2 doses of a single EV protein, A33, could partially protect BALB/c mice from death with a lethal dose of ECTV by footpad (101). By combining EV and MV targets, protein vaccinations with A33, B5, and L1, were able to fully protect against an intranasal ECTV challenge with only mild disease symptoms observed (110).

A monkeypox model of poxvirus infection has also been studied using protein vaccination. This model is important because monkeypox represents a known human pathogen, and it is believed that if monkeys can be protected from monkeypox it is likely that a similar immune response in humans could provide similar protection. Due to the expense of non-human primate studies and the need to have a model with a reproducible outcome of death in unvaccinated controls, the monkeypox model in non-human primates has focused on a high dose intravenous challenge (104). There are obvious disadvantages of this model. One disadvantage is that the high dose intravenous challenge bypasses the natural acquisition and spread of the virus in the host and is thought to reproduce mainly the stage of secondary viremia. Thus, this type of challenge sets a very high hurdle for a vaccine to show protection since natural acquisition of infection is likely caused by a

much lower dose that may be more easily controlled by vaccination. Of equal concern is that an intravenous challenge may accentuate the protection of vaccines that rely mainly on antibody responses that neutralize the incoming virus. Nevertheless, protein vaccination has been shown to protect monkeys from challenge. Heraud, et al., injected the monkeypox orthologs of A27, A33, B5, and L1 into rhesus macaques and found that these monkeys were completely protected from death with a lethal intravenous challenge with monkeypox, though they exhibited varying degrees of morbidity (149). Similarly, a small pilot study with the vaccinia virus A33, B5, and L1 proteins showed protection from severe disease after monkeypox challenge (105). Future studies using the monkeypox model will need to examine vaccine protection using more natural modes of challenge and will have to determine if adjustments in the vaccine formulation could enhance protection.

Protein vaccination, in general, requires proper formulation in order to induce an effective immune response to the injected antigens. Varying the source of protein, amounts of protein, site of injection, and adjuvant can all play a role in the ability of the protein vaccination to elicit a potent and effective immune response. Live vaccinia virus vaccination with a fully protective vaccine like Dryvax resulted in Th1-type cellular and humoral responses (98,259). For protein vaccination, appropriate adjuvants that skew the immune response towards a Th1-type response were shown to produce the best protection from both morbidity and death (105,110,149,168).

While live vaccinia virus vaccination provides cross-protection against various orthopoxvirus infections, there is concern that a subunit smallpox vaccine based on vaccinia virus proteins might miss important epitopes present in the variola virus ortholog

proteins. Compared to live virus vaccination, the small differences of just a few amino acids between the vaccinia virus and variola virus proteins may be amplified in a subunit vaccine that relies on just a few proteins to confer protection. For example, anti-B5 monoclonal antibodies have revealed that there are protective epitopes on the vaccinia B5 protein that are not present on the variola B5 ortholog (167). Similar findings have been reported with differences between the vaccinia virus A33 protein and the monkeypox A33 ortholog (254). Thus, another strategy that is being pursued by many groups is to use the variola virus (VARV) protein orthologs. For example, vaccination with smallpox orthologs of the vaccinia virus A27, B5, and D8 proteins provided complete protection from vaccinia virus challenge (162). Importantly, in this study it was found that the antibodies induced were at least as efficient at binding VARV protein as their vaccinia virus counterparts. Further studies will be needed to determine if VARV proteins can provide greater protection against smallpox virus than vaccinia virus proteins can confer.

DNA-based subunit vaccines

DNA vaccination involves the introduction of recombinant DNA plasmids that encode relevant protein antigens (260). The DNA plasmid is introduced into mammalian cells at the injection site, where the protein is then expressed. This is thought to have a number of advantages over simply vaccinating with a purified protein: (1) Using the normal host cell machinery to produce the protein, rather than using bacterial or baculovirus produced proteins, may create a more antigenetically authentic protein to what would be made during a natural infection; (2) By producing the protein within cells, it may generate a stronger T cell response through normal MHC class I presentation; (3)

Multiple gene targets can be easily included in a vaccine; and (4) Lyophilized DNA can be stored at room temperature for long periods of time without degradation. These advantages have led a number of laboratories to pursue subunit DNA smallpox vaccines.

Galmiche, et al. were the first to demonstrate that protective responses could be generated by A33R or B5R DNA vaccines (109). Similar to what they found with protein vaccination, intramuscularly injecting DNA encoding either the A33 or B5 protein (but without the need for additional adjuvants), resulted in 100% survival of vaccinia virus challenged mice. This work led Hooper, et al. to create a bivalent DNA vaccine encoding both the MV and EV proteins L1 and A33. Using a “gene gun”, a device created to inject DNA coated on gold beads, they were able to show 100% survival and only mild disease symptoms after challenge by the intraperitoneal route with a lethal dosage of vaccinia virus (160). Hooper, et al. later expanded upon this work by including the A27L and B5R genes to make a tetravalent vaccine (138). While they found that a bivalent A27L and B5R DNA vaccine did not give complete protection, the tetravalent vaccine (A27L, A33R, B5R, L1R) gave complete protection from a lethal intraperitoneal vaccinia virus challenge with only mild disease symptoms (138). Pulford, et al. used DNA vaccines against single MV and EV vaccinia virus protein targets to determine if they could provide protection from an intranasal challenge with vaccinia virus (169). In addition to showing that the B5R DNA vaccine offered 100% protection from challenge, they also demonstrated that smallpox subunit DNA vaccines could induce an IFN γ response and a memory response mediated by a CD4⁺ T cell population (169). To determine if additional antigen targets would be beneficial in a polyvalent vaccine, Sakhatsky et al. (153) added a fifth gene, D8L, to the tetravalent DNA vaccine formulation used by Hooper, et al.

(138). They found that adding the D8L gene to the other four vaccinia virus genes offered better protection in an intranasal model of challenge than without it, though protection was not 100% (153). Additionally, Sakhatsky et al. determined that using the VARV homologs of A27L, B5R, and D8L partially protected mice from a lethal intranasal vaccinia virus challenge (162). As discussed previously, the additional use of VARV sequence to construct a vaccine may be important to ensure immune reactivity in the face of a smallpox challenge.

The lack of complete protection from morbidity seen by some investigators with the polyvalent DNA vaccines (Table 2-2) could be due to the method of DNA delivery and the type of immune response that was generated. To determine if this was the case, Hooper, et al. used a novel method of skin electroporation to deliver their DNA vaccine (161). They found that this method of delivery improved the efficacy of their tetravalent DNA vaccine (A27L, A33R, B5R, L1R), and provided complete protection from challenge in an intranasal model of infection. They found that skin electroporation mimicked to a greater extent the type of antibodies produced during Dryvax vaccination, by inducing more mouse IgG2a antibodies (Th1 response), than the gene gun method of DNA delivery (161). This finding makes the skin electroporation method a more attractive method of DNA delivery than the gene gun method. The mode of vaccination was further highlighted by work in non-human primates. Hooper, et al. found that they could generate complete protection from an intravenous monkeypox virus challenge using their tetravalent (A27L, A33R, B5R, and L1R) formulation delivered by a gene gun (106). However, Heraud et al. found that when the monkeypox homologs of A27L, A33R, B5R, and L1R were injected as naked DNA, there was no protection from

monkeypox challenge (149). Going forward, DNA vaccines will need to be administered in a way best able to generate a Th1-type immune response that includes both neutralizing antibodies and strong T cell responses.

Vector-based subunit vaccines

Vectored vaccines utilize a non-pathogenic virus or bacteria to deliver a desired antigen. Because protein and DNA vaccinations have been shown to require multiple vaccinations to achieve protective immunity, vectored vaccines have been pursued as a way to generate a smallpox vaccine that can offer protection in a single vaccination. In a smallpox outbreak setting, it would be important to induce protective immunity as rapidly as possible to avoid spread of the virus. The first laboratory to explore vector subunit smallpox vaccines utilized replicon particles of Venezuelan equine encephalitis virus (256). By expressing A27, A33, and B5, they generated a strong mouse IgG2a antibody response (Th1-type response) and protected mice from a sublethal dose of cowpox virus. Kaufman et al, utilized replication incompetent recombinant Adenovirus serotype 35 (rAd35) vectors expressing A27, A33, B5 and L1 antigens (246). By delivering a single immunization with all four rAd35 vectors, they were able to achieve complete protection in mice from a lethal intranasal vaccinia virus challenge. The rAd35 vaccine generated strong MV neutralizing antibodies that were balanced between mouse IgG2a and IgG1 antibodies (Th1 and Th2 response) (246). Vectored vaccines so far appear to be a promising delivery method for subunit smallpox vaccines, but much work is still needed to determine the immunogenicity and safety profile in non-human primates and humans.

Conclusions

While stockpiling of a live vaccinia virus vaccine grown in cell culture has been successful, significant concerns about the minor and major complications from this vaccine remain, especially in populations that have contraindications for vaccination. More attenuated live vaccinia virus vaccines, which will be much safer to give to a diverse population, will likely be the next new generation smallpox vaccine that gains regulatory approval. However, growing and maintaining a stock of a live virus vaccine, as well as the potential for adverse events, are limitations that fuel the continued pursuit of future generation smallpox vaccines. Subunit vaccines are showing great success. Many possibilities for protective vaccines exist and future efforts to directly compare different vaccination strategies will be needed. For example, Barefoot, et al. chose a single immunogen, B5, and compared multiple vaccination strategies for generating immune responses and examined the level of protection from challenge (257). They found that a heterologous prime-boost combination of recombinant vesicular stomatitis virus (rVSV) expressing B5 and recombinant Venezuelan equine encephalitis virus replicons (VRP) expressing B5 as the most synergistic regimen. A possible scenario is that the best protection from challenge may incorporate a combination of strategies, such as a DNA prime and protein boost. Heraud, et al. found that this particular strategy offered the best protection from a monkeypox challenge when compared to either DNA or protein vaccination alone (149). While subunit vaccines have mainly focused on production of antibody responses, subunit smallpox vaccines expressing vaccinia virus immunodominant T-cell epitopes have been shown to protect mice from orthopoxvirus challenge (261). Thus, another strategy is to identify epitopes that are critical for T-cell

mediated protection from smallpox and provide these to the host immune system (262). Many of the current subunit vaccines under development have only been shown to produce short-term protection from challenge, on the order of three to four weeks after the last vaccine dose. Two studies have examined the ability of a subunit smallpox vaccine to protect long-term (3-6 months after the final vaccine dose) against lethal (and sublethal) challenge with vaccinia virus. These studies showed that protection against death was incomplete and mice developed significant to severe symptoms (110,257). Live vaccinia virus vaccination offers long-term immunity, on the order of decades (92) that likely may protect against death from smallpox (263). Subunit vaccines will likely not achieve this impressive benchmark, but may be improved with continued formulation refinement. To protect against smallpox outbreaks that may be many months or years apart, it may be necessary to boost individuals who were previously vaccinated with only a subunit vaccine. An alternative strategy might be to use subunit vaccines as a way to prime diverse populations to allow safer vaccination with live vaccinia virus vaccines (264).

**Chapter Three: Antibody Against Extracellular Vaccinia Virus (EV) Protects Mice
Through Complement and Fc Receptors**

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Abstract

Protein-based subunit smallpox vaccines have shown their potential as effective alternatives to live virus vaccines in animal model challenge studies. We vaccinated mice with combinations of three different vaccinia virus (VACV) proteins (A33, B5, L1) and examined how the combined antibody responses to these proteins cooperate to effectively neutralize the extracellular virus (EV) infectious form of VACV. Antibodies against these targets were generated in the presence or absence of CpG adjuvant so that Th1-biased antibody responses could be compared to Th2-biased responses to the proteins with aluminum hydroxide alone, specifically with interest in looking at the ability of anti-B5 and anti-A33 polyclonal antibodies (pAb) to utilize complement-mediated neutralization in vitro. We found that neutralization of EV by anti-A33 or anti-B5 pAb can be enhanced in the presence of complement if Th1-biased antibody (IgG2a) is generated. Mechanistic differences found for complement-mediated neutralization showed that anti-A33 antibodies likely result in virolysis, while anti-B5 antibodies with complement can neutralize by opsonization (coating). In vivo studies found that mice lacking the C3 protein of complement were less protected than wild-type mice after passive transfer of anti-B5 pAb or vaccination with B5. Passive transfer of anti-B5 pAb or monoclonal antibody into mice lacking Fc receptors (FcRs) found that FcRs were also important in mediating protection. These results demonstrate that both complement and FcRs are important effector mechanisms for antibody-mediated protection from VACV challenge in mice.

Introduction

In the 1970s, the World Health Organization led a successful campaign to eradicate smallpox using live vaccinia virus (VACV) vaccines (3). However, recent concern over the intentional or accidental release of variola virus has led some of the world's nations to stockpile live VACV vaccines (20-22). With the risk of variola virus release minimal, concerns regarding live VACV vaccine's rare but serious side effects and many contraindications (12,226,265) have led to the pursuit of safer smallpox vaccine strategies (23,266,267). Modified vaccinia virus Ankara (MVA), a highly attenuated VACV-derived vaccine, has been under development and will likely soon become a safer alternative (268,269). However, subunit vaccination is an approach that does not rely on production of a virus. We evaluated the efficacy and mechanism by which a protein-based subunit vaccine can protect against orthopoxvirus infection.

After vaccination, protection from orthopoxvirus disease heavily depends on antibody responses in animal models (100,107,109) and humans (92,93). Many of the responses are directed against viral surface proteins on the two virion forms, mature virus (MV) and extracellular virus (EV). The MV form is the most abundant virion form in infected cells (33) and is believed to mediate spread between hosts. The EV form mediates dissemination within an infected host (34,42,270,271). The MV form contains a large set of surface proteins, while the EV form contains an extra membrane and an additional, unique subset of surface proteins. Antibody against certain proteins of either form can be partially protective, such as L1 on MV (96,98,106,147,160) and B5 or A33 on EV (96,98,101,109,159,168), though optimal protection is seen when antibodies are directed against both forms (96-98,106,110,160). Subunit protein vaccination including

target antigens from both forms achieves protection from lethal orthopoxvirus challenge in mouse and non-human primate challenge models (98,105,108,110,149). In theory, antibody generated against the MV form would act to neutralize a portion of the initial infectious dose and antibody against the EV form could then prevent some spread of progeny virus within a host. Having these antibody responses present at the time of challenge could then allow the host time to generate additional immune responses and provide protection from lethal disease.

Serum from vaccinated animals or humans is capable of efficiently neutralizing the MV form of VACV (98,108,110,132,272); however, direct antibody neutralization of the EV form has been suboptimal at even high concentrations of anti-EV antibody (44,109,111,113,155,159). Therefore, understanding the mechanism by which anti-EV antibodies provide protection has been of interest. Recent mouse studies have elucidated that an IgG2a isotype monoclonal antibody (mAb) against the B5 protein called B126 can neutralize EV in the presence of complement (C') and utilizes C' to partially mediate protection in vivo (124,125). This evidence suggests that antibody against EV would be more effective if it was of an isotype that mediated effector functions such as activation of C' and/or Fc receptor (FcR) dependent activity (e.g. antibody dependent cellular cytotoxicity (ADCC)). Previous studies of antibody responses to protein vaccination found that formulations that included adjuvants that produced higher titers of IgG2a antibody in mice and IgG1 antibody in non-human primates were more effective at mediating protection than vaccines formulated without these adjuvants (105,108). This suggests that antibody with specific Fc activities might be beneficial for protection.

By utilizing a high PFU luciferase reporter EV neutralization assay, we find that polyclonal antibody responses against the EV proteins A33 and B5 utilize C' to neutralize virus in vitro, though in mechanistically different ways. These findings shed light on how differing viral proteins dictate the requirements for the host to neutralize incoming virus with C'. Additionally, we show that antibody against B5 utilizes C' and FcRs to protect mice from lethal VACV challenge. These findings add to our understanding of how antibody can protect against orthopoxvirus disease and highlights the importance of understanding antibody effector functions necessary for protection to aid in the rational design of anti-viral vaccines and therapeutic antibodies.

Materials and Methods

Proteins and vaccine formulations

Proteins used in the vaccine formulations were purified recombinant baculovirus-expressed proteins that were previously described (108). Protein vaccines were prepared and used as described previously (110). Briefly, proteins (each at 2 µg/mouse) and adjuvant(s) were prepared in sterile PBS and a final volume of 50 µL was injected intramuscularly into the hind leg of ketamine/xylazine-anesthetized mice. For vaccines adjuvanted with alum only (Alhydrogel, Accurate Chemical, Westbury, NY), formulations included the alum at 200 µg aluminum ion/mouse. For vaccines formulated with alum and CpG, the alum was at 100 µg aluminum ion/mouse and the phosphorothioate B class mouse specific CpG ODN 1826 (sequence 5'-TCC ATG ACG TTC CTG ACG TT-3'; Coley Pharmaceutical Group, now Pfizer Inc.) was used at 50 µg/mouse. On the day of vaccinations, vaccine formulations were prepared, mixed at

room temperature for 2-3 hours, and loaded into 0.3 mL insulin syringes with a 29-gauge needle (Becton Dickinson).

Mice, immunizations, and challenge

BALB/c mice and C57BL/6 mice were purchased from Charles River and Jackson Laboratory, respectively. Fc-receptor knockout mice (FcRKO) mice on the BALB/c background were purchased from Taconic Farms. Complement component C3 knockout mice (C3KO) on the C57BL/6 background (originally provided by J. D. Lambris, University of Pennsylvania) were bred at the University of Pennsylvania. Active immunizations were performed as previously described (110). Briefly, mice were primed by intramuscular vaccination, boosted 2 weeks later, and bled 1 day prior to challenge (approximately 3 weeks after the boost) to assess successful antibody production and isotype analysis. In some instances, additional mice were terminally bled prior to challenge for serum to be used in *in vitro* EV neutralization assays. Passive immunizations were performed using the anti-B5 rabbit polyclonal antibody (pAb) R182 (96) (2 mg of purified total rabbit IgG /mouse) or the anti-B5 monoclonal antibody (mAb) B126 (125) (100 μ g of purified mouse IgG /mouse; generously provided by Kyowa Hakko Kirin Co. and S. Crotty, La Jolla Institute for Allergy and Immunology). Antibodies, diluted in sterile saline at a final volume of 300 μ L/mouse for R182 or 100 μ L/mouse for B126, were injected intraperitoneally (i.p.) one day prior to challenge. Control mice were given sterile saline only. Vaccinated mice were challenged as described previously (110). Briefly, VACV (strain WR) was grown in BSC-1 cells (ATCC[®] Number CCL-26[™]) and virus from cell lysates isolated by ultracentrifugation

through two sequential 36% sucrose cushions. Three weeks after the boost protein vaccination or one day after passive immunization with antibody, mice were anaesthetized with ketamine/xylazine and challenged intranasally with a lethal dose of VACV in a total volume of 20 μ L (10 μ L/nostril) in sterile PBS. Challenge doses were confirmed by titering on BSC-1 cells the day of challenge and indicated in the figure legends. Mice were weighed and monitored each day and mice that reached >30% starting weight or met end point criteria were humanely euthanized. Experiments were performed under a protocol that was approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC), Animal Welfare Assurance number A3079-01. To minimize pain, all viral challenges and intramuscular vaccinations were performed under ketamine/xylazine anesthesia. To minimize suffering after viral challenge, mice were monitored and humanely euthanized when end point criteria were met.

Antibody ELISA

Antibody ELISA was performed as previously described (108,110). Briefly, plates were coated overnight at 4°C with 0.5 μ g/mL non-his tagged recombinant A33, B5, or L1 protein in bicarbonate/carbonate coating buffer. After blocking with 5% non-fat dry milk in PBS, 2-fold serial dilutions of mouse sera were added and incubated for 1.5 hrs at 37°C. After washing, HRP-conjugated rabbit anti-mouse IgG (Abcam, Cambridge, MA) secondary antibody was added at 1:4000 in blocking buffer and incubated at 37°C for 1 hr. Color development was performed using ABTS substrate (Sigma) for 20 minutes at room temperature. The reaction was stopped using 1% SDS in

distilled water. IgG isotype was assessed similarly, using HRP-conjugated rat anti-mouse IgG1 or IgG2a (BD Biosciences Pharmingen), or HRP-conjugated goat anti-mouse IgG2c (SouthernBiotech, Birmingham, AL) secondary antibody diluted 1:1000, 1:1000, and 1:4000 respectively in blocking buffer and incubated for 1 hr at 37°C.

In vivo complement depletion

C' was transiently depleted using native Cobra Venom Factor (CVF) from *Naja naja kaouthia* (Quidel Corporation) as previously described (125). Briefly, 10 µg (~4 units) of CVF in sterile saline (100 µL total volume) was administered i.p to mice on days -1, 2, and 5 of challenge. Complement depletion was confirmed on days 0, 3, and 6 by C3 western blotting (273), C3 ELISA (125), and CH50 assay (274,275) (Fig. 3-1). We found that C' was fully depleted on days 0 and 3, but C' activity and C3 protein were at ~50% the levels of undepleted sera on day 6 confirming previous findings of transient depletion (125). Intranasal challenge was performed on day 0.

EV production

RK-13 cells (ATCC® Number CCL-37™) were plated in 6-well plates 2 days prior to use and used at 100% confluency. To produce EV, RK-13 cells were infected with vaccinia IHD-JvFire (276) (generously provided by B. Moss, NIH) in serum-free MEM at MOI of 0.5. Two days after infection, supernatant was harvested, clarified by centrifugation at 450 × g at 4°C, and the virus remaining in the supernatant was immediately titered in the presence of MV neutralizing monoclonal antibody 2D5 (1:500 dilution of ascites fluid) (277) . In general, based on titering of supernatants in the

presence or absence of 2D5, >80% of virus in the supernatants was EV. Clarified media was stored on ice at 4°C and used within a week of isolation. For EV expressing specific human complement regulators, the same protocol for EV preparation and titering was followed except that simian virus 40-transformed aortic rat endothelial cells (SVAREC) stably transfected with plasmids expressing human CD55 or human CD59 were used (46,278) (a generous gift of G. L. Smith, Imperial College of London). We confirmed expression of human CD55 and human CD59 in these cell lines by western blotting using polyclonal rabbit anti-human CD55 and anti-human CD59 antibodies (Santa Cruz Biotechnology) (Fig. 3-2). The parental and stably transfected SVAREC cells were maintained in DMEM with 10% FBS and 100 µg/mL hygromycin B.

EV neutralization in the presence of complement

Serum from vaccinated mice or rabbit pAb or mAb was serially diluted 1:3 in triplicate in serum free MEM in a round-bottom 96-well plate. Serum free MEM was added to all wells so that each well had 50 µL total volume. Next, $\sim 5 \times 10^4$ PFU of EV containing a 1:500 final dilution of anti-MV mAb 2D5 in MEM was added to each well (22 µL/well) so that each well had a total volume of 72 µL. Baby rabbit complement (C') (Cedarlane Laboratories, Burlington, NC) at a final dilution of 10% (8 µL) was added to each well so that the final volume in each well was 80 µL. Alternatively, heat inactivated baby rabbit C' (iC') was added as a negative control. The 96-well plates containing virus, antibody, and C' were incubated at 37°C for 30 minutes after which the contents from each well was transferred to Costar 96-well white clear-bottom tissue culture treated plates (Corning) containing a monolayer of BSC-1 cells. The BSC-1 cells were prepared

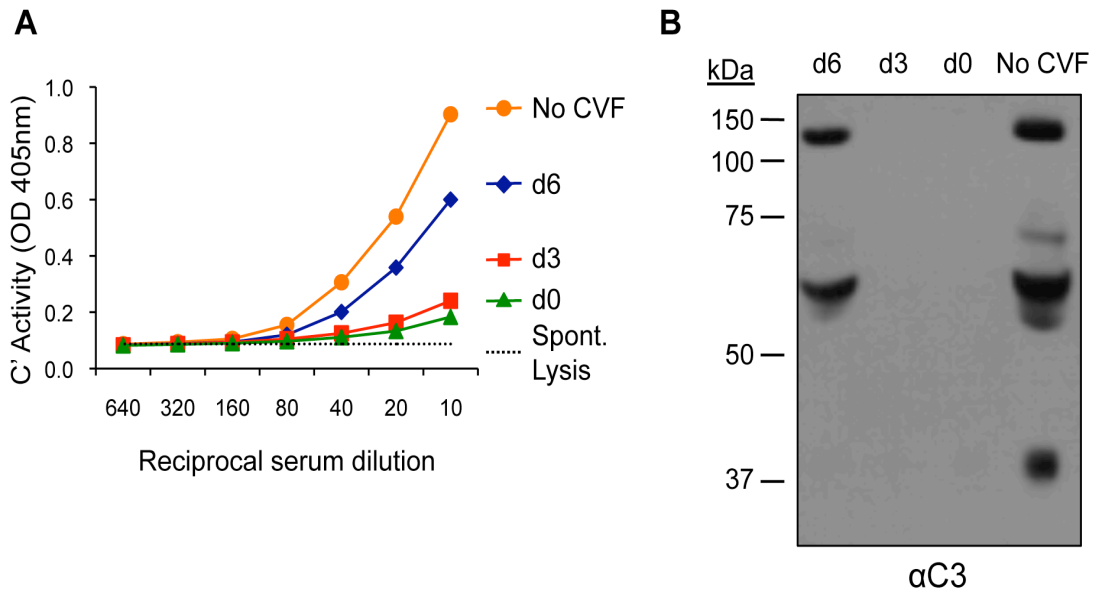


Figure 3-1. Cobra Venom Factor (CVF) treatment of BALB/c mice transiently depletes complement.

To assess the degree of C' depletion after CVF treatment, groups of 11- to 12-week old female BALB/c mice (2 mice per group) were treated with CVF and then terminally bled the following day. One group was treated on day -1 and bled the next day (d0). A second group was treated on days -1 and +2 and bled the next day (d3). A third group was treated on days -1, +2, and +5 and bled the next day (d6). A group of untreated mice was used a control (No CVF). (A) CH50 assay using rabbit erythrocytes (Complement Technology, Tyler, TX) sensitized with goat anti-rabbit erythrocyte antibody (MP Biomedicals, Solon, OH) was performed with sera from mice treated or not treated with CVF. Complement activity levels on days 0 and 3 were low, while some complement activity returned by day 6. Note that sensitized rabbit erythrocytes were used because sensitized sheep erythrocytes are resistant to lysis by mouse complement. (B) Western blot of C3 protein in sera of mice treated or not treated with CVF. Serum (2 μ l) from the indicated group of mice was loaded on to a 10% polyacrylamide gel. After blotting, HRP-conjugated goat anti-mouse C3 antibody (MP Biomedicals, Solon, OH) at 1:10,000 was used to probe for the presence of C3. C3 protein was not detected on days 0 and 3, while some C3 protein was detected on day 6.

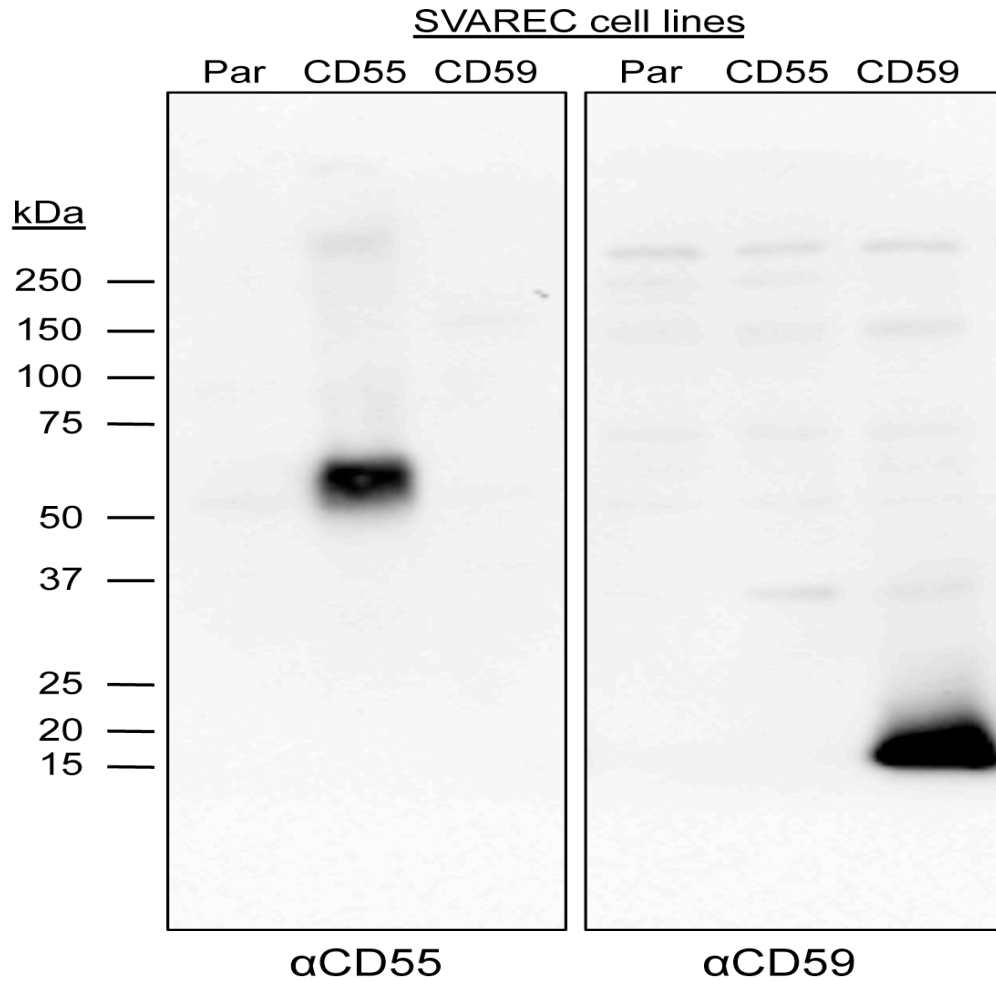


Figure 3-2. CD55 and CD59 are detected in stably transfected SVAREC cell lines.

SVAREC cell lines stably transfected to express CD55 and CD59 (46,278) were grown under selective pressure as described in materials and methods. Western blotting for either CD55 (α CD55) or CD59 (α CD55) was performed on lysates from the parental SVAREC cell line expressing no human complement regulators (Par), a SVAREC cell line expressing CD55 (CD55), and a SVAREC cell line expressing CD59 (CD59). Rabbit polyclonal anti-human CD55 and anti-human CD59 antibodies were used at a dilution of 1:250. CD55 was only detected in the CD55-expressing cell line at its expected size of ~70kDa and CD59 was only detected in the CD59-expressing cell line at its expected size of ~20kDa.

on these plates 48 hours before use and subsequently used at 100% confluency. Once infected, the plates were incubated at 37°C for ~20 hours. Luciferase production was measured by adding 100 µL of SuperLight™ Luciferase Reporter Gene Assay Reagent (BioAssay Systems, Hayward, Ca) directly to each well and relative light unit (RLU) measurements obtained on a MLX Revelation microtiter plate luminometer (Dynex Technologies). To relate RLU readings to PFU, known amounts of EV were serially diluted on the same BSC-1 plate to generate a standard curve. Linear fit was calculated and RLU readings were converted to PFU.

Neutralization of EV with human C' (Sigma-Aldrich, St. Louis, MO) or human C' depleted of C1q (Complement Technology, Tyler, TX) or C5 (Sigma-Aldrich, St. Louis, MO) was performed using the same luciferase assay as above with the following modifications. Prior to the addition of C', virus and antibody were incubated for 1 hour. After the addition of human C' (used at a concentration of 20%), the plate was incubated for an additional 1 hr. These changes were made because we found that neutralization using human C' with the rabbit and mouse antibodies was less efficient than with baby rabbit C'. Percent neutralization was calculated by dividing luciferase RLU readings from wells containing antibody by RLU readings of control wells containing no antibody for each serially diluted antibody. Percent neutralization by virolysis was calculated by the following formula: $100 - [(\%NAb - \%NAb \text{ with } C5\text{def}C') / (\%NAb - \%NAb \text{ with } hC') * 100]$ where %NAb is percent neutralization with antibody, C5defC' is C5 deficient human C' and hC' is complete human C'. This formula controls for any neutralization with antibody alone and determines the contribution of the lytic pathway.

Statistics

Statistical significance was determined using Prism 5.0 software. Differences in percent neutralization and weight loss were calculated using an unpaired 2-tailed t-test. Differences in survival were calculated using Kaplan-Meier analysis and log-rank test. *P* values of less than 0.05 were considered significant.

Results

Protection from lethal VACV challenge requires the inclusion of CpG adjuvant and correlates with the induction of IgG2a antibody.

We have previously shown that vaccinating mice and non-human primates with a combination of VACV proteins and CpG and aluminum hydroxide (alum) protects from a lethal poxvirus infection (108,110). Using different adjuvant systems (MPL+TDM or QS21), Fogg et al. showed that vaccination with combinations of proteins provided better protection than individual proteins (98). Here, we investigated the protection of BALB/c mice vaccinated with both combination and individual proteins with adjuvants CpG/alum or alum alone (Table 2-1). Mice that received a vaccine adjuvanted only with alum succumbed to infection, regardless of the combination of proteins given. Mice receiving protein(s) with CpG/alum were able to survive infection to varying degrees, with combination proteins achieving 100 percent survival. The combination protein vaccines achieved 100 percent protection due to antibody generated against both MV and EV. L1/CpG/alum and B5/CpG/alum showed partial protection. Notably, while A33/CpG/alum showed no survival at this challenge dose, the addition of A33 to L1/CpG/alum resulted in less post-challenge weight loss than mice vaccinated with only

Table 3-1. Summary of survival and maximum weight loss after challenge with 4×10^6 pfu VACV

Vaccination group ^a	Percent survival	Maximum percent average weight loss ^b
ABL/CpG/alum	100	10
AL/CpG/alum	100	2
BL/CpG/alum	100	12
L1/CpG/alum	80	23
B5/CpG/alum	40	24
A33/CpG/alum	0	N/A
ABL/alum	0	N/A
AL/alum	0	N/A
BL/alum	0	N/A
L1/alum	0	N/A
B5/alum	0	N/A
A33/alum	0	N/A
unvaccinated	0	N/A

a. A, B, L: A33, B5, L1, respectively. Alum: aluminum hydroxide

b. N/A: not applicable. Since these groups had 0% survival, average weight loss of the group is not reported since mice were sacrificed when they had 30% weight loss or died prior to reaching this degree of weight loss.

L1/CpG/alum (2% vs. 23% weight loss; $p=0.0003$) again demonstrating that combinations of proteins provided better protection than individual proteins.

Others have previously shown that the addition of CpG adjuvant biases the immune response towards Th1 in mice (105,279-282). As expected, IgG2a antibody was only produced in BALB/c mice given the CpG/alum combination adjuvant (Fig. 3-3). IgG1 was produced in both CpG/alum and alum only groups, with varying titers to the individual proteins.

Sera from mice vaccinated with A33 or B5 /CpG/alum can neutralize large numbers of EV particles in the presence of complement.

Given the correlation seen between the appearance of Th1-biased antibodies (IgG2a in mice) and protection, as well as previous studies showing that Th1-biased antibodies are more protective than Th2-biased antibodies and can neutralize EV in the presence of C' (105,108,124,125), we next determined if sera from vaccinated mice could neutralize EV in the presence of C'. Previously, plaque reduction assays with small numbers of EV (~50-150 PFU) were used to show neutralization; however, we wished to test the ability of antibody and C' to neutralize large numbers of EV particles that are more likely present during an infection. To do this, we developed an EV neutralization assay with a recombinant VACV that expressed a luciferase reporter protein. For this assay we generated standard curves with known amounts of EV, which allowed for conversion of RLU to PFU. In this assay, anti-L1 mAb 2D5 (277) was always added to neutralize contaminating MV in the EV preparation. To confirm the functionality of this assay, we tested B126, an anti-B5 mAb with IgG2a isotype previously shown to

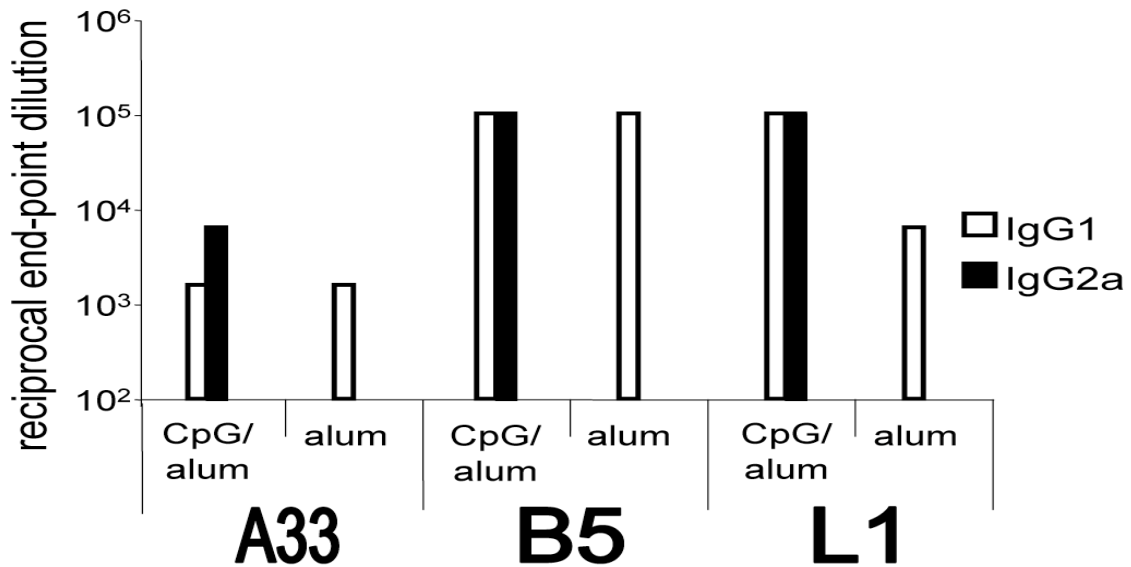


Figure 3-3. Antibody isotypes produced in BALB/c mice after vaccination. Groups of 6-week old female BALB/c mice (9 mice/group) vaccinated individually with A33, B5, or L1 adjuvanted with alum +/- CpG were bled three weeks after the boost vaccination. Equal volumes of heat-inactivated serum from individual mice in each group were pooled. Shown are reciprocal end-point dilutions for antibody isotypes IgG1 (white bars) and IgG2a (black bars) as measured by ELISA reactivity with proteins A33, B5, or L1. Vaccinations without CpG produced no detectable IgG2a response.

neutralize EV in the presence of C' (125), as well as VMC-30, an anti-B5 mAb from a previously characterized panel of anti-B5 mAbs (166). As shown in Fig. 3-4, B126 was capable of neutralizing 97% of $>10^4$ EV particles in the presence of C'; however, VMC-30 was not. Neutralization was abrogated if C' was first heat inactivated.

Using this luciferase-based high EV particle neutralization assay, we found that anti-A33 and anti-B5 sera from mice vaccinated with protein and CpG/alum could neutralize large numbers of EV particles in the presence of C' (Fig. 3-5). Neutralization was largely abrogated if C' was first heat inactivated. Anti-B5 sera was better at C'-mediated neutralization than anti-A33 sera, though this might be explained by the higher titer of IgG2a anti-B5 antibody compared to anti-A33 antibody (Fig. 3-3). Interestingly, while anti-A33 antibodies have the ability to neutralize EV in the presence of C', there was a lack of protection in mice vaccinated with A33/CpG/alum.

Anti-A33 C'-mediated antibody neutralization requires steps that could lead to EV outer envelope lysis, while anti-B5 C'-mediated antibody neutralization can occur by opsonization.

To elucidate why the A33/CpG/alum vaccination was not as effective as B5/CpG/alum at protecting mice from lethal challenge despite neutralizing EV in the presence of C', we asked whether the pathway of C'-mediated neutralization was playing a role. Lustig, et al. demonstrated that anti-A33 polyclonal rabbit sera (pAb) resulted in C'-mediated lysis of the outer membrane of EV allowing anti-MV neutralizing antibody access to the MV particle within (97). Benhnia et al. showed that anti-B5 mouse mAb

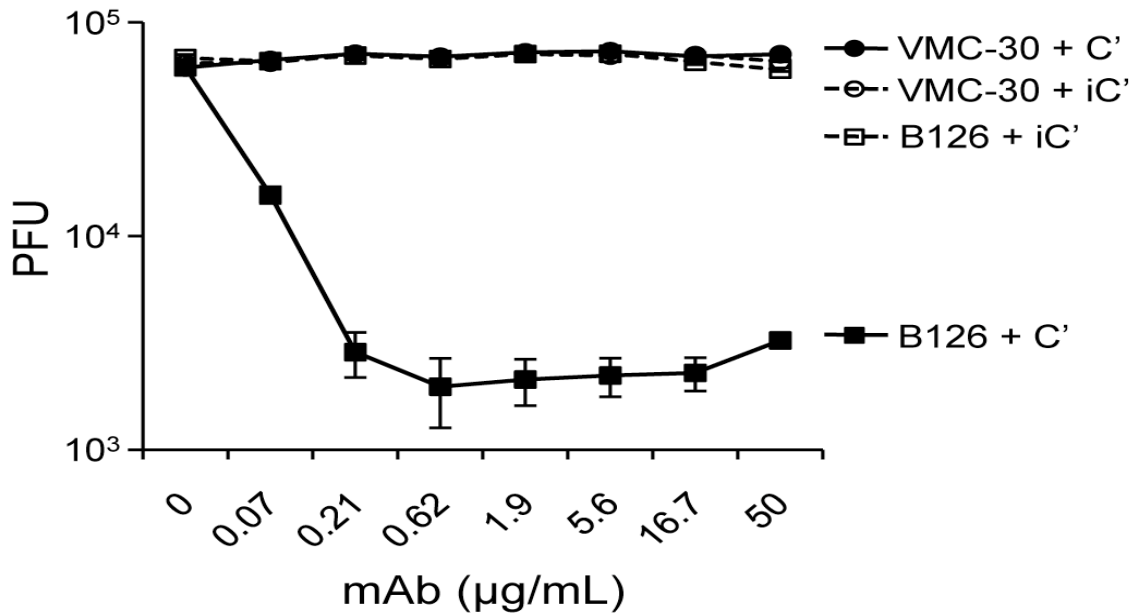


Figure 3-4. Neutralization of large numbers of EV particles using anti-B5 mAbs and complement.

Anti-B5 mAb B126 (squares) and anti-B5 mAb VMC-30 (circles) were used in a luciferase-based high PFU EV neutralization assay. $\sim 5 \times 10^4$ pfu of EV was incubated with increasing concentrations of mAb in the presence of 10% baby rabbit C' (solid symbols) or heat inactivated baby rabbit C' (iC') (open symbols). Luciferase units were converted to PFU by linear regression of a standard curve using known numbers of EV. Neutralization was performed in triplicate for C' and duplicate for iC' and represents two independent experiments. Error bars represent standard deviation.

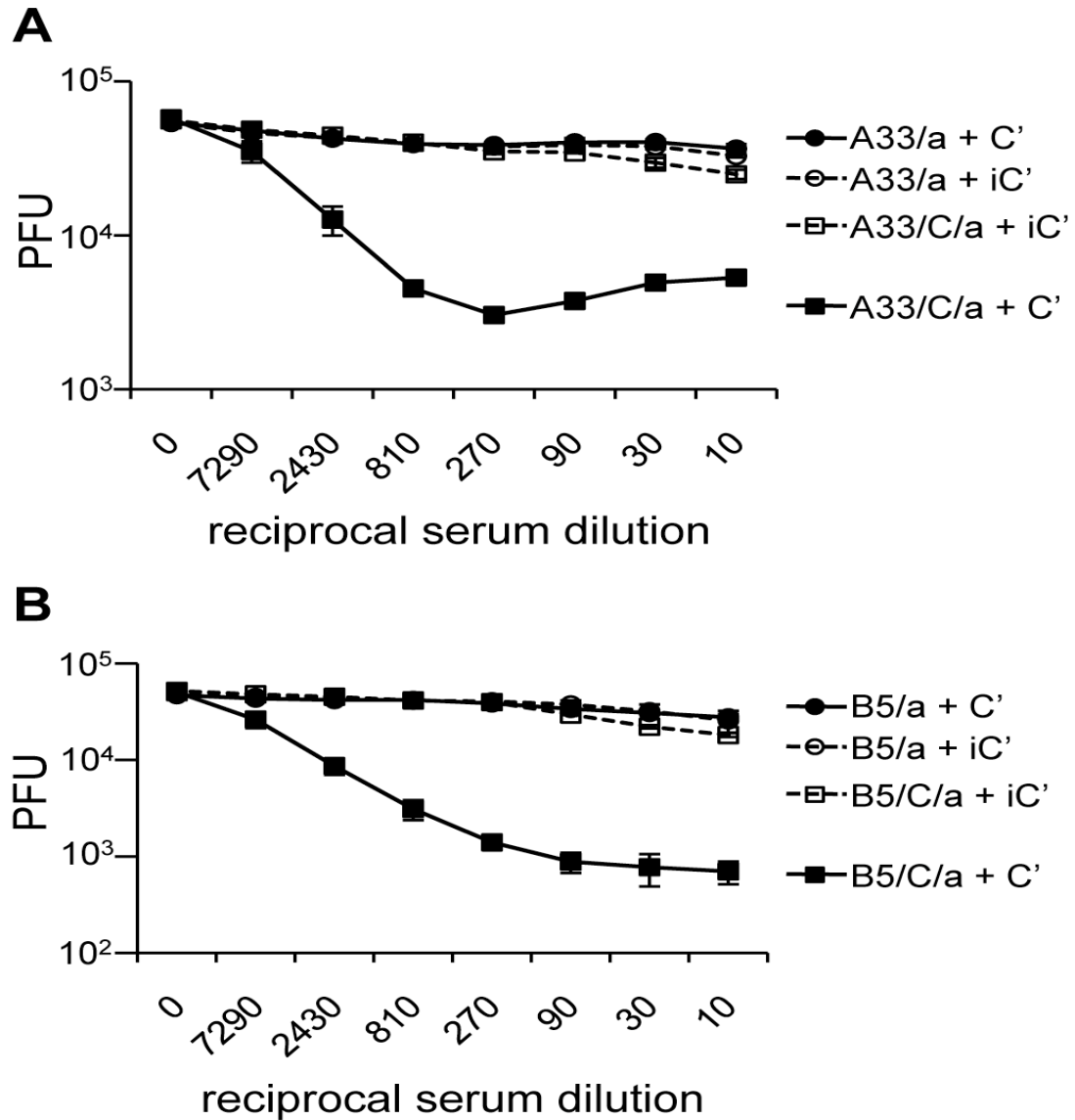


Figure 3-5. Neutralization of large numbers of EV particles using sera from mice vaccinated with A33 or B5 in CpG/alum or alum only.

Sera from BALB/c mice vaccinated with A33 (A) or B5 (B) adjuvanted with CpG/alum (squares) or alum only (circles) were collected 3 weeks post boost vaccination. Equal volumes of heat-inactivated serum from groups of mice were pooled (9 mice/group). $\sim 5 \times 10^4$ pfu of EV was neutralized with increasing amounts of sera in the presence of 10% baby rabbit C' (closed symbols) or heat inactivated baby rabbit C' (iC') (open symbols). Luciferase units were converted to PFU by linear regression of a standard curve using known numbers of EV. Neutralization was performed in triplicate for C' and duplicate for iC' and represents three independent experiments. Error bars represent standard deviation.

B126 neutralized EV by opsonizing particles with complement and could neutralize in the absence of membrane attack complex formation and without an anti-MV antibody present (125). However, the question remained whether these observations with anti-A33 and anti-B5 antibodies were due to the differing EV target proteins or if the differences in C'-mediated neutralization was due to inherent differences in the two studies (e.g., pAb vs. mAb, rabbit vs. mouse antibodies, and different adjuvants used during generation of the antibodies). To determine if different C'-mediated neutralization pathways might be used for different EV protein targets, sera from mice vaccinated with either A33 or B5 /CpG/alum were used with human C' depleted of C5 or C1q (Figs. 3-6A-C). Depletion of either C1q or C5 from sera significantly reduced the ability of anti-A33 mouse sera to efficiently neutralize EV (Fig. 3-6A) indicating that steps that lead to formation of the membrane attack complex were required for neutralization. However, only the sera depleted of C1q affected the ability of anti-B5 mouse sera to neutralize EV (Fig. 3-6B) indicating that membrane attack complex does not need to form for successful EV neutralization. Similar results were obtained when rabbit pAb against A33 and B5 was used (Figs. 3-6D and E). These results are consistent with the previously described findings that the mechanism of C'-mediated neutralization for A33 and B5 differ(97,125), with anti-A33 antibody relying on virolysis and anti-B5 antibody able to neutralize by opsonization (Figs. 3-6C and F).

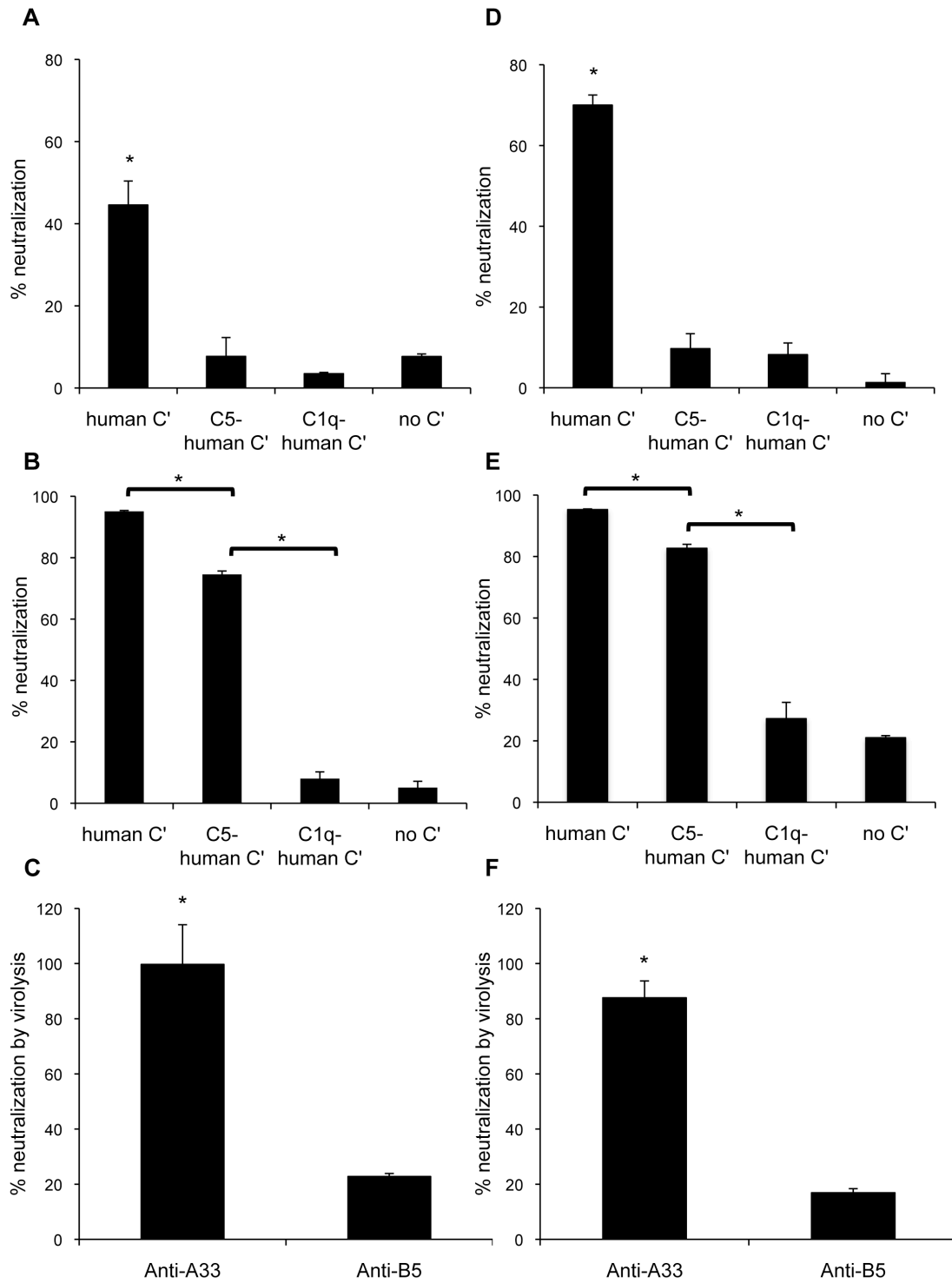


Figure 3-6. Contribution of steps leading to the formation of the membrane attack complex in complement-mediated neutralization of EV.

Graphed is the percent neutralization of EV in the presence of antibody and 20% human C' or with human C' depleted of component C5 or C1q. Sera from BALB/c mice vaccinated with A33 or B5/CpG/alum was used at a dilution of 1:20 (A-C). Rabbit pAb against A33 and B5 was used at 50 µg/mL (D-F). Data shown in A, B, D, and E are percent neutralization of no antibody control. Data in C and F show the specific EV neutralization dependent on C5 (virolysis) and was calculated as described in materials and methods. Neutralizations were performed in triplicate. Error bars represent standard deviation. * p<0.05.

(The following paragraph and figure are unpublished.)

The inability of anti-A33 antibody to neutralize EV in the presence of C5 depleted sera suggested that C' activation steps downstream of C5 were needed; however, the possibility remained that a C5-dependent, MAC-independent mechanism of neutralization was being utilized by anti-A33 antibody and complete C'. Such a mechanism has been reported for C'-mediated neutralization of HSV (194). To confirm that MAC formation was required, we measured the ability polyclonal rabbit antibody against A33 to neutralize EV in the presence of human C' depleted of C8. In the presence of complete human C', anti-A33 antibody was able to neutralize EV, but not in the presence of C5, C8, or C1q depleted human C' (Fig. 3-7). The percent neutralization by virolysis dependent on C8 was calculated as described in materials and methods using C8 instead of C5 and found to be 93.9%. This finding confirms that C' activation steps downstream of C5 and C8, likely formation of the MAC, are needed for neutralization of EV in the presence of C'.

Benhnia hypothesized that antibody alone was unable to fulfill the basic occupancy model for EV neutralization because of the amount of B5 protein on the EV surface and that antibody-induced C' coating of the EV membrane allowed for the occupancy model to succeed (124). To examine this further, we varied the anti-B5 antibody concentration as well as used EV that incorporated different C' regulators into its outer envelope. These C' regulators (CD55 and CD59) have been shown to be present on the EV envelope (46). CD55, also known as decay-accelerating factor, inhibits stable formation of the C3 convertase and down-modulates the amount of C3b/C4b deposition as well as the downstream steps in the C' cascade (199) and thus could alter the ability of

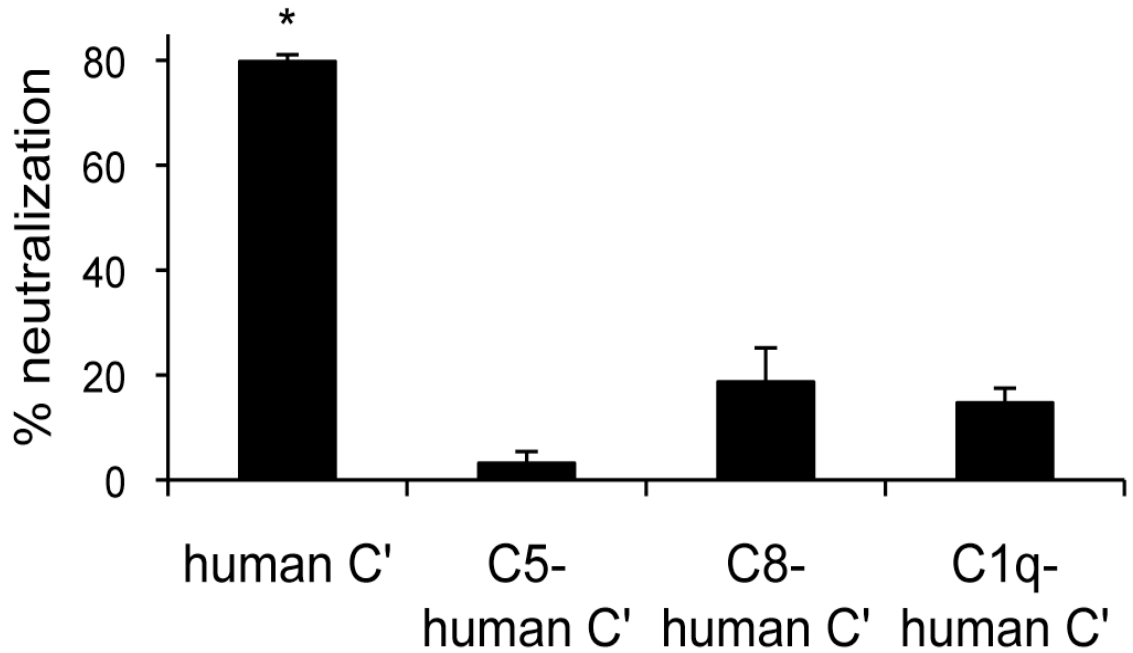


Figure 3-7. Contribution of complement component C8 in complement-mediated neutralization of EV.

Graphed is the percent neutralization of EV in the presence of antibody and 20% human C' or with human C' depleted of component C5, C8, or C1q. Rabbit pAb against A33 was used at 50 µg/mL. Data shown is percent neutralization of no antibody control. Neutralizations were performed in triplicate. Error bars represent standard deviation. * p<0.05. (Unpublished)

C' to opsonize EV. CD59, or protectin, prevents formation of the membrane attack complex and could block virolysis of the EV membrane. We produced EV that had each C' regulator on its surface using previously described SVAREC cell lines that expressed no human C' regulators or were stably transfected to express human CD55 or CD59(46,278). At high concentrations of anti-B5 sera (1:80), EV produced in the cell line expressing CD55 showed some protection from C'-mediated neutralization (Fig. 3-8, striped bar), while EV produced in the cell line expressing CD59 showed no protection from C'-mediated neutralization (Fig. 3-8, white bar). This data supports a model where virolysis is not needed since the presence of CD59 did not alter the ability to neutralize EV at the relatively high concentration of antibody. However, at relatively low concentrations of anti-B5 sera (1:640), EV produced in the cell lines expressing CD55 or CD59 were equally protected from C'-mediated neutralization (striped and white bars). The finding that CD59 provides protection equal to that of CD55 when antibody is at low concentration indicates that virolysis becomes the predominant pathway for neutralization. Therefore, if the amount of antibody on the surface of EV is limited, virolysis is required for neutralization as was seen with anti-A33 sera.

Complement is partially responsible for the protection seen in mice after passive or active immunization.

To determine what effector functions of antibody were important for in vivo protection, we used immunizations that targeted B5. Benhnia et al. found that passive immunization with mAb B126 was less protective in vivo if mice were first transiently depleted of C' using cobra venom factor (CVF) (125). However, mice still recovered and

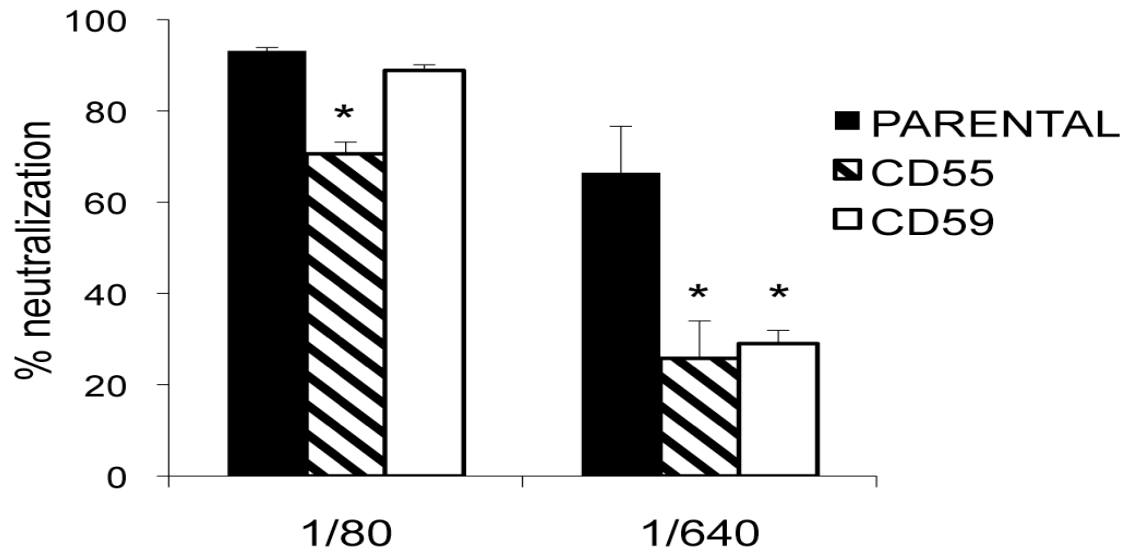


Figure 3-8. Protection of EV by human regulators of complement from complement-mediated neutralization at high and low amounts of antibody.

Sera from BALB/c mice vaccinated with B5/CpG/alum was used to neutralize EV produced in SVAREC cells expressing CD55 (striped bars), CD59 (white bars), or no human regulators of C' (black bars) in the presence of 20% human C'. While a full range of antibody dilutions were tested, shown is a representative low dilution (1/80) and high dilution (1/640) of antibody and the effect on complement-mediated neutralization of EV containing CD55 or CD59. The full range of antibody dilutions revealed that EV was partially protected by CD59 at dilutions between 1/80 and 1/640, but not as protective as CD55 at those dilutions. Data is shown as percent neutralization of no antibody control. Error bars represent standard deviation. * p<0.05.

were more protected than naïve mice. To first confirm the contribution of C' in protection by a polyclonal anti-B5 antibody response, we passively transferred pAb rabbit anti-B5 antibody (R182) into C3 knockout (C3KO) and wild-type mice (C57BL/6) and intranasally (i.n.) challenged them with a lethal dose of VACV (Fig. 3-9). C3KO mice lost significantly more weight than C57BL/6 mice indicating that C' contributed to protection. However, similar to Benhnia et al. (125), when compared to the controls that were not treated with antibody, we did note partial protection in the absence of C'. Next, to determine if antibodies produced during active immunization protected in a similar fashion, we vaccinated C3KO and wild-type C57BL/6 mice with B5/CpG/alum. Interestingly, despite a few reports that C3KO mice were defective in making antibody responses (273,283-285), we found that our vaccine resulted in total IgG and IgG2c responses comparable to the wild type C57BL/6 mice (Fig. 3-10A). After vaccinations, mice were challenged i.n. with VACV and weight loss was monitored. Vaccinated C57BL/6 mice lost minimal weight and fully recovered by day 8 post-infection. Conversely, vaccinated C3KO mice lost significant weight similar to C57BL/6 naïve and C3KO naïve mice (Fig. 3-10B) again indicating a role of C' in protection from challenge.

Fc receptors protect mice passively transferred with anti-B5 antibody in the absence of complement.

In the work by Benhnia, et al (125), it was unclear whether the recovery of normal levels of C' after CVF depletion or effector functions mediated through Fc receptors (FcRs) were responsible for the protection afforded by mAb B126 even after C' depletion. Based on our findings in C3KO mice, it was evident that additional

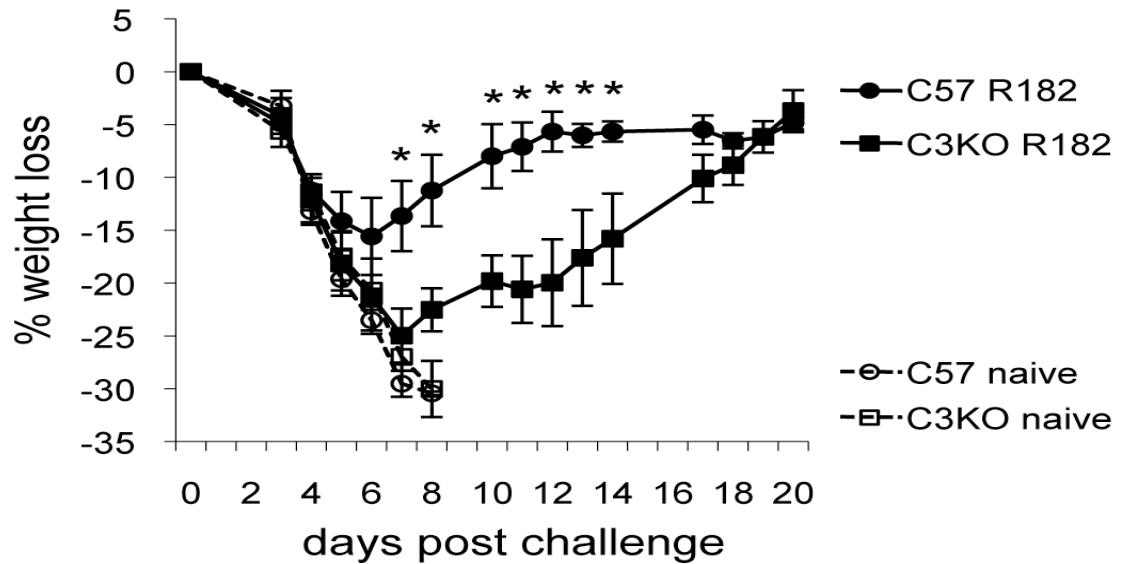


Figure 3-9. Protection from vaccinia virus challenge by anti-B5 rabbit pAb in C57BL/6 and C3KO mice.

Anti-B5 rabbit pAb (R182; 2 mg/mouse) were passively transferred by the i.p. route into 9-week old female C57BL/6 (circles) and C3KO (squares) mice. Groups of mice that did not receive antibody treatment were included (dashed lines and open symbols). Twenty-four hours after antibody treatment, mice were i.n. challenged with $\sim 9 \times 10^4$ pfu of vaccinia virus. Weight loss was monitored and the percent weight loss calculated against each mouse's starting weight. Five of 5 mice in each R182 treated group survived challenge while 4 of 4 naïve C57BL/6 and 3 of 4 naïve C3KO mice did not. Error bars represent standard error. * $p < 0.05$.

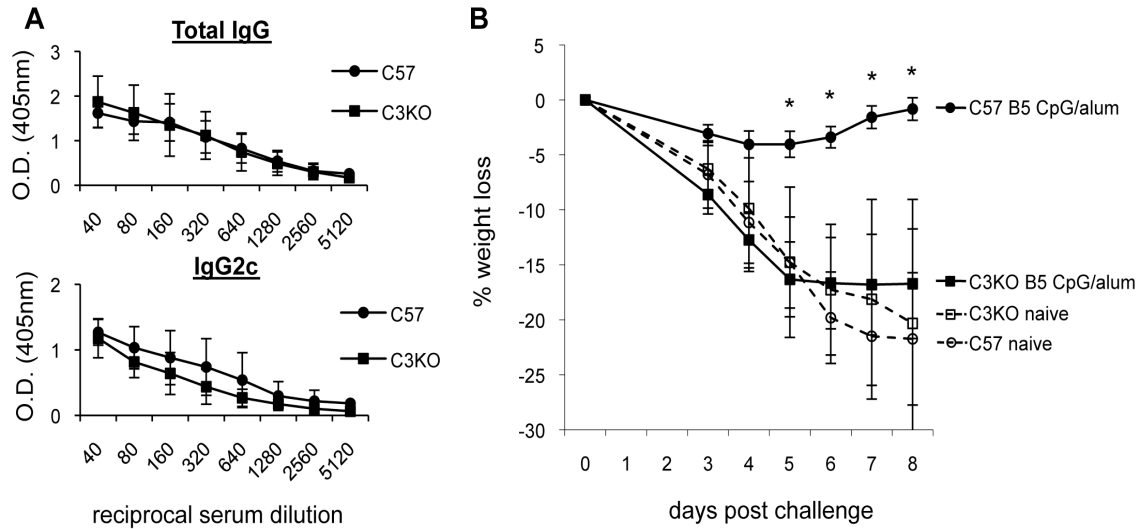


Figure 3-10. Protection from vaccinia virus challenge by B5/CpG/alum vaccination in C57BL/6 and C3KO mice.

9-week old male C57BL/6 (circles) (5/group) and C3KO (squares) (6/group) mice were vaccinated with B5/CpG/alum. (A) Anti-B5 total IgG and IgG2c were measured by ELISA from sera taken from vaccinated mice 3 weeks after boost vaccination. Because C57BL/6 mice do not have the gene for IgG2a, IgG2c was measured and is known to have similar effector functions (284,286,287). (B) Three weeks after the boost vaccination, mice were i.n. challenged with $\sim 2 \times 10^5$ pfu of vaccinia virus. Unvaccinated naïve C57BL/6 and C3KO groups were included (3 mice/group) (dashed lines and open symbols). Weight loss was monitored and the percent weight loss calculated against each mouse's starting weight. One of the B5/CpG/alum vaccinated C57BL/6 mice lost significantly more weight than the other 4 mice in its group and was removed from analysis based on Grubbs' test for outlier detection. Error bars represent standard error. Data shown is representative of two independent experiments. * $p < 0.05$. At time of challenge, the mice were ~ 14 weeks old and at this challenge dose in the C57BL/6 background only about half of the unvaccinated mice required euthanasia. Thus, mortality between groups was not statistically significant.

mechanisms were playing a role in protecting mice in the absence of C'. Thus, we passively transferred FcRKO mice with anti-B5 pAb (R182) and i.n. challenged them with a lethal dose of VACV (Fig. 3-11). These mice lost significant weight (~25%) but all survived challenge. However, if FcRKO mice were transiently depleted of C' with CVF before challenge, all mice succumbed to infection indicating that both FcR and C' play a role in protection (Fig. 3-11B). Given the finding that FcRs play a role in protection with rabbit polyclonal anti-B5 antibody, we sought to determine if mAb B126 also used FcRs to protect mice from lethal challenge. B126 was passively transferred into FcRKO or wild type BALB/c mice followed by i.n. challenge with a lethal dose of VACV (Fig. 3-12). By day 6, significant differences were seen in the weight loss of these BALB/c and FcRKO mice treated with B126. By day 8, B126 treated FcRKO mice had succumbed to infection while the wild type BALB/c mice that received B126 had already started to recover and ultimately survived challenge again indicating an important role for FcRs in protection after passive immunization.

Discussion

Vaccine induced antibodies have been shown to be critical for protection from orthopoxvirus challenge (103,107,245,288). Likewise, protection afforded by protein vaccination is thought to heavily depend on antibody responses generated and often these responses are measured and reported as a possible correlate of protection (98,101,105,108-110,168). However, besides direct pathogen neutralization, these antibody responses could protect through various effector mechanisms such as activation of C' to neutralize virus or lyse infected cells and activating cellular responses through

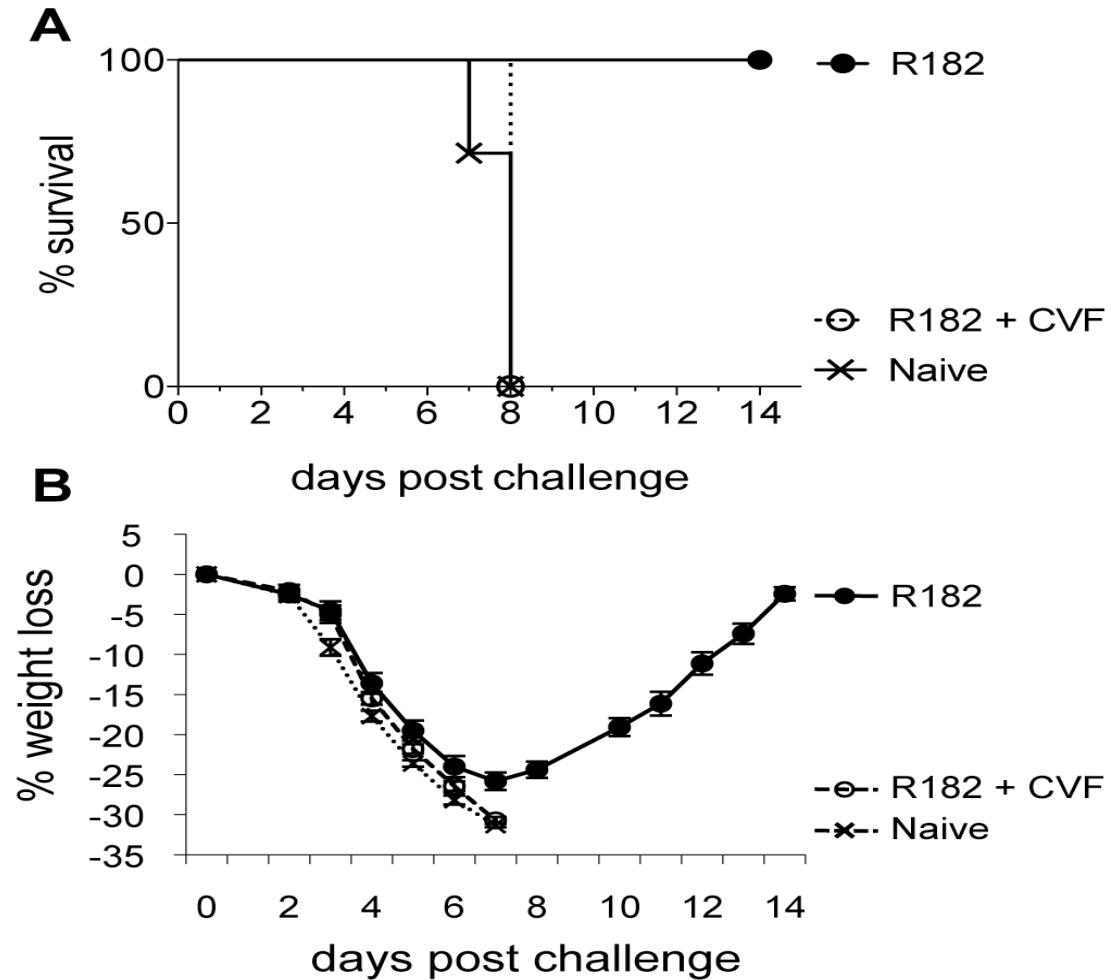


Figure 3-11. Protection from vaccinia virus challenge by anti-B5 rabbit pAb in FcRKO mice.

Anti-B5 rabbit pAb (R182; 2 mg/mouse) were passively transferred by the i.p. route into 7- to 13-week old female FcRKO mice (8 mice/group; circles). Approximately 4 units of cobra venom factor were delivered on days -1, 2, and 5 by the i.p. route to one group of mice that received R182 (open circles). A group FcRKO mice that did not receive antibody or CVF was included (crosses). Twenty-four hours after antibody treatment, mice were i.n. challenged with $\sim 3 \times 10^5$ pfu of vaccinia virus. (A) Survival differences between the FcRKO mice treated with R182 and those treated with R182 and CVF were statistically significant; $p=0.0084$ (Log-rank Test). (B) Weight loss was monitored and percent weight loss calculated against each mouse's starting weight. Error bars represent standard error. Data shown is representative of two independent experiments.

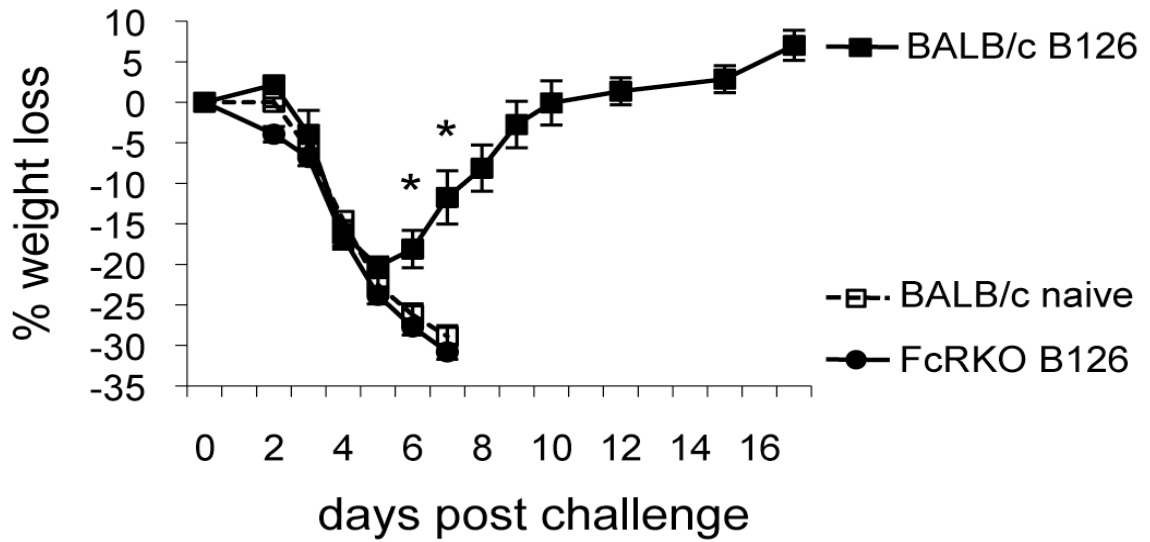


Figure 3-12. Protection from vaccinia virus challenge by anti-B5 mAb B126 is FcR dependent.

Anti-B5 mAb (B126; 100 μ g) was passively transferred by the i.p. route into 8- to 9-week old female FcRKO (closed circle) and 8-week old female BALB/c mice (closed squares) (4 mice/group). A naïve female BALB/c control group was included (dashed line, open square). Twenty-four hours after antibody treatment, mice were i.n. challenged with $\sim 4 \times 10^5$ pfu of vaccinia virus. Weight loss was monitored and percent weight loss calculated against each mouse's starting weight. Error bars represent standard error. Data shown is representative of two independent experiments. * $p < 0.05$. All BALB/c mice given B126 survived challenge, while FcRKO mice given B126 and untreated BALB/c mice all succumbed to infection and/or reached end-point criteria for euthanasia.

FcRs to lyse and kill infected cells. Here, we examined the functionality of antibody responses generated after vaccination with individual VACV proteins to better understand the type of antibody response needed to confer protection. Recently, Benhnia et al. showed that a mouse anti-B5 mAb named B126 required C' to neutralize VACV EV and mediate in vivo protection (125). Therefore, we examined whether the same was true for pAb responses after active or passive immunization against the VACV EV protein B5.

To do this, we initially set up a new luciferase based assay to measure the neutralization of large numbers of EV particles. B126 was confirmed to neutralize EV in the presence of C' (Fig. 3-4) as had been previously reported (125). We also tested another anti-B5 mAb (VMC-30; (166)) and found that it was unable to neutralize EV in the presence of C' (Fig. 3-4). Benhnia et al, reported that B126 was an IgG2a isotype and this afforded it the ability to activate C' as mAbs of IgG1 isotype did not (125). Interestingly, VMC-30 is an IgG2b isotype (166), which should also be capable of activating C' and other effector functions similar to IgG2a (186-188,208). However, in this case, the isotype of the mAb did not predict functional activity in vitro and therefore highlights the importance of testing functional activity of mAb and not relying solely on the prediction of isotype analysis. When passively transferred into mice, VMC-30 also did not protect against challenge (data not shown), again demonstrating the need for effector function for protection in vivo. We confirmed the isotype of VMC-30 and speculate that it may have been unable to neutralize VACV in the presence of C' due to potential amino acid changes in the Fc region of the mAb which could abrogate functional activity (170,174). However the IgG2b isotype could interact with Fc

receptors, so other factors like affinity may be playing a role in its inability to protect. The role of IgG2b in mediating protection from vaccinia virus infections in vivo is currently unknown. This may be interesting to examine further in the future.

As others had previously found in BALB/c mice (105), we observed that protection in vivo by protein vaccination was correlated with the production of IgG2a antibodies (Table 3-1 and Fig. 3-3). Therefore, we predicted that these antibodies would neutralize EV in the presence of C' similar to B126, but we needed to fully examine this given the lack of C'-fixing activity with mAb VMC-30. We found that sera containing IgG2a from mice vaccinated with A33 or B5/CpG/alum could utilize C' to neutralize large numbers of EV particles in vitro. Sera lacking IgG2a antibody from mice vaccinated with proteins and alum only was unable to neutralize virus in the presence of C' (Fig. 3-5). This confirms the importance of isotype and strengthens the correlation between protection of mice and production of IgG2a isotype antibodies.

Further examination of the mechanism of C'-mediated neutralization revealed that anti-A33 and anti-B5 antibody responses utilized C' to neutralize EV in different ways (Fig. 3-6). In agreement with previous reports (97,124,125), A33 antibody required C' activation steps that could lead to virolysis, while B5 antibody and C' could neutralize through opsonization. Benhnia et al. provided a model of anti-B5 antibody- C' mediated neutralization whereby B5 protein was not in high enough density on the surface of EV to allow for the basic occupancy model of antibody neutralization to succeed (124,125). They reasoned that deposited C' components enhanced the footprint of antibody bound to B5 protein on the virus surface to assist in neutralization. This model explained why virolysis was not needed. Lustig et al. provided a model of C' assisted EV neutralization

for anti-A33 antibody (97). In this model, C' lyses the outer envelope of EV, which provides anti-MV antibody access to the MV virion within. An anti-MV antibody was required to be present during the assay for C' assisted neutralization to occur. Because our luciferase based EV neutralization assay always contains an anti-MV antibody to eliminate contaminating MV in the EV preparations, we were unable to examine neutralization in the absence of an anti-MV antibody. Despite this, our data suggests that both mechanisms are correct for each protein target and not due to differences in antibody species, clonality, or adjuvant used to generate the antibody.

We hypothesized that disparity in A33 and B5 protein density on the EV surface contributed to the difference in mechanism. Galmiche et al. showed that total EV lysate had A33 and B5 protein amounts of <5 µg/mg and 30 µg/mg, respectively (109). The reduced amount of A33 protein on the EV surface could decrease the amount of antibody bound to EV to the point where coating with C1q and C3b/C4b in the area around the bound antibody is still insufficient to completely opsonize the EV virion. Under this scenario, formation of even one or two membrane attack complexes (MAC) on the EV virion could be enough to disrupt the outer membrane and allow access of neutralizing MV antibody. This model would predict that further limiting the amount of anti-B5 antibody bound to the EV surface (equivalent to a lower density of protein) would switch the mechanism of C'-mediated neutralization from opsonization to lysis. To test this hypothesis, we used a novel approach whereby EV was generated with the incorporation of different human regulators of C' (Fig. 3-8). We found that when EV was generated in cells that would result in the inclusion of CD59 (an inhibitor of MAC formation) on EV, CD59 could not provide additional protection from C'-mediated neutralization at high

concentrations of anti-B5 antibody as neutralization could occur through opsonization. However, at low concentrations of anti-B5 antibody, CD59 was protective against C'-mediated neutralization to the same degree as EV containing CD55 (an inhibitor of C3 convertase formation), likely indicating the mechanistic switch from opsonization to lysis. Additionally, we found that under the right experimental conditions, human regulators of C' on the VACV EV surface can block C' activation by antibody, and not just activation by C' alone (46). These findings provide new insight into interactions of antibody, C', and viral protein and how those interactions impact neutralization of virus.

The finding that A33 requires virolysis for C'-mediated neutralization while B5 does not may also explain differences in protection we observed after vaccinating with A33 or B5/CpG/alum. At the challenge doses we used, the ability of B5 to provide at least partial protection could be explained by the ability to neutralize EV in the absence of an anti-MV antibody response, which A33 is incapable. A33 antibody and C' would simply release MV particles which could propagate the infection, albeit that some anti-A33 effect could be gained by allowing C' free access to the C' sensitive MV particle or A33 antibody-dependent lysis of infected cells. This may also explain why a vaccine that adds L1 to A33 improves protection from disease compared to A33 or L1 alone (Table 3-1).

To examine more closely which effector functions of antibodies are important for protection in vivo, we studied the role of C' and FcRs in the protection we observed with B5 antibody. The rabbit anti-B5 pAb used in neutralization experiments had been previously shown to be protective in vivo by passive immunization (96) and the ability to neutralize EV in the presence of C' potentially contributed to this observation. To

confirm this, we examined the ability of anti-B5 antibody to protect mice in the absence of the central C' component C3 (Figs. 3-9 and 3-10). We found that both passive immunization with rabbit anti-B5 antibody and active immunization with B5/CpG/alum partially relied on C' for protection. Similar to previously reported studies that transiently depleted C' in challenged animals (125,289), we found that antibody could still provide partial protection even in the genetic absence of C3, which abrogates the function of the C' system (Figs. 3-9 and 3-10B). Somewhat unexpectedly, we found that vaccinated C3KO mice generated antibody responses similar to that of wild-type mice (Fig. 3-10A). Binding of the B cell antigen receptor/co-receptor by C3d-antigen complexes lowers the threshold for B cell activation by 10- to 100-fold (290) and provides an important survival signal to B cells (291). CpG has been shown to directly stimulate B cells and enhance IgG secretion (292,293). Inclusion of CpG in our vaccine may stimulate B-cells in a way that overcomes the requirement of C' activation for B-cell priming, activation, and survival.

Because we observed partial protection in the absence of C', we examined whether FcRs may be playing a role in protection as was previously suggested by Benhnia et al. (125). FcRKO mice were partially protected by passive transfer of rabbit anti-B5 pAB, but not if C' was transiently depleted with CVF first (Fig. 3-11). Likewise, anti-B5 mAb B126 was heavily reliant on FcRs for its protective effects (Fig. 3-12). This finding indicates that both C' and FcRs can contribute to protection and that both are important effector functions that mediate protection by pAb anti-B5 responses in vivo.

In summary, we found that after active vaccination, pAb responses against the EV form of VACV utilize C' and FcRs to mediate protection. C' plays an important role in

neutralization and the protein target can alter the mechanism through which this neutralization occurs. FcRs contribute to protection in vivo likely through Fc mediated phagocytosis and/or ADCC. Together these effector functions cooperate to provide protection from challenge. Importantly, we suggest the need to evaluate antibody effector function requirements for protection in vivo to any pathogen, especially if monoclonal antibodies are to be used. Advances in the understanding of the molecular basis for effector functions of antibody allows for customization. By altering the Fc region amino acid sequence one can impart or abrogate specific effector functions (170). By understanding the mechanism by which antibodies provide protection against a given pathogen and understanding how to manipulate antibody effector functions, vaccines and other therapeutic antibodies can be designed to specifications that activate C' or FcRs as necessary.

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Author Contributions

Conceived and designed the experiments: MEC and SNI. Provided reagents: RJE and GHC. Performed or analyzed the experiments: MEC, YX, SNI. Wrote the paper: MEC, RJE, GHC and SNI.

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**Chapter Four: A Protein-based Smallpox Vaccine Generates a Protective Th1-
biased Antibody Response in Non-human Primates**

Portions of this chapter were published as part of:

*“A protein-based smallpox vaccine protects non-human primates from a lethal
monkeypox virus challenge”*

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Abstract

Concerns about infections caused by orthopoxviruses, such as variola and monkeypox viruses, drive ongoing efforts to develop novel smallpox vaccines that are both effective and safe to use in diverse populations. A subunit smallpox vaccine comprising vaccinia virus membrane proteins A33, B5, L1, A27 and aluminum hydroxide (alum) ± CpG were administered to non-human primates, which were subsequently challenged with a lethal intravenous dose of monkeypox virus. Alum-adjuvanted vaccines provided only partial protection but the addition of CpG provided full protection that was associated with a more homogeneous antibody response and stronger IgG1 responses. These results indicate that it is feasible to develop a highly effective subunit vaccine against orthopoxvirus infections as a safer alternative to live vaccinia virus vaccination.

Introduction

Smallpox was eradicated worldwide by a 1970s campaign led by the World Health Organization (3). However the possibility of accidental or purposeful re-introduction of variola virus has led governments to stockpile live vaccinia virus (VACV) vaccines like ACAM2000™ derived from the Dryvax® vaccine (223,224). Serious side effects can accompany live vaccinia-based vaccines, especially in immunocompromised people and those with common skin diseases. This has encouraged efforts to develop safer smallpox vaccines (216-219). Modified vaccinia Ankara (MVA), a highly attenuated smallpox vaccine under development (268), may be safer, but requires high doses. Zoonotic human infections with monkeypox virus (MPXV) in the USA (6) further

illustrate the need for safe and effective vaccines against other poxviruses. Our approach has been to develop and test the efficacy of a subunit protein-based vaccine.

Identification of target antigens for a subunit vaccine is challenging because poxviruses encode hundreds of proteins and their complex life cycle produce two infectious forms: mature virus (MV) and extracellular virus (EV) (33,34). MV is an enveloped virus with many surface proteins required for infectivity (33). EV has an additional membrane surrounding the MV particle with another set of unique membrane proteins (33,34). Both forms are important in viral acquisition and spread. Subunit vaccines under development usually contain antigens from both envelopes (98,105,106,110,138,149,153,160,246,255,256,294). Here we report that vaccination of non-human primates (NHP) with purified protein ectodomains of A33 and B5 derived from EV plus L1 and A27 derived from MV, combined with the adjuvants Alhydrogel and CpG, provided full protection of NHPs from lethal intravenous challenge with MPXV. Our results clearly show the feasibility of developing safe and effective subunit vaccines for human use against smallpox and monkeypox.

Materials and Methods

Non-human primates

Two separate NHP vaccination and challenge studies were performed in cynomolgus macaques (*Macaca fascicularis*) with three- or two-dose regimens. The 3-dose study involved 30 macaques (16 females, 14 males) obtained from Three Spring Scientific (Perkasie, PA) then vaccinated and challenged at Southern Research Institute (SRI, Frederick, MD). The 2-dose study involved 12 macaques (7 females, 5 males)

obtained from Charles River Laboratories (Reno, NV), quarantined (3 months) and vaccinated at University of Maryland School of Medicine (Baltimore, MD) then challenged at SRI. All animal facilities are approved by the Association for the Assessment and Accreditation of Laboratory Animal Care (AAALAC) International and procedures were in accordance with relevant federal policies and guidelines, and protocols were approved by Institutional Animal Care and Use Committees.

VACV proteins

For this study, recombinant proteins were produced by infecting whole insect larvae with recombinant baculoviruses (details in a manuscript in preparation). Briefly, recombinant A33, B5, L1, and A27 (145,166,167) were produced by infecting insect larvae (cabbage looper moth, *Trichoplusia ni*) (295,296) with recombinant baculoviruses (*Autographa californica* multiple nuclear polyhedrosis virus) expressing individual histidine-tagged VACV proteins. After metal affinity chromatography, additional ion exchange polishing and formulation chromatographic steps were added to ensure very high purity proteins (>98% by digitized Coomassie image analysis) that were largely free of contaminating host proteins / proteases. The protein preparations were verified to have endotoxin levels below FDA guidance levels.

Vaccinations

Vaccines comprised three or four antigens (20 or 100 µg each) adsorbed to aluminum hydroxide at 8.25 or 16.5 µg aluminum ion/µg protein (Alhydrogel, Accurate Chemical & Scientific Corp., Westbury, NY). Some formulations included B-Class CpG

oligodeoxynucleotide TLR9 agonists (500 µg/vaccine dose), namely CPG7909 (sequence 5'-TCG TCG TTT TGT CGT TTT GTC GTT-3') and CpG10104 (sequence 5'-TCG TCG TTT CGT CGT TTT GTC GTT-3') (Coley Pharmaceutical Group, now Pfizer Inc.), in the 3-dose and 2-dose studies respectively. These CpG's differ by a single nucleotide and have similar activity in mice and NHP (H. Davis, unpublished). The to change to CpG 10104 in the second study occurred because CPG 7909 had been dedicated for immune therapy in oncology indications by Pfizer. For the 3-dose study, monkeys (5/group; Table 4-1) were intramuscularly vaccinated at 0, 4, and 12 weeks with 1 mL of vaccine containing 3 proteins (ABL, 100 µg each) plus CpG/alum or 4 proteins (ABLA, 20 or 100 µg) plus alum or CpG/alum. In the 2-dose study, monkeys (3/group; Table 4-2) were vaccinated at 0 and 4 weeks with 4 proteins (ABLA, 100 µg) plus CpG/alum (2 different alum ratios). Both studies included a non-vaccinated control group (CpG/alum without proteins) and a positive control group receiving Dryvax[®] (Lot # 4020075; CDC) administered at a single time (day 0) by scarification (~2.5x10⁵ pfu live VACV) with 15 pricks between the shoulder blades using bifurcated needle. (At the time of initiation of these studies, Dryvax was the only FDA approved vaccine for smallpox. ACAM2000 has since replaced Dryvax and is considered to have similar immunogenicity. All monkeys were bled prior to vaccination, 2 weeks after each vaccination, and just prior to challenge for immunogenicity measures.

MPXV challenge

Anesthetized monkeys were challenged 5 weeks (3-dose study) or 4 weeks (2-dose study) after the last subunit vaccine dose by intravenous infusion into the saphenous vein of

Table 4-1. Three-dose vaccination study (5 monkeys/group)

Vaccine formulation				Monkey number	PRNT ₅₀ ⁴	Mean / Median number of lesions		
Group ¹	Protein ²	Protein amount (µg/protein)	Adjuvant ³			Number of lesions in individual monkeys		
						Day 6	Day 9	Day 12
1. Control	-	-	CpG/alum	4342	<10	TNTC ⁵	TNTC ⁵	TNTC ^{5b}
				4348	<10	116	TNTC ^{5a}	
				4354	<10	TNTC ⁵	TNTC ^{5a}	
				4357	<10	TNTC ⁵	TNTC ⁵	TNTC ^{5c}
				4364	<10	TNTC ⁵	TNTC ^{5a}	
2. Dryvax	Dryvax	-	-			7/2	0/0	0/0
				4347	208	2	0	0
				4353	140	0	0	0
				4362	351	0	0	0
				4369	<10	31	0*	0
4371	313	2	2	0				
3. ABLA (100)	ABLA	100	CpG/alum			11/3	32/17	3/0
				4345	1,895	3	26	0*
				4351	17,501	15	7	4*
				4360	297	1	17	0*
				4363	1,847	0	2	0*
4367	132	34	108	10*				
4. ABLA (20)	ABLA	20	CpG/alum			11/3	25/21	12/0
				4346	6,613	3	49	0*
				4352	1,836	0	0	0
				4361	3,744	3	12	4*
				4368	281	6	21	0*
4370	326	45	99	54*				
5. ABLA (alum only)	ABLA	100	alum only			35/15	95/73	100/14 ⁶
				4344	544	4	7	13*
				4350	1,728	0	2	16*
				4356	455	128	312	TNTC ⁷
				4359	279	15	73	14*
4366	123	29	80	8*				
6. ABL (100)	ABL	100	CpG/alum			34/19	167/179	180/224
				4343	287	19	180	224*
				4349	1,054	46	323	12*
				4355	247	10	179	404*
				4358	30	2	2	0
4365	193	95	152	258*				

1. Group number and abbreviations of vaccine type used in text
 2. Protein where A, B, L, A stands for A33, B5, L1, A27, respectively.
 3. Adjuvant where CpG/alum stands for CPG 7909 and aluminum hydroxide (Alhydrogel).
 4. PRNT₅₀, pre-challenge (day 121) 50% plaque reduction neutralizing titer against MPXV.
 5. TNTC, too numerous to count. a. Three control animals needed to be euthanized on day 11; b. 1 on day 13; and c. 1 on day 15.
 6. For calculation of mean and median value, the lesion count was set at 500 for the monkey with TNTC lesions on day 12.
 7. One in ABLA/alum only needed to be euthanized on day 13.
- * Lesions present are mostly or only scabs

Table 4-2. Two-dose vaccination study (3 monkeys/group)

Vaccine formulation				Monkey number	PRNT ₅₀ ⁴	Number of lesions in individual monkeys		
Group ¹	Protein ²	Protein amount (µg/protein)	Adjuvant ³			Day 6	Day 10	Day 14
A. Control	-	-	CpG/alum	4372	<10	TNTC ⁵		
				4376	<10			
				4380	<10			
B. Dryvax	Dryvax	-	-	4381	383	13	0*	0*
				4382	102	21	0	0
				4383	497	18	0*	0
C. ABLA (8.25)	ABLA	100	CpG/alum (8.25)	4377	9,387	0	5	0*
				4378	151	5	11	0*
				4379	1,440	0	13	0
D. ABLA (16.5)	ABLA	100	CpG/alum (16.5)	4373	1,800	36	0*	0*
				4374	7,235	24	21*	0*
				4375	1,984	0	0	0

1. Group letter and abbreviations of vaccine type used in text

2. Protein where ABLA stands for A33, B5, L1, A27

3. Adjuvant where CpG/alum stands for CpG 10104 and aluminum hydroxide (Alhydrogel), used at either 8.25 µg of aluminum ion/ µg of protein as in the study in Table 4-1 or at 16.5 µg of aluminum ion/ µg of protein to increase the amount of aluminum hydroxide.

4. PRNT₅₀, pre-challenge 50% plaque reduction neutralizing titer against MPXV.

5. TNTC, too numerous to count. The three control animals needed to be euthanized on day 8.

* Lesions present are mostly or only scabs

1.0 mL media containing 2×10^7 pfu MPXV (NR523, BEI Research Resources Repository, Manassas, VA). The challenge inoculum, which was back titered on Vero E6 cells using a plaque assay to confirm dose, was selected to be lethal and indeed all control animals and one monkey receiving ABLA/alum required euthanasia upon meeting a predetermined set of criteria of disease progression. Post-challenge, blood was drawn every third day and DNA extracted from 200 μ l of blood for viral load (VL) determination by real-time PCR (149). The detection limit of the viral load assay was 5000 genome copies/mL blood. Animals were monitored for activity, temperature, weight, appetite, and development of pock lesions.

Antibody ELISA

Standard ELISA determinations used as capture antigens either purified VACV (0.6 μ g/mL in PBS) or recombinant proteins (A33, B5, or L1 at 2.5 μ g/mL; or A27 at 0.625 μ g/mL) in PBS coated overnight at 4 °C. After blocking, 2-fold dilutions of sera were incubated for 1 hr at 37 °C. After extensive washes, the secondary antibody, HRP-conjugated goat anti-monkey IgG (KPL) at 1:2000 was incubated for 1 hr at 37 °C followed by color development with ABTS substrate (Sigma) for ~20 min at RT. IgG isotypes were determined by coating plates with non-his tagged versions of B5 or L1 at 1 μ g/mL and after incubation with sera, HRP-conjugated anti-human total IgG, IgG1, IgG2, IgG3, or IgG4 (Binding Site, Birmingham, UK) diluted at 1:1000 was added and incubated for 1 hr at 37 °C.

Depletion of sera of anti-L1 antibodies

Recombinant L1 protein (1 mg) was coupled to 0.5 grams of CNBr-activated Sepharose 4B beads (Amersham Biosciences) following the manufacturer's instructions. Mock-coupled beads were processed similarly except without protein addition. Beads were washed and resuspended in 500 μ L PBS. Equal volumes of sera from the five monkeys in each of the ABLA/CpG/alum or ABL/CpG/alum groups in the 3-dose study were combined, heat inactivated, and 20 μ L of pooled serum was incubated with 180 μ L of L1-coupled or mock-coupled sepharose beads overnight at 4°C. Beads were pelleted, the supernatant collected, and L1 depletion confirmed by L1 or A27 ELISA as above, except plates were coated with each protein at 1 μ g/mL. Supernatants, stored at 4 °C, were used for neutralization experiments.

Virus neutralization

For MXPV 50% plaque neutralization reduction titers (PNRT₅₀), 4-fold dilutions of sera (in 225 μ l of media) were mixed with 225 μ l purified MPXV MV (450 pfu/well). After overnight incubation at 37 °C, 100 μ l was removed and plated in triplicate on Vero cells for titering. VACV (WR) MV was used in neutralization studies with the L1-depleted and control depleted sera. MV neutralization assays were carried out in triplicate in a final volume of 100 μ l containing ~200 pfu of MV, dilutions of sera from individual monkeys at 1:20 to 1:2000, and incubated for 2 hr at 37 °C. Samples of 90 μ l were titered on confluent wells of a 6-well tissue culture plate. After 36 to 48 hrs, wells were stained with crystal violet, plaques counted, and normalized to control wells that contained MV incubated in media alone. EV neutralization was carried out with VACV EV. EV was

isolated from VACV (strain IHDJ) infected RK13 cells in MEM (without FBS) as previously described (113). The media from infected cells was clarified by low speed centrifugation and EV was titered in the presence of the MV neutralizing monoclonal antibody (mAb) 2D5. EV neutralization assays were carried out in triplicate in a final volume of 100 μ l containing ~200 pfu EV, the indicated dilution of sera from individual monkeys, the anti-L1 mAb 2D5 (to neutralize contaminating MV), and with or without 10 μ l of guinea pig complement (Complement Technology, Inc., Tyler, Texas) for 30 min at 37 °C. 90 μ l of each sample were then titered.

Statistical analysis

For the 3-dose study (5/group), statistical analyses were performed with SASTM Version 9.1 using an alpha =0.05, except where indicated. The dilution ratios for ELISA proteins at an optical density (OD) =0.2 were calculated by point-point regression between ELISA OD readings immediately <0.2 and >0.2 OD. The dilution values calculated at OD =0.2 were used for statistical analyses. A dilution of 0 was given where ELISA optical density readings were <0.2. Group median dilutions, PRNT₅₀ levels, and viral loads were compared by Kruskal-Wallis ANOVA. Pair-wise comparisons for significant differences in viral loads across groups were performed for Groups 1-6 (Dunn's test). Significant changes in PRNT₅₀ levels from a baseline level at day 98 were tested by Wilcoxon Rank Signed Test. Kaplan-Meier survival plots were calculated for vaccination groups following MPXV challenge (days 0-27) and survival curves for each vaccination group were compared by Cox-Mantel test; Spearman's rank-order correlation test was used on the log[PRNT₅₀] versus lesion numbers and viral loads at day 6 and day

9 post-challenge (GraphPad Prism™, Version 5). The effect of vaccination group on weight and temperature were tested with a repeated measures analysis of variance (PROC MIXED, SAS™). For MV neutralization assays, statistical differences in plaque number were calculated with the Student's t-test.

Results

Vaccine safety

The adjuvanted subunit vaccines were well tolerated by all animals with no adverse reactions.

Challenge with MPXV

Negative and positive vaccine controls.

We analyzed clinical symptoms and survival post-challenge (Fig. 4-1A), lesion counts (Fig. 4-1B), and viral loads (Fig. 4-2) in both vaccinated and non-vaccinated animals. The non-vaccinated control animals (Group 1) exhibited typical signs of MPXV disease and experienced depression, lethargy, and pock lesions after challenge that became too numerous to count (TNTC) by day 6 (Table 4-1, Fig. 4-1B). Peak viral loads (VL) were between 10^7 and 10^9 genome copies/mL by day 9 or 12 (Fig. 4-2). These animals met endpoint criteria for euthanasia between days 11 and 15 (Fig. 4-1A). Animals vaccinated with the positive control gold standard Dryvax vaccine (Group 2), all survived and exhibited little or no clinical signs of infection; the few pock lesions that developed healed quickly (Fig. 4-1B), and had no measurable VL at any time except for animal #4353 with a VL of $\sim 2 \times 10^4$ genome copies/mL on day 3 post-challenge (p.c.)

(Fig. 4-2). When we compared Group 2 with Group 1, there were statistically lower viral loads in Group 2 at all time points (Dunn's multiple comparison test; $P < 0.05$). Similar results were seen for negative and positive control groups in the smaller 2-dose study where all non-vaccinated control monkeys (Group A) met endpoints that required euthanasia on day 8 p.c. and the Dryvax-vaccine protected all the monkeys (Group B) from MPXV disease (Table 4-2).

Subunit vaccine provides protection.

All animals vaccinated with three doses of subunit vaccines containing CpG/alum (Groups 2, 4, & 6) survived ($\chi^2_{(1,10)} = 10.03$; $P = 0.0015$ relative to control Group 1), and all but one in Group 5 (ABLA/alum) survived ($\chi^2_{(1,10)} = 6.872$; $P = 0.0088$) (Fig. 4-1A). Groups vaccinated with ABLA/CpG/alum also had statistically lower VL than controls at days 3, 5, and 12 p.c. (Dunn's multiple comparison test; $P < 0.05$; Fig. 4-2, Groups 3 & 4). In all groups, monkeys with the highest VL exhibited the most lesions.

Benefit of CpG.

In the 3-dose study, addition of CpG to the tetravalent vaccine resulted in more consistent control of infection than with alum as the sole adjuvant as indicated by fewer lesions (Table 4-1, Fig. 4-1B) and lower VL (Fig. 4-2; Groups 3 & 4 versus 5). Indeed, with just alum, the VL at all time points p.c. were not significantly different than non-vaccinated controls (Dunn's multiple comparison test; $P > 0.05$). Also, a dose of 20 $\mu\text{g}/\text{protein}$ with CpG and alum gave equivalent results for survival, lesions, and VL as a dose of 100 $\mu\text{g}/\text{protein}$ (Groups 3 versus 4) indicating that lower antigen doses can be

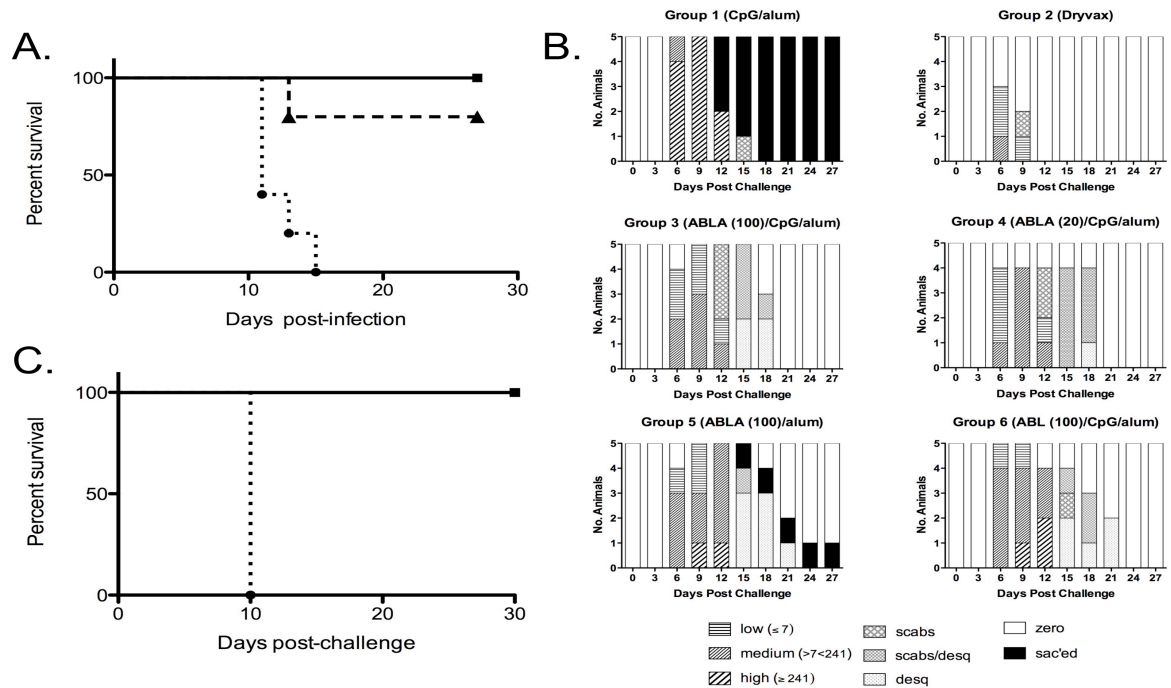


Figure 4-1. Kaplan-Meier survival analyses and pock lesion summary.

A. Kaplan-Meier survival plots following MPXV challenge for the 3-dose study with 5 monkeys/group. Solid line with squares: Dryvax (Group 2), ABLA/CpG/alum (Groups 3 & 4), and ABL/CpG/alum (Group 6); Dashed line with triangles: ABLA/alum (Group 5); Dotted line with circles: CpG/alum control (Group 1). B. Frequency of each lesion category for vaccination groups at days 0-27 post-challenge. The upper and lower quartiles of lesion frequencies for numeric lesion data were calculated and plotted. The 25th (1 to ≤ 7 lesions) and 75th percentiles (≥ 241 lesions) were used to recode the numeric lesion data into low ($\leq 25^{\text{th}}$), medium (25^{th} - 75^{th}) and high ($\geq 75^{\text{th}}$) lesion categories. For this analysis, lesion counts that were recorded as “TNTC” (too numerous to count) were set at ≥ 500 lesions. High lesions (≥ 241) were observed for all animals in Group 1 by day 9 post-challenge. High lesions were observed in 1 of 5 animals in Group 5 by day 9 and 2 of 5 animals in Group 6 by day 12. All animals in Group 3 experienced low (≤ 7) to medium (< 241) lesions by day 9. All lesions of surviving animals in Groups 2-6 were healed by day 24, post-challenge. C. Kaplan-Meier survival plots following MPXV challenge for the 2-dose study with 3 monkeys/group. Solid line with squares: Dryvax (Group B) ABLA/CpG/alum (Groups C & D); Dotted line with circles: CpG/alum control (Group A).

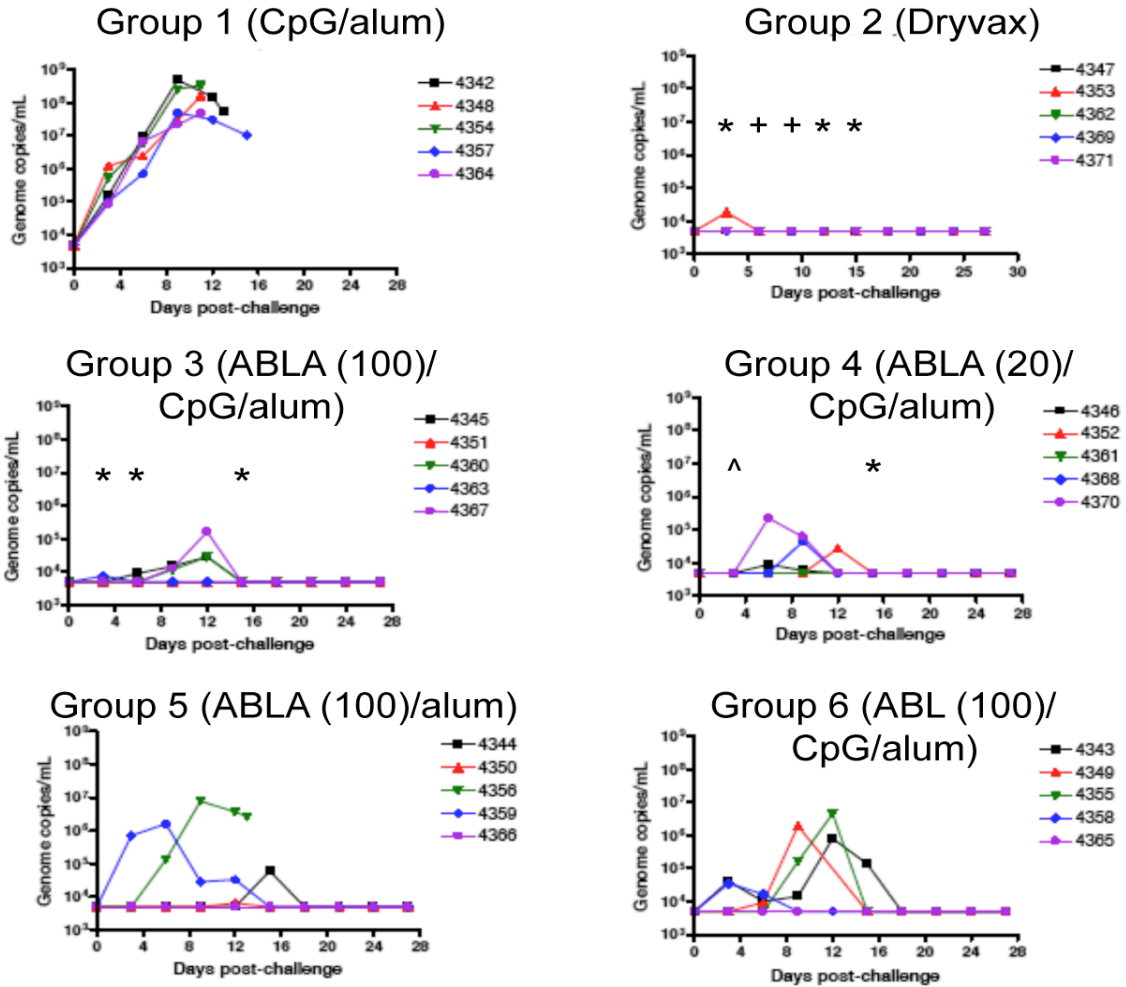


Figure 4-2. Viral load on days 0-27 post-challenge.

Graphed are the post-challenge viral load by group and monkey. The detection limit of the viral load assay was 5000 genome copies/mL blood. Median VL and inter-quartile ranges were determined and Dunn's multiple comparison test was performed on vaccination pairs where a significant main effect of vaccination groups was found. A higher viral load was found for Group 1 compared to (i) Group 2 (Dryvax) at days 3-15 ($P < 0.05$); (ii) Group 3 (ABLA(100)/CpG/alum) at days 3, 6, and 15 ($P < 0.05$); and (iii) Group 4 (ABLA(20)/CpG/alum) at day 3 and 15 ($P < 0.05$). * $P < 0.05$, ^ $P < 0.01$, + $P < 0.001$

used when CpG is included. In the 2-dose study we compared CpG containing vaccines with two doses of alum, either 8.25 aluminum ion/ μg of protein (as used in the 3-dose study) or 16.5 (Table 4-2, Groups C and D). We found no difference in protection (Fig. 4-1C, Table 4-2), although only 3 monkeys per group may be inadequate to detect differences.

Trivalent versus tetravalent protein vaccines and three versus two-dose regimen.

The CpG/alum adjuvanted trivalent vaccine protected animals from death, as did the tetravalent vaccines (Fig. 4-1B, Groups 3, 4, and 6), but there were fewer lesions and lower VL with ABLA (20 or 100 μg) than with ABL (100 μg), suggesting a role for A27 in protection. In the 2-dose study, the tetravalent vaccine (100 μg /protein) with CpG/alum provided excellent protection. One monkey showed no lesions at any time tested (Fig. 4-1C, Table 4-2). While this study was run separately from the 3-dose study, the results were similar for both.

Vaccine immunogenicity and correlation with protection.

Since protection from secondary poxvirus infections is primarily antibody-mediated (103,107,149), we focused on evaluating antibody responses. In the 3-dose study, animals vaccinated with a single Dryvax scarification developed VACV-specific antibodies early (day 14) but as expected, titers did not increase at later times (data not shown). Monkeys vaccinated with subunit vaccines had no detectable VACV-specific antibody responses post-prime but exhibited strong responses by day 42 (2 weeks post 1st boost). These titers increased further by day 98 (2 weeks post 2nd boost). While there

were no significant differences between groups in antibody titers to individual proteins, due to high variability and small number of animals, we did note trends indicating that 100 µg/protein of the tetravalent vaccine was better than 20 µg/protein, and CpG/alum was better than alum alone, with the highest titers being found with ABLA(100)/CpG/alum (data not shown).

Neutralization activity correlates with protection.

The tetravalent vaccines adjuvanted with CpG/alum (Groups 3 & 4) were the only formulations that yielded better pre-challenge neutralization titers against MPXV MV than non-vaccinated controls ($P<0.01$) (data not shown). The 100 µg antigen dose developed the best pre-challenge responses, which did not increase appreciably post-challenge. In contrast, while pre-challenge PRNT₅₀ values for Dryvax were not “statistically significant” (but at similar levels to published studies (222,297)), the post-challenge (day 9, 18 and 27 p.c.) PRNT₅₀ values with Dryvax increased over pre-challenge values ($P<0.05$) and were significantly higher than non-vaccinated controls ($P<0.001$). They were also higher than the titers of the ABL/CpG/alum group ($P<0.05$). There was an inverse relationship between prechallenge PRNT₅₀ and post-challenge VL or lesion numbers (Fig. 4-3).

Antibody isotype contributes to protection.

CpG, a Th1 adjuvant, had a clear effect on protection (Table 4-1, Groups 3 & 4 vs. 5). Although antibody isotype is a less clear indicator of Th-bias in primates than mice, we examined anti-B5 and anti-L1 specific responses after the third vaccine dose

(day 98). We found IgG1 and IgG2, but not IgG3 and IgG4, which possibly were not detected due to use of human reagents. The anti-B5 isotype response was more homogeneous in vaccines formulated with CpG/alum than with alum alone. Moreover, the CpG/alum adjuvanted vaccine showed a consistent IgG1:IgG2 ratio >2 (Fig. 4-4A), whereas the ratio of IgG1:IgG2 for alum alone was closer to one (Fig. 4-4B).

Importantly, IgG1 responses alone were consistently high in all NHPs vaccinated with CpG/alum adjuvanted vaccine, while only 2 of 5 NHPs vaccinated with alum only showed high IgG1 titers. Antibody isotype determines function, and IgG1 antibodies are known to activate complement (189). When we used concentrations of sera that did not efficiently neutralize EV, addition of active complement consistently enhanced EV neutralization in animals vaccinated with ABLA(100)/CpG/alum but had an enhancing effect in only 2 of 5 animals that received ABLA(100)/alum (Fig. 4-4C).

Anti-A27 antibodies do not neutralize virus.

While the trivalent and tetravalent vaccines adjuvanted with CpG/alum protected NHP from death after lethal challenge with MPXV, we were surprised that pock lesion counts with the trivalent vaccine (Group 6) were much higher than with the tetravalent vaccine (Group 3, Table 4-1). In mice, A27 could not substitute for L1 in a trivalent vaccine, possibly because the anti-A27 antibodies do not neutralize MV (110). To determine whether the monkeys developed a neutralizing antibody response to A27, we depleted anti-L1 antibodies by passing pooled sera from the vaccinated groups through L1-coupled (or uncoupled) sepharose beads. ELISA showed no remaining anti-L1 titers, but titers to A27 were unchanged (data not shown). We found that if L1 antibodies were

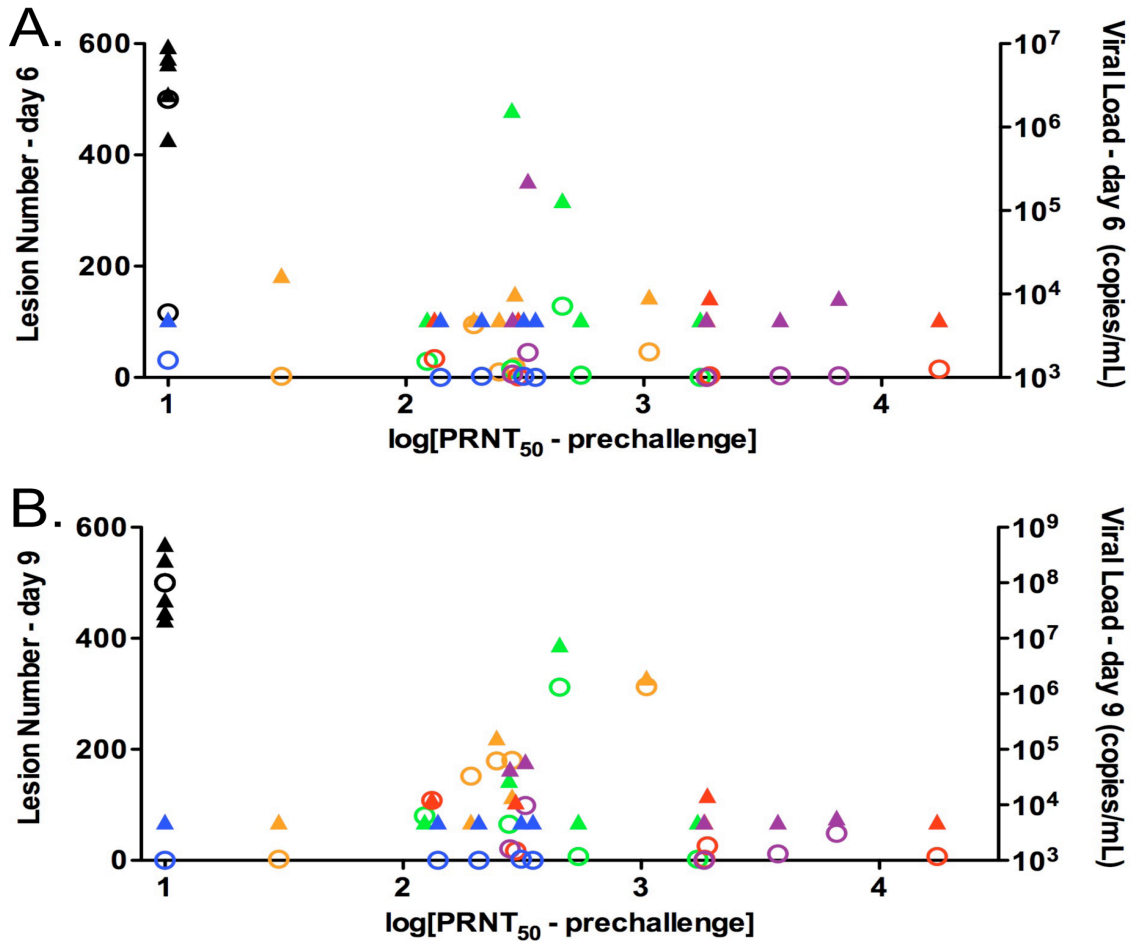


Figure 4-3. Relationship between PRNT50, lesion number, and viral load. Post-challenge lesion counts (open circles) and viral loads (solid triangles) at day 6 (A) and day 9 (B) are plotted against the log of the pre-challenge PRNT₅₀. Various colored symbols represent each different vaccination group: Group 1 (adjuvant only) black; group 2 (Dryvax) blue; group 3 (ABLA(100)/CpG/alum) red; group 4 (ABLA(20)/CpG/alum) purple; group 5 (ABLA/alum) green; group 6 (ABL/CpG/alum) orange. For this analysis, “TNTC” lesions were set at 500 lesions. Correlations between prechallenge neutralization (log[PRNT₅₀]) and day 6 lesions ($r = -0.5433$ $P=0.0019$) and viral loads ($r = -0.3997$ $P=0.0287$) and day 9 lesions ($r = -0.3929$ $P=0.0318$) and viral loads ($r = -0.3928$ $P=0.0318$) were statistically significant.

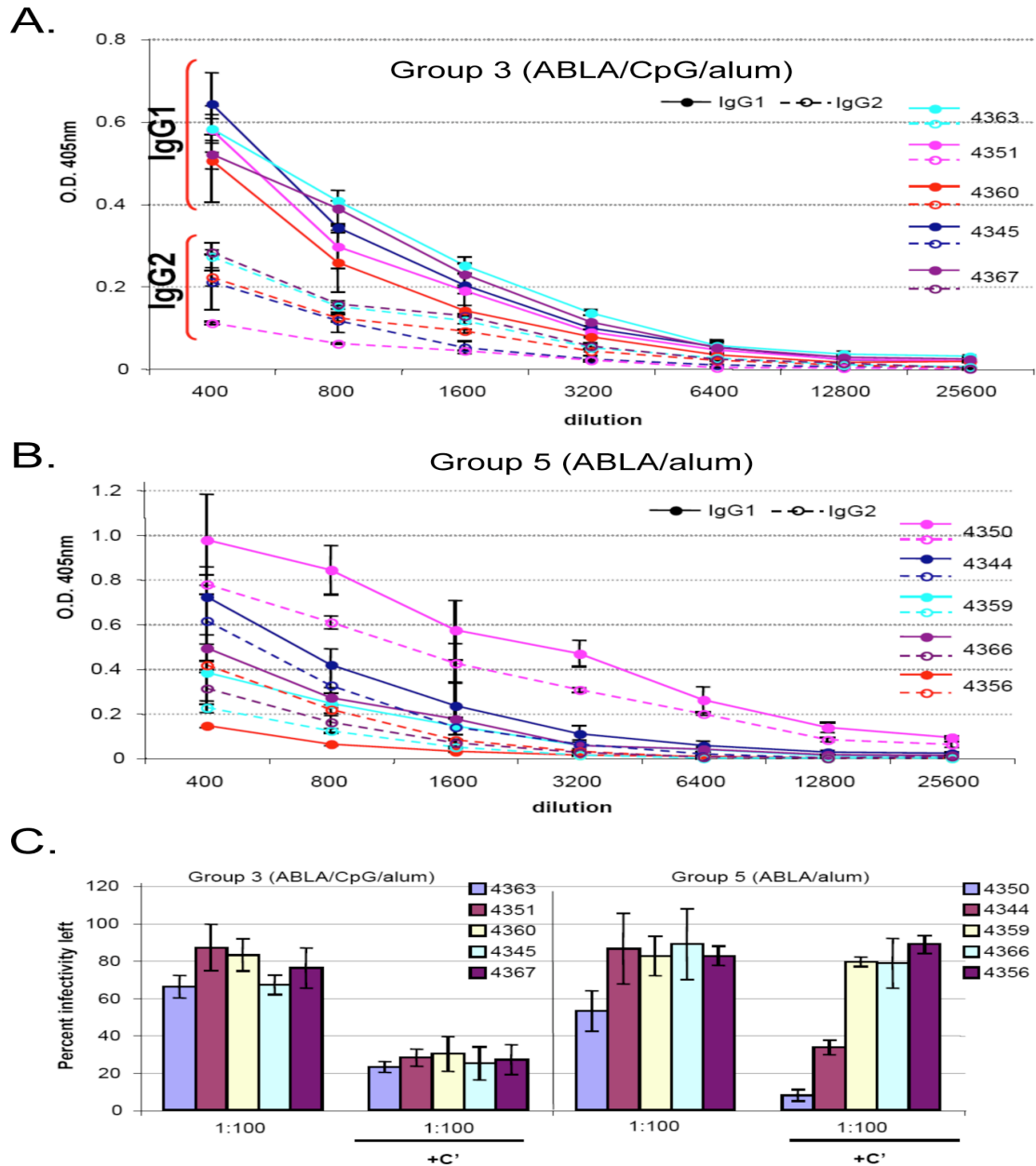


Figure 4-4. Protein vaccines adjuvanted with CpG/alum provide higher IgG1 to IgG2 ratios that result in more consistent complement-enhanced neutralization of EV.

A. IgG1 and IgG2 isotype responses to B5 in Group 3 (ABLA(100)/CpG/alum) at day 98 (2 weeks after the second boost). B. IgG1 and IgG2 isotype responses to B5 in Group 5 (ABLA/alum) at day 98 (2 weeks after the second boost). Solid lines with solid symbols: IgG1 responses. Dashed lines with open symbols: IgG2 responses. C. EV neutralization by NHP sera on day 98 (2 weeks after the second boost) in the absence and presence of complement (C'). Error bars represent standard deviation.

depleted, sera no longer was able to neutralize MV (Fig. 4-5), indicating that the monkeys did not develop a neutralizing antibody response to A27.

Discussion

Recent life-threatening complications (298,299) with the FDA approved live VACV vaccine highlight the need for safer vaccines against orthopoxviruses, for which the threat of infection exists through zoonosis (e.g., monkeypox) or inadvertent or purposeful release of smallpox. The highly attenuated MVA, which does not produce infectious virions in human cells, is thought to be a safer attenuated VACV vaccine (268).

However, it continues to rely on a live virus and requires 1000-times the dose of the current live VACV vaccine. Subunit vaccines comprising recombinant protein antigens may be safe for all individuals and could be a useful future alternative, particularly if live virus vaccines are no longer acceptable at those times. Such a subunit vaccine would be useful in the pre-event setting to provide baseline immunity should newly introduced orthopoxviruses cause serious human infections. Also, a protein vaccine might enhance the safety of a live vaccinia vaccine in the event that a fully replication competent vaccine be deemed necessary to control a significant smallpox outbreak. Alternatively, it could also be useful to boost responses in the older population who had received childhood smallpox vaccines, leaving potentially limited supplies of live vaccine for those at greatest risk. Herein, we have shown the ability of a trivalent (A33, B5, and L1 (ABL)) or tetravalent (ABL+A27 (ABLA)) protein-based adjuvanted vaccine to elicit humoral immune responses in monkeys that protect against lethal MPXV challenge.

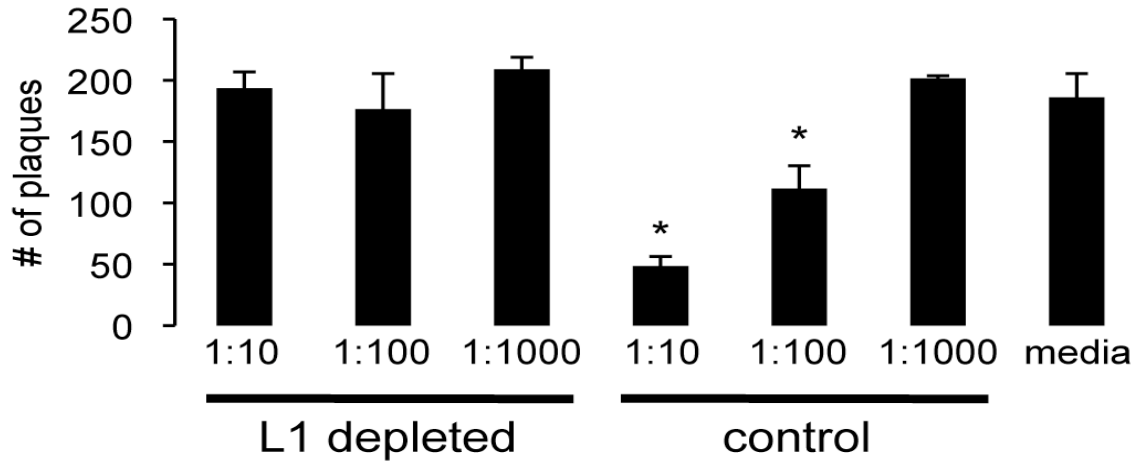


Figure 4-5. Antibody against A27 does not neutralize MV.

MV neutralization by NHP sera on day 98 from group 3 (ABLA(100) CpG/alum). Sera were passed through L1-coupled sepharose beads or control beads. It was then used at dilutions of 1:10, 1:100, and 1:1000. Only control treated sera was still capable of neutralizing MV at 1:10 and 1:100 dilutions when compared to input virus treated with media alone. * $p < 0.05$.

While a direct correlate of immunity to poxvirus infections is still unknown, our data suggest that the best results are obtained when the protein vaccine elicits a high amount of IgG1 isotype antibodies that are able to neutralize EV in the presence of complement. These results are consistent with findings by the Crotty group showing the importance of the Fc domain in antibody protection of poxvirus infections (124,125). They found that anti-B5 mAbs (that poorly neutralized EV on their own) were quite potent at neutralizing EV in the presence of complement. Furthermore, treatment of mice with anti-B5 mAbs with complement fixing isotypes were better at protecting mice from VACV challenge. In future studies of smallpox vaccines in humans or NHPs, inducing a high titer of IgG1 antibodies may correlate with improved protection for the vaccine and thus IgG1 titers should be evaluated.

Our earlier work in mice indicated that ABL and ABLA (both formulated with CpG/alum) provided similar protection from intranasal challenge with VACV or ectromelia virus (110). Thus we were surprised that the tetravalent vaccine clearly outperformed the trivalent protein vaccine for clinical outcomes (pock lesions and VL) in NHPs. The role of A27 is not clear since we found that anti-A27 antibodies did not neutralize MV. It is noteworthy that a recent report from the Moss group found neutralizing antibodies to A27, but the protein was still less protective than the L1 protein (137). T-helper epitopes within A27 may improve responses to other antigens that enhance protection. Another possibility is that the addition of A27 resulted in physiochemical changes in the tetravalent vaccine formulation that enhanced protection. Studies to better understand this are underway. Our results are consistent with others using the same antigens. ABL/CpG/alum provided similar protection to that reported by

the Moss group with ABL/QS21 (a saponin adjuvant) (105), although it is important to note several differences in study design. These include adjuvant formulation, vaccination schedules (dose number and time between doses), the number of NHP, and relative lethality of challenge dose. Likewise, our ABLA results are similar to those obtained in a smaller study by the Franchini group using MPXV orthologs of the same four VACV proteins used here (149).

Aluminum hydroxide, a well-established adjuvant, provides reasonably good protection, but adding CpG provides additional benefit and improved protection. CPG7909 is a B-Class CpG oligodeoxynucleotide that has proven in clinical studies to significantly enhance (~5 to 10-fold) humoral responses against several different antigens (300,301). CpG10104, a related molecule with similar pharmacological effects, is currently under development as an adjuvant for infectious disease vaccines. While not compared side-by-side in the same study, our results support their similar activities. The Franchini study involved DNA prime/protein boost and a slightly higher MPXV challenge dose, but two groups were vaccinated with protein adjuvanted with either aluminum hydroxide (n=3) or CpG (2 mg/animal of CPG7909, also known as CpG2006; n=4). More than 25 lesions were seen in 3 of 3 (protein/alum) and 3 of 4 (protein/CpG) challenged monkeys in the Franchini study and 3 of 5 (protein/alum) monkeys in our study, but only 4 of 16 (protein/CpG/alum) challenged monkeys in our studies. Thus, there appears to be a benefit for including both alum and CpG in subunit vaccine formulations.

Intravenous challenge has been widely used to study smallpox therapies and vaccines because it more closely mimics some aspects of smallpox disease (104-

107,149,240,241,294,302-305). However, there are disadvantages. The challenge dose is high and essentially starts the disease process at a stage closer to the secondary viremic phase of smallpox, therefore setting a very high bar for a preventative vaccine.

Conversely, the outcome of intravenous challenge may depend more heavily on antibody responses against MV. Other NHP challenge models, like respiratory challenge, are being developed (233,239,306,307) and should be useful to further test effectiveness of subunit orthopoxvirus vaccines. For FDA approval of future generation smallpox vaccines by the “animal rule”, multiple animal models with various modes of challenge will likely be needed.

In conclusion, we found that as few as 2 doses of an adjuvanted protein-based subunit vaccine protected NHP from a lethal MPXV challenge. Such a vaccine would be valuable in a setting where it is difficult to screen large populations to identify those with increased risk of complications from live VACV vaccination. It could also be used to safely provide base-line poxvirus immunity and for immunization of individuals refusing VACV.

Acknowledgements

We thank the veterinarians and the animal care technicians at SRI and University of Maryland for their work with the NHPs. We also thank Manuel Ponce de Leon and Chwang Hong Foo for the L1-coupled sepharose beads. This work was supported by Public Health Service grants UC1-AI067129, U54-AI057168 (Middle Atlantic Regional Center of Excellence in Biodefense and Emerging Infectious Diseases), and U01-AI077913 from the National Institute of Allergy and Infectious Diseases.

Chapter Five: Concluding Remarks

A summary of results contained within, future directions, and implications for future vaccine design and licensure

Introduction

Antibody plays a critical role in protection from secondary poxvirus infection. This thesis focused on antibody against two different EV protein targets: A33 and B5. The EV form of the virus, responsible for dissemination within a host, is relatively resistant to direct neutralization by antibody. Therefore, antibodies against proteins found on EV utilize Fc-dependent mechanisms to enhance their protective effects. These Fc-dependent mechanisms include the activation of complement to neutralize EV particles and lyse infected cells and the interaction with FcRs to direct an innate cellular response against EV particles or infected cells. In chapter two, I reviewed future generation smallpox vaccines. Many of the future generation subunit vaccines rely on antibody responses to confer protection. In chapter three, we showed that production of IgG2a isotype antibody (Th1-biased immune response) by protein vaccination of mice correlated with protection from lethal challenge with VACV. We then showed that IgG2a containing polyclonal antibody against A33 or B5 could neutralize a large input of virus only in the presence of active complement. Additionally, we showed that neutralization by complement occurred by virolysis for antibody against A33 and could occur by opsonization (coating) with antibody against B5. We attributed this difference to the different amount of protein expressed on the surface of EV and showed that manipulation of the amount of B5 antibody bound to EV could change the activation steps by which complement neutralizes EV (Fig. 5-1). We went on to show in chapter three that both complement and FcRs play a role in the ability of antibody to protect mice from VACV challenge. In chapter four, we showed that vaccination with a protein-based smallpox

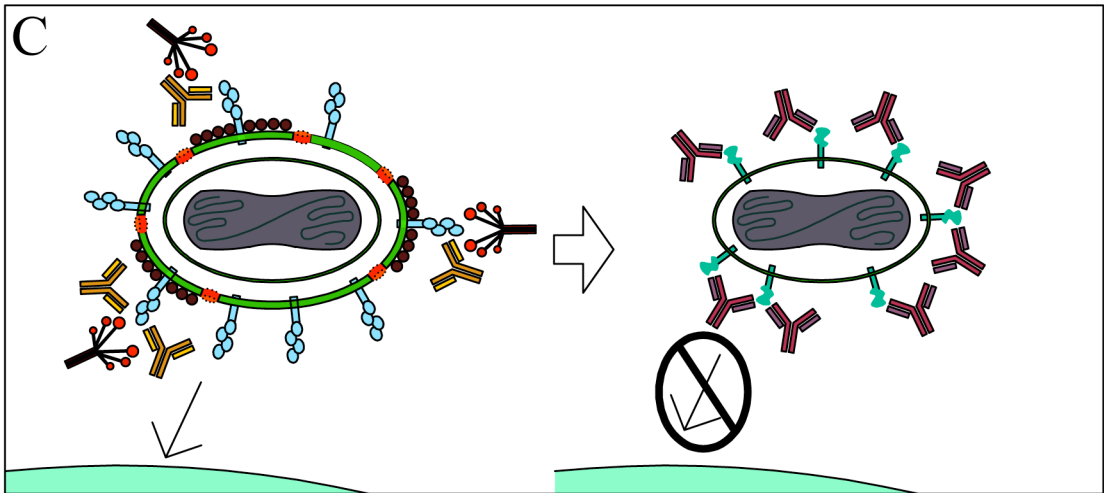
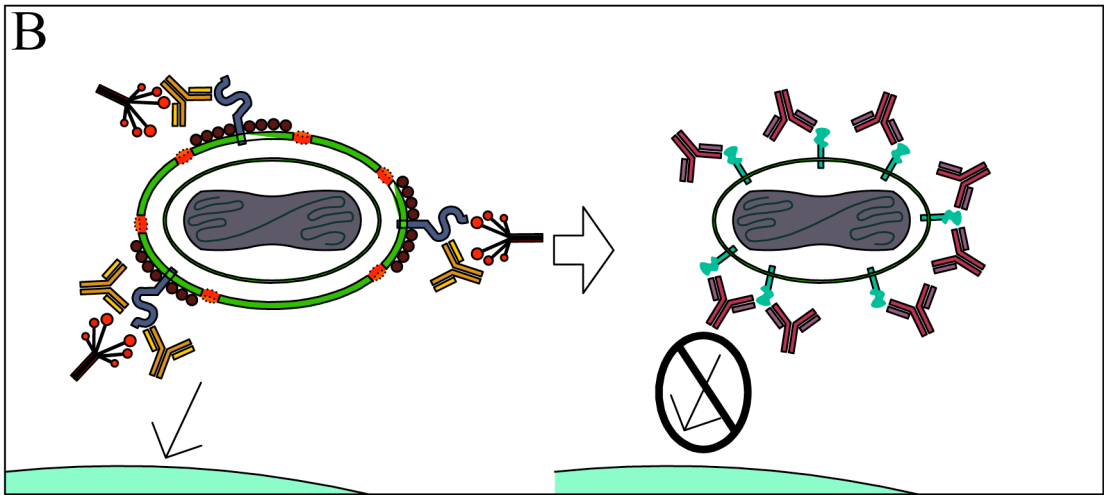
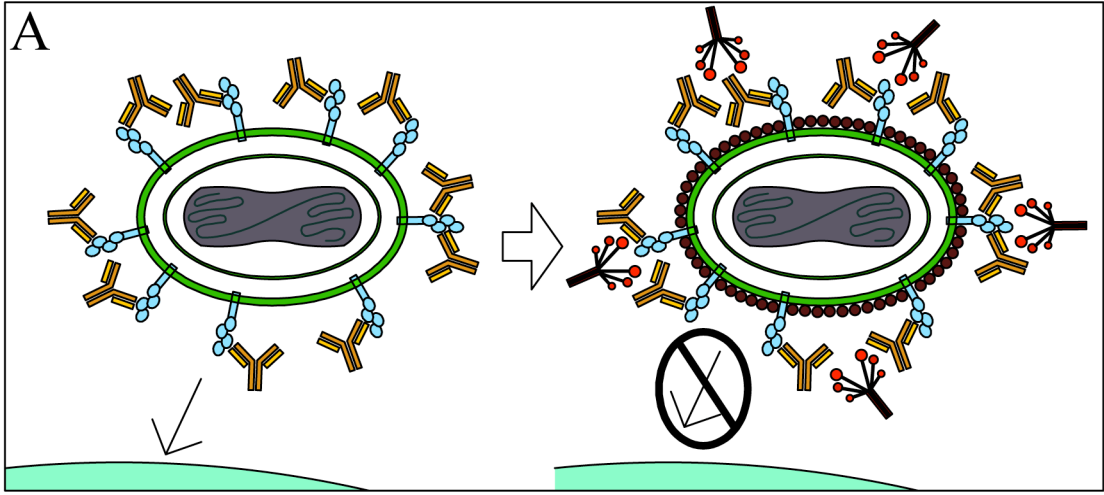
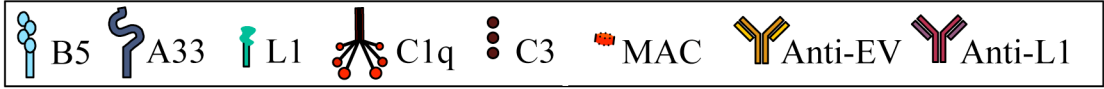


Figure 5-1. Model of complement-dependent EV neutralization by anti-B5 or anti-A33 antibody.

Antibody against the surface proteins B5 and A33 can neutralize EV more effectively after activation of complement. We hypothesized that the amount of antibody bound to the EV surface accounts for differences in the required complement activation steps. (A) Anti-B5 antibody binds the B5 surface proteins on EV, but does not efficiently block infection, due to an insufficient number of B5 proteins available for antibody to bind and completely coat the particle. Activation of complement by binding of C1q to the Fc portion of the antibody and subsequent efficient deposition of C3 on the surface of the EV virion can block infection and neutralize virus. (B) A33 protein is found at lower levels than B5 protein on EV virions. Anti-A33 antibody binds the A33 surface proteins on EV, but does not block infection, again due to an insufficient number of A33 proteins available for antibody to bind. Activation of complement by antibody binding C1q and deposition of C3 alone is also insufficient to block infection. We hypothesize that this is due to the smaller numbers of antibody bound to EV and, therefore, the inability to efficiently activate complement and deposit enough C3 on the EV virion surface. Instead, the complement activation needs to proceed to the insertion of the MAC and lysis of the outer membrane of EV. Anti-MV neutralizing antibody such as anti-L1, if present, is then capable of interacting with the revealed MV virion and neutralization can occur. (C) At lower concentrations of anti-B5 antibody, a smaller number of the B5 proteins on the EV surface are bound by anti-B5 antibody. This scenario recapitulates the smaller numbers of anti-A33 antibody bound to EV as found in panel B. As with anti-A33 antibody, C1q binding and C3 deposition is inefficiently activated and cannot occupy the entire EV surface. For neutralization to occur, complement activation needs to proceed to insertion of the MAC and lysis of the outer EV membrane. MV neutralizing antibody such as anti-L1, if present, can then neutralize the released MV virion.

vaccine protected NHP from MPXV challenge and viral loads/lesion counts were inversely correlated with MV neutralizing antibody titers (Fig 4-3). The inclusion of CpG in the protein vaccine formulation induced a more homogeneous Th1 antibody response against B5, characterized by an IgG1:IgG2 ratio >2 and these sera were capable of neutralizing EV in the presence of complement. This may have partially accounted for the better protection from morbidity and mortality for those NHP receiving CpG as part of their vaccine formulation. Lastly, we found that protein vaccination formulations that included A27 enhanced protection in NHPs, but this protection was not due to the development of a neutralizing antibody response against A27. In this section, I will review the major conclusions from chapters three and four and discuss their implications. Additionally, I will discuss potential future directions.

Complement-dependent EV neutralization

EV is relatively resistant to direct neutralization by antibody. At high concentrations, some monoclonal and polyclonal antibody raised to B5 can directly neutralize EV when assays use small numbers of EV (44,109,113,166). Interestingly, while A33 has been shown to be an important target of protective antibody responses, anti-A33 antibody has not been shown to directly neutralize EV. One proposed mechanism of the protective effect of anti-A33 antibody is the antibody preventing the release of EV from the infected cell (34). Another mechanism might be by complement-enhanced neutralization. Polyclonal rabbit antibody against A33 in the presence of complement was found to lyse the outer membrane of EV, exposing the MV virus within. If MV neutralizing antibody was present, these virions were neutralized (97).

Complement can also enhance EV neutralization by anti-B5 antibody. Recent work by Benhnia, et. al. described an anti-B5 monoclonal antibody that could not directly neutralize EV, but could neutralize in the presence of C'. However, they found that EV neutralization did not require lysis of the EV outer membrane (125). Because C'-dependent neutralization of EV by anti-A33 antibody appeared to require virion lysis and anti-B5 antibody and complement could neutralize by opsonization and were studied using small numbers of EV particles in the presence of a large amount of antibody generated by different methods, we examined the ability of anti-A33 and anti-B5 polyclonal antibody generated after active vaccination of mice to neutralize large numbers of EV in the presence of complement. We found that polyclonal sera were able to neutralize EV in presence of active complement resulting in approximately a log reduction in infectivity (Fig. 3-5). Because these experiments were performed in the presence of a neutralizing MV antibody, we used complement depleted of C5 to determine that indeed anti-A33 antibody required virolysis for neutralization but anti-B5 antibody did not. This finding helps explain our observation that vaccinating with B5 protein was more protective than vaccinating with A33 protein alone (Table 3-1). This finding also suggests that the use of anti-A33 antibody therapeutically or in a vaccine formulation would benefit from the inclusion of a neutralizing MV antibody or protein target to neutralize released MV after C'-mediated lysis of the EV outer envelope. To date, the other proteins found on EV have not been found to generate neutralizing antibody or to protect mice if given as a vaccine (109,169). It would be interesting to determine if antibody to these targets could neutralize EV in the presence of complement and explore why they have not been shown to be protective *in vivo*.

The ability of anti-B5 antibody to neutralize in the absence of C5 suggested to us that something about the B5 protein allowed for complement to opsonize (coat) the particle and block the ability of EV to infect cells. We hypothesized that because B5 protein was found at higher amounts on the surface of EV than A33 protein, antibody could more completely cover the surface of EV and therefore result in more efficient deposition of complement on the EV surface and subsequent neutralization by opsonization. Conversely, the lower amount of A33 protein on the EV surface results in less antibody on the EV surface and the inability to induce complement deposition on the entire surface. EV neutralization would then require the formation of MAC pores and the lysis of the outer envelope of EV resulting in the release of MV that is then neutralized by MV-neutralizing antibody (Fig. 5-1). In support of this model, we found that by reducing the amount of anti-B5 antibody bound to EV, we could force the need of the MAC lytic pathway of complement-dependent neutralization. This finding suggests that the amount of antibody available to activate complement is important to drive the efficient deposition of complement and neutralization of the virion. To further confirm our hypothesis, one could overexpress A33 in an attempt to provide more antibody targets on the surface of EV. We would expect that anti-A33 antibody along with complement could then neutralize EV by opsonization (coating). Additionally, it would be interesting to determine the importance of formation of the MAC *in vivo* for protection from challenge by anti-A33 and anti-B5 antibody. To examine this, mice genetically deficient in the C5 complement component could be used, albeit other functions of C5 would need to be considered. This would also be a way to begin to examine the importance of direct complement-dependent virus neutralization vs. the ability of

complement to lyse infected cells. The presence of host regulators of complement found on EV particles (46) and a viral complement regulator on infected cell surfaces (193) suggests that both mechanisms are important for protection from infection. Overall, our findings demonstrate how the relative expression levels of different viral surface proteins can alter an important host-pathogen interaction.

Fc-dependent protection by antibody in mice

Active or passive immunization against the EV proteins A33 or B5 can provide protection from challenge in mice (96,98,105,106,108-110,124,125,138,149,153,159-162,166-169). Because antibody is inefficient at directly neutralizing EV, Fc-dependent effector mechanisms must play a role in protection. Benhnia, et al. found that a complement fixing anti-B5 monoclonal antibody could protect mice and that protection was partially dependent on complement (125). We hypothesized that protection of mice by polyclonal antibody against B5 would also be partially dependent on complement and that interaction of antibody with FcRs would account for protection seen in the absence of complement. Indeed, mice deficient in the central complement component C3 were still partially protected from challenge after active B5 protein vaccination or passive vaccination with polyclonal B5 antibody, though not as protected as wild-type mice (Figs. 3-9 and 3-10). If polyclonal B5 antibody was passively transferred to FcR knockout mice, mice were protected from challenge. However, if complement was depleted, protection was completely lost (Fig. 3-11). We additionally examined the monoclonal anti-B5 antibody (B126) that Benhnia, et. al. used and found that FcRs were important for the in vivo protective effects of this antibody (Fig. 3-12). Together, these

results suggest that both complement and FcRs are important for mediating protection by antibody against the EV protein B5.

While we found a role for FcRs, we did not determine the type of FcR expressing cells important for mediating protection. Because we found that IgG2a was important for protection in mice, and not IgG1 (Fig. 3-3 and Table 3-1), it's likely that Fc γ RIV expressing cells are important for protection. These include macrophages, neutrophils, monocytes, and dendritic cells. Macrophages have previously been shown to be important for protection from primary challenge (211), so it would be interesting to determine if they are also important when an antibody response is present. NK cells may also help mediate protection through the expression of the low affinity Fc γ RIII and interaction with IgG1 or IgG2a/b. Additionally, these innate cells are known to secrete proinflammatory cytokines in response to FcR stimulation, but the cytokines produced after infection in the presence of a protective antibody response are currently unknown. The cell types involved in FcR-mediated protection could be examined *in vitro* and *in vivo*. *In vitro* studies could include the examination of the ability of peripheral lymphocyte and myeloid cells taken from wild-type and FcRKO mice to kill infected cells in the presence of anti-B5 antibody and/or measure their ability to secrete cytokines in response to FcR engagement by flow cytometry and ELISA. *In vivo* studies could determine the ability of anti-B5 antibody to protect after the depletion of innate cell subsets, such as macrophages or NK cells.

Anti-A33 antibody has been found to neutralize EV in the presence of complement (and anti-MV neutralizing antibody) (97) and could induce complement-dependent lysis of infected cells (160). However, we did not examine the relative

importance of complement or FcRs in the ability of anti-A33 antibody to protect mice from lethal challenge. While we found that anti-A33 antibody was not as protective as B5 antibody, others have shown that anti-A33 antibody can be protective at lower challenge doses (98,101,109) and can enhance the protection afforded by anti-MV antibodies against L1 (98,105,160). It would be interesting to determine if complement and FcRs are important in mediating that enhanced protection, as anti-A33 antibody has been shown to be able to inhibit the release of CEV virions from the infected cell surface in comet inhibition assays. By better understanding the antibody functional requirements necessary for protection, we can better design and test new therapeutics and vaccines.

Antibody isotype and function in NHPs

Generation of a Th1 immune response is important for recovery from viral infections. Therefore, it may be beneficial for viral vaccines to not only elicit antibody, but also elicit antibody isotypes driven by a Th1-biased response. In mice this is IgG2a/b/c, and in NHP or humans this is IgG1. However, knowing the isotype does not always indicate the specific functionality of the response. As we found in chapter three, an IgG2b monoclonal antibody did not activate complement like the isotype would suggest. Therefore, it's important to measure both the isotype and the function of the antibody response, especially when analyzing the immune response to a vaccine in individually genetically diverse NHPs or humans. In Chapter 4, we found that including the Th1 stimulating adjuvant CpG in our protein-based vaccine induced an immune response that was more Th1-like. While some NHPs that were vaccinated with formulations that did not include CpG developed this type of immune response, others

did not. By including CpG, we found that vaccination of NHPs generated a homogeneous immune response characterized by higher IgG1 than IgG2. We then found that NHPs that generated a higher IgG1 response had sera that could fix complement to neutralize EV, including all of the NHPs that had CpG as part of their vaccine formulation. In future analysis of new smallpox vaccines in NHPs or humans, IgG1 responses should be analyzed and functional activity measured. This is especially important for smallpox vaccines, as it's impossible to test the effectiveness of a new vaccine against variola virus in humans. In order to receive FDA approval, we must have rigorous animal models to test the vaccine efficacy as well as suitable analysis of the immune response to the vaccine in order to have the best and most accurate assessment of how the vaccine might protect humans from a smallpox infection.

Additionally, we found that including A27 protein in the protein vaccine added protection from MPXV challenge, but this was not due to production of neutralizing antibody. Whether the A27 protein contributed to the stabilization of the vaccine formulation, induced a CD4+ or CD8+ immune response, or enhanced MV neutralization in a complement dependent fashion is unknown. In the future, it would be beneficial to better understand the contributions of each protein in the vaccine for protection of NHPs. This would allow for the optimization of the vaccine and allow for better assessment of protective immune responses at the clinical testing stages.

In conclusion, this thesis has provided evidence that antibody isotype and functional activity are critical for efficient neutralization of EV and contribute to protection from lethal poxvirus challenge in mice and NHP. We have also provided a

benchmark to further analyze future smallpox vaccine candidates in mice, NHPs, and ultimately humans.

**Appendix: Preliminary results on the production and characterization of a
recombinant VACV with β -lactamase fused to the core protein A4**

Introduction

Poxviruses enter cells by fusion at the plasma membrane or macropinocytosis followed by low-pH induced fusion within macropinosomes (29,51-56). These entry mechanisms lead to the poxvirus core penetrating into the cytoplasm of the infected cell. Efforts to study the entry of poxviruses would be enhanced with an easy to use, high-throughput assay that strictly measure the ability of the virus core to enter the cytoplasm.

The most commonly used assay relies on measuring reporter gene expression (e.g., luciferase or β -galactosidase under the control of an early VACV promoter) (308). Therefore, it is a surrogate measure for VACV entry as entry, uncoating, early gene expression, and protein translation must all occur. While these reporter assays are easy to use, and allow for high-throughput testing, it does not strictly measure entry because of the multiple additional steps after entry that are needed for successful reporter gene expression.

Another assay used to measure entry of VACV is called the “core penetration assay” (309). This assay has been used to quantitatively assess the ability of VACV to bind and enter cells and does not rely on expression of any genes. Instead, an antibody that only recognizes VACV cores that have entered the cytoplasm is used to probe recently infected cells. A second antibody against a viral envelope protein is used to detect virions that did not enter the cell. Confocal immunofluorescence microscopy is then used to manually count the number of viral cores that have entered the cytoplasm compared to viral particles that remained outside the cell. While this assay accurately assesses entry, it has some limitations. If more than ten to twenty cores enter a single cell, it is impossible to accurately count the fluorescent particles. Thus, the number of viral

particles that one can infect each cell with is limited. Additionally, the core penetration assay is technically difficult, time consuming, and uses potentially limited, precious reagents. These factors make it unsuitable for use in a high-throughput format.

As an initial step to develop a high-throughput assay that strictly measures the penetration of VACV cores into the cytoplasm, we designed and produced a recombinant VACV that has β -lactamase fused to the core protein A4. This assay is similar to one initially used in HIV entry research, where β -lactamase is fused to the Vpr protein (310). Subsequently, this assay has been adapted to study the entry of other viruses through the use of pseudotyped HIV virions (311,312) as well as the creation of other viral β -lactamase fusion proteins (313,314). We chose to fuse β -lactamase to the N-terminal domain of the VACV A4 core protein because a recombinant VACV with GFP fused to the N-terminal domain of A4 had previously been made and was viable (29). A4 has been shown to be one of the most abundant proteins found in mature virus (MV) (315,316) and would likely incorporate enough β -lactamase enzyme per virion to be detectable in cells. Additionally, substrates that measure β -lactamase activity in live cells are available and can quantitatively measure the ability of the core to penetrate into the cytoplasm of live infected cells. The following appendix chapter describes the construction and isolation of this virus and its initial characterization.

Methods, Results, and Discussion

Plasmid Construction.

To create a recombinant virus expressing β -lactamase fused to the N-terminus of A4, a plasmid was constructed using splicing by overlap extension (SOEing) PCR. The

resulting plasmid contains the left flanking non-coding sequence of the A4L gene, followed by the β -lactamase gene (without a stop codon) fused in frame with the A4L coding sequence (with the start codon removed), and finally flanking sequence to the right of A4. Additionally, we included an *E. coli gpt* (guanine phosphoriboxyltransferase) selection cassette outside of where homologous recombination was to take place to increase the ability to isolate a recombinant VACV with β -lactamase fused in frame to the A4L gene (Fig. 6-1). To construct this plasmid, SOEing PCR was used to generate three separate PCR fragments from VACV WR strain genomic DNA or pUC19 DNA (β -lactamase gene) using the primers in Table 6-1. The three PCR products generated were: (1) A 465 base-pair PCR product containing the left-flank non-coding A4L sequence and the first part of the β -lactamase gene was amplified using oLMC192 and oLMC193 from VACV WR strain genomic DNA. (2) A 789 base-pair PCR product containing the β -lactamase gene (without stop codon) was amplified using oLMC194 and oLMC195. (3) A 1230 base-pair PCR product containing the last part of the β -lactamase gene (without a stop codon), a linker that would encode three glycines, the A4L coding sequence (without a start codon), and the right flank non-coding sequence of A4L was amplified using oLMC196 and oLMC197. These three PCR products were then mixed in a single PCR reaction and the full length ~2.4-kb fragment was amplified using oLMC192 and oLMC197. The final product was cloned blunt-end into pCR-BLUNT II-TOPO (Invitrogen), confirmed by sequencing the full 2.4-kb insert, and named pMC243.

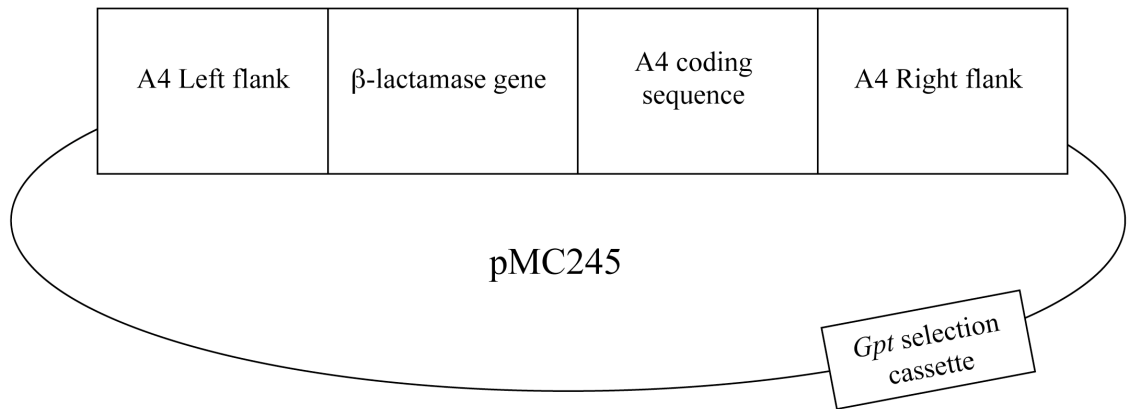


Figure 6-1. Diagram of pMC245.

Shown is the 2.4-kb insert for recombination of the β -lactamase-A4 gene into wild-type VACV. The A4 left flank includes the natural A4 promoter sequence before the β -lactamase gene. The β -lactamase gene has a start codon, but no stop codon so that there is read-through to the A4 coding sequence. The A4 coding sequence open reading frame does not have a start codon, but does have the natural A4 stop codon. The flanking sequence to the right of A4, along with the left flanking sequence drives homologous recombination into the virus. A *gpt* selection cassette driven by the VACV P7.5 promoter was ligated into the plasmid to aid in the enrichment of recombinants after the initial recombination event. It is located outside of the area of recombination so that the final recombinant virus will not contain the *gpt* cassette after a second recombination event that selects against *gpt*.

Table 6-1. Primers used in β -lactamase-A4 plasmid pMC243 and pMC245 construction

Primer Name	Primer Nickname	Primer Sequence	Notes
oIMC192	Fwd A4 left flank	5'-CTCCGTTCTTTTCGAT GACTATAGGACAAGA-3'	
oIMC193	Rev A4 left flank	5'-TTTCACCAGCGTTTCT GGGTCC <u>ATTTA</u> AGGCTT TAAAATTGAATTGCG-3'	Contains the beginning of the β -lactamase gene (initial codon underlined)
oIMC194	Fwd beta-lac	5'-GACCCAGAAACGCTG GTGAAAG-3'	
oIMC195	Rev beta-lac	5'-CCAATGCTTAATCAG TGAGGC-3'	
oIMC196	Fwd A4 right flank	5'-GCCTCACTGATTAAG CATTGGGGCGGAGGCGA CTTCTTTAACAAGTTCTC ACAGGGG-3'	Contains the end of the β -lactamase gene and a three glycine linker (underlined)
oIMC197	Rev A4 right flank	5'-CGTACTCCAATCATGT GTAGATGCTACTTCGTC GATGG-3'	
oIMC199	<i>Gpt</i> FWD primer	5'-AACTGCAGCAGCTGC ATATGTAAAAGTT-3'	Contains 5' PstI restriction site (underlined)
oIMC200	<i>Gpt</i> REV primer	5'-AACTGCAGGCGGCCG CGTCGACCCGGG-3'	Contains 5' PstI restriction site (underlined)

To increase the ability to isolate a recombinant virus with β -lactamase fused in frame to the A4L gene, we added the *E. coli gpt* selection cassette to pMC243. *Gpt* was amplified by PCR from the plasmid pel-P1-*gpt* using the primers oIMC199 and oIMC200. The *gpt* PCR product was then inserted into pMC243 to create the plasmid pMC245 (Fig. 6-1) and insertion of the *gpt* selectable marker was confirmed by sequencing.

Detection of β -lactamase-A4 fusion protein expression and activity from plasmid pMC243.

Before creating a recombinant virus, we wished to confirm that the β -lactamase-A4 fusion protein would be produced and stable inside infected cells. Therefore, we transfected RK-13 cells with pMC243 and subsequently infected them with VACV WR for 48 hours. Cells receiving only pMC243 or VACV WR were used as controls. To confirm expression of β -lactamase-A4 from pMC243, we performed western blots with cell lysates and probed using anti-A4 antibody at 1 μ g/mL (R236, polyclonal rabbit anti-VACV A4 peptide antibody generously provided by Gary Cohen and Roselyn Eisenberg). Cells that were transfected with pMC243 and infected with VACV WR for 48 hours produced a recombinant β -lactamase-A4 fusion protein at a predicted MW of ~60-70 kDa, while cells receiving either plasmid or virus alone did not (Fig. 6-2). This indicates that the recombinant protein was produced in cells and is not rapidly degraded.

To determine if the recombinant β -lactamase-A4 fusion protein had β -lactamase activity, we again transfected cells with pMC243 and infected them with VACV WR for 24 or 48 hours. Cells receiving plasmid or virus only were used as negative controls.

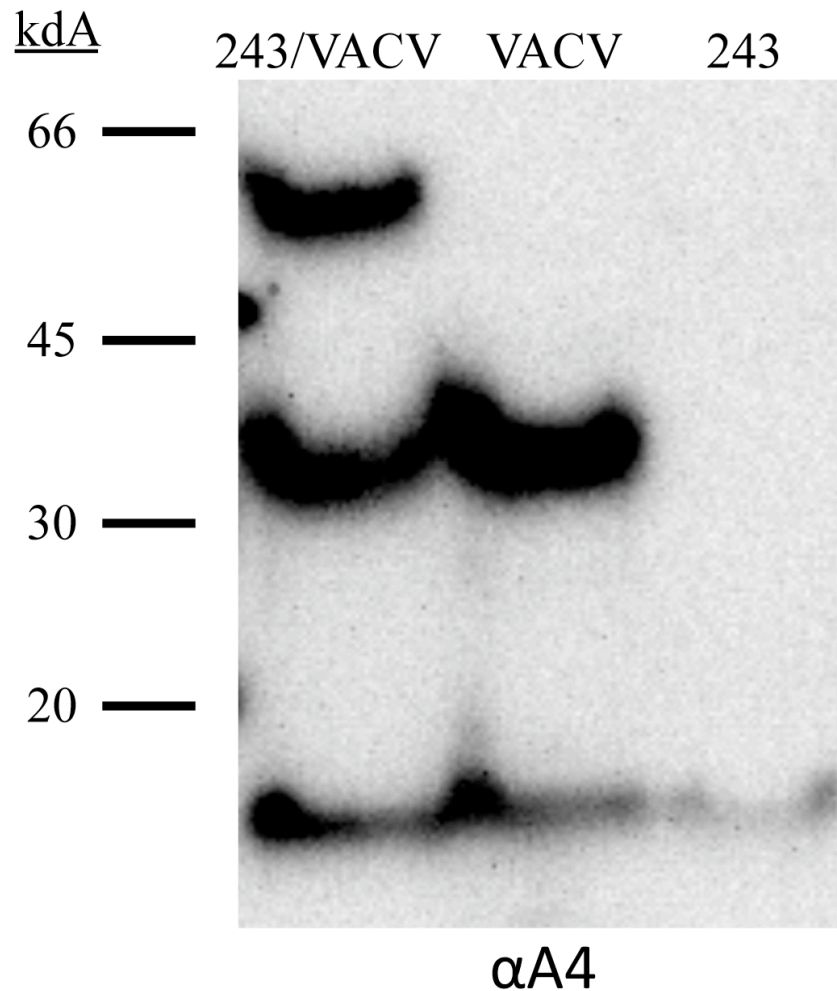


Figure 6-2. Protein expression of β -lactamase-A4 fusion protein from pMC243 transfected cells infected with VACV.

RK-13 cells were transfected with pMC243 and infected with VACV WR (lane 243/VACV). Control cells were either just infected with VACV WR (lane VACV) or just transfected pMC243 (lane 243). At 48 hours post infection, cells were harvested, pelleted, and lysed. Western blots were performed using cell lysate under denaturing/reducing conditions and probed using purified $\alpha A4$ rabbit polyclonal antibody. Wild-type A4 was detected at 39 kDa in both of the VACV infected cell lysates, but not in cells only transfected with pMC243 as expression from the endogenous A4 promoter requires VACV infection. Transfected/infected cell lysate also contained a larger protein at the predicted size for the β -lactamase-A4 fusion protein (~60-70kDa: β -lactamase is 31.5 kDa and A4 is 39 kDa).

Cells were lysed and cell lysate was mixed with nitrocefin (EMD Biosciences, La Jolla, CA), a yellow substrate that turns red after being hydrolyzed by β -lactamase. We found that cell lysates that were transfected with pMC243 and infected with VACV WR had β -lactamase activity (Fig. 6-3). Therefore, the β -lactamase-A4 fusion protein retains β -lactamase activity.

Recombinant virus isolation.

A confluent T-25 flask of CV-1 cells were infected at an MOI of ~ 0.5 with VACV WR for 2 hours and then transfected with pMC245. After 48 hours, the cells were harvested, freeze/thawed three times, and sonicated. To isolate recombinant virus with the β -lactamase-A4 fusion protein, we used both positive and negative *gpt* selection. First, three rounds of plaque picking under positive drug selection was used to isolate a recombinant that had *gpt* inserted into its genome. This was done by growing virus in media containing mycophenolic acid, xanthine, and hypoxanthine (317) and picking plaques from wells where small numbers of plaques formed. After the third plaque pick, this virus was used to do two growths of virus under *gpt* positive selection to amplify the virus and obtain a larger pool of recombinant virus that contained the β -lactamase-A4 fusion protein and *gpt* cassette. During these growths, the presence of the β -lactamase-A4 fusion protein and *gpt* cassette in recombinant virus was confirmed using PCR and β -lactamase activity was assayed using nitrocefin with cell lysates as before. Once we felt we had a pool of virus that was enriched with recombinant viruses, we used negative *gpt* selection to remove the *gpt* cassette while screening for plaques that retained β -lactamase activity. To do this, recombinant virus underwent two rounds of plaque purification in the

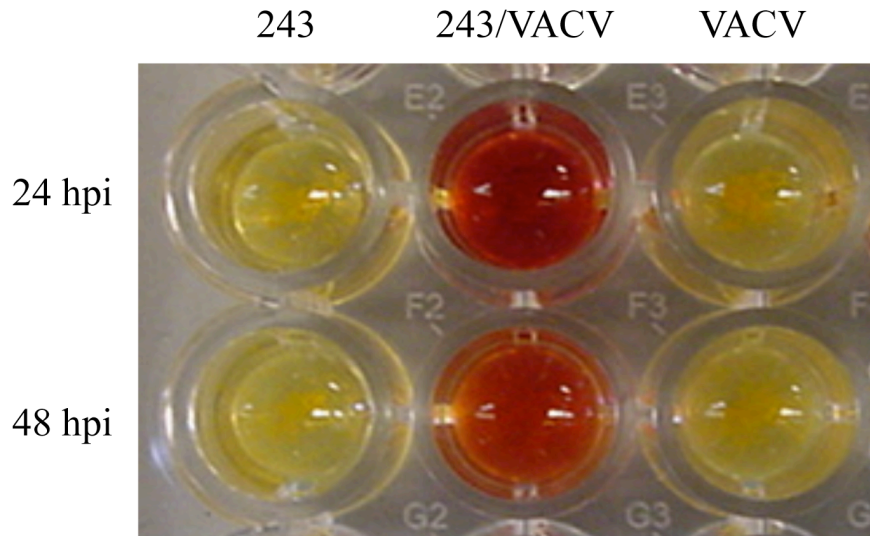


Figure 6-3. β -lactamase activity in pMC243 transfected and VACV infected cell lysates.

BSC-1 cells were transfected with pMC243 and infected with VACV WR (243/VACV). Control cells were either transfected pMC243 (243) or infected with VACV WR (VACV). At 24 and 48 hours post infection (hpi), cells were harvested, pelleted, and lysed. Cell lysates were mixed 1:1 with yellow nitrocefin β -lactamase substrate (1 mM solution). Cell lysate from transfected/infected cells caused the nitrocefin to turn red indicating the presence of β -lactamase and that β -lactamase-A4 fusion protein retained β -lactamase activity. Nitrocefin remained yellow in the presence of transfected or infected cell lysate indicating that β -lactamase was not present.

presence of 6-thioguanine, a drug that selects against the inserted *gpt* selection cassette when virus is plaqued on a hypoxanthine-guanine phosphoribosyltransferase-negative cell line of mouse fibroblasts (318). During this process, plaques were also screened using CCF2/AM live-cell β -lactamase substrate (Invitrogen) to identify and pick plaques that fluoresce blue indicating that β -lactamase-A4 was present. Once plaques were obtained that no longer contained the *gpt* selection cassette but continued to fluoresce blue in the presence of CCF2/AM, we performed an additional 7 rounds of plaque purification using CCF2/AM live-cell β -lactamase substrate to ensure no wild-type virus was present. The resulting plaque purified virus was added to BSC-1 cells in limiting dilutions so that a well on a 24-well plate only had 1 plaque on it. This plaque was allowed to grow until the entire well was infected (~6 days). The resulting recombinant virus was plaqued again and confirmed to only make plaques that fluoresced blue in the presence of CCF2/AM. Four of these plaques were picked and labeled A through D. The resulting plaque purified virus (vMC211A and vMC211C) underwent successive rounds of amplification by growth in increasing amounts of BSC-1 cells. A final large growth underwent virus purification by pelleting through a sucrose cushion. The presence of β -lactamase-A4 fusion protein and absence of wild-type A4 protein and *gpt* cassette in the resulting recombinant virus stocks was confirmed by PCR and sequencing. Virus was confirmed to have β -lactamase activity using CCF2/AM live-cell β -lactamase substrate on infected cells and nitrocefin β -lactamase substrate on infected cell lysates.

Detection of β -lactamase-A4 fusion protein in purified recombinant virions.

To confirm incorporation of the β -lactamase-A4 fusion protein in virions, we performed western blots using purified virions. Blots were probed with the rabbit anti-A4 antibody at 1 μ g/mL. Indeed, β -lactamase-A4 fusion protein (~60-70kDA) was found in purified recombinant virions (Fig. 6-4), though we have yet to compare relative amounts of β -lactamase-A4 fusion protein to A4 found in wild type virions.

When growing vMC211, we observed smaller plaque sizes and slower growth than normally seen with wild-type virus (data not shown). Preliminary results of the optical density of purified recombinant and wild-type viruses may indicate that the recombinant virus has a higher particle to pfu ratio than wild type virus (data not shown). Because of the growth characteristics of the virus, the recombinant virions may be different than wild-type virions. Thus, there will be concern that its entry may be different than wild-type. However, this can be tested using the core penetration assay. The core penetration assay, along with electron microscopy, can also help confirm the difference in particle to pfu ratio between the recombinant and wild type viruses. Additionally, we will confirm that other proteins important for entry are incorporated normally into the recombinant virion.

In the future, experimental conditions for both rapid in-plate and FACS entry inhibition assays utilizing the live-cell CCF2/AM β -lactamase substrate can be pursued. These assays would be powerful tools to have to study poxvirus entry and to rapidly screen entry inhibitors.

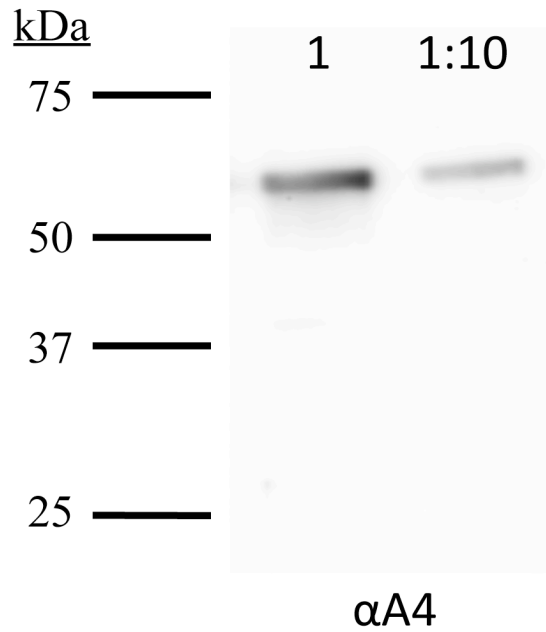


Figure 6-4. β -lactamase-A4 fusion protein is incorporated into recombinant virions.

Western blots were performed using purified recombinant β -lactamase-A4 expressing virions under reducing/denaturing conditions and probed using purified α A4 rabbit polyclonal antibody. 1 μ L of purified virions was run on the gel along with a 1:10 dilution. β -lactamase-A4 fusion protein was detected between 60-70kDa indicating that the recombinant protein was incorporated into virions.

Bibliography

1. Stetton Jr. D (1978) Victory over variola. *ASM News*: 639-644.
2. Eichner M (2003) Analysis of historical data suggests long-lasting protective effects of smallpox vaccination. *Am J Epidemiol* 158: 717-723.
3. Fenner F, Henderson D, Arita I, Jezek Z, Ladnyi I (1988) *Smallpox and its eradication*. Geneva: World Health Organization.
4. Hutin YJ, Williams RJ, Malfait P, Pebody R, Loparev VN, et al. (2001) Outbreak of human monkeypox, democratic republic of congo, 1996 to 1997. *Emerg Infect Dis* 7: 434-438.
5. Mwanbal PT, Tshioko KF, Moudi A, Mukinda V, Mwema GN, et al. (1997) Human monkeypox in Kasai Oriental, Zaire (1996-1997). *Euro Surveill* 2: 33-35.
6. Reed KD, Melski JW, Graham MB, Regnery RL, Sotir MJ, et al. (2004) The detection of monkeypox in humans in the Western Hemisphere. *N Engl J Med* 350: 342-350.
7. Hazel SM, Bennett M, Chantrey J, Bown K, Cavanagh R, et al. (2000) A longitudinal study of an endemic disease in its wildlife reservoir: cowpox and wild rodents. *Epidemiol Infect* 124: 551-562.
8. Baxby D, Bennett M, Getty B (1994) Human cowpox 1969-93: a review based on 54 cases. *Br J Dermatol* 131: 598-607.
9. Kretzschmar M, Wallinga J, Teunis P, Xing S, Mikolajczyk R (2006) Frequency of adverse events after vaccination with different vaccinia strains. *PLoS Med* 3: e272.
10. Lane JM, Ruben FL, Neff JM, Millar JD (1969) Complications of smallpox vaccination, 1968. *N Engl J Med* 281: 1201-1208.
11. Amorosa VK, Isaacs SN (2003) Separate worlds set to collide: smallpox, vaccinia virus vaccination, and human immunodeficiency virus and acquired immunodeficiency syndrome. *Clin Infect Dis* 37: 426-432.
12. Fulginiti VA, Papier A, Lane JM, Neff JM, Henderson DA (2003) Smallpox vaccination: a review, part II. Adverse events. *Clin Infect Dis* 37: 251-271.
13. Cono J, Casey CG, Bell DM (2003) Smallpox vaccination and adverse reactions. Guidance for clinicians. *MMWR Recomm Rep* 52: 1-28.
14. Halsell JS, Riddle JR, Atwood JE, Gardner P, Shope R, et al. (2003) Myopericarditis following smallpox vaccination among vaccinia-naive US military personnel. *Jama* 289: 3283-3289.
15. CDC (2003) Update: cardiac-related events during the civilian smallpox vaccination program--United States, 2003. *MMWR Morb Mortal Wkly Rep* 52: 492-496.
16. Henderson DA, Inglesby TV, Bartlett JG, Ascher MS, Eitzen E, et al. (1999) Smallpox as a biological weapon: medical and public health management. Working Group on Civilian Biodefense. *Jama* 281: 2127-2137.
17. Gibson DG, Benders GA, Andrews-Pfannkoch C, Denisova EA, Baden-Tillson H, et al. (2008) Complete chemical synthesis, assembly, and cloning of a *Mycoplasma genitalium* genome. *Science* 319: 1215-1220.

18. Cello J, Paul AV, Wimmer E (2002) Chemical synthesis of poliovirus cDNA: generation of infectious virus in the absence of natural template. *Science* 297: 1016-1018.
19. Domi A, Moss B (2002) Cloning the vaccinia virus genome as a bacterial artificial chromosome in *Escherichia coli* and recovery of infectious virus in mammalian cells. *Proc Natl Acad Sci U S A* 99: 12415-12420.
20. Bradbury J (2001) USA to increase smallpox vaccine stockpile. *Lancet Infect Dis* 1: 290.
21. Moore ZS, Seward JF, Lane JM (2006) Smallpox. *Lancet* 367: 425-435.
22. Nalca A, Zumbun EE (2010) ACAM2000: the new smallpox vaccine for United States Strategic National Stockpile. *Drug Des Devel Ther* 4: 71-79.
23. Cohen ME, Isaacs SN (2009) Improved Smallpox Vaccines in New Generation Vaccines. In: Levine MM, editor. *New Generation Vaccines*. 4th ed. New York: Informa Healthcare USA, Inc. pp. 838-850.
24. Moss B (2007) Poxviridae: the viruses and their replication. In: Knipe DM, editor. *Fields Virology*. 5th ed. Philadelphia, Pa: Wolters Kluwer Health/Lippincott Williams & Wilkins. pp. 2905–2946.
25. Hsiao JC, Chung CS, Chang W (1998) Cell surface proteoglycans are necessary for A27L protein-mediated cell fusion: identification of the N-terminal region of A27L protein as the glycosaminoglycan-binding domain. *J Virol* 72: 8374-8379.
26. Chung CS, Hsiao JC, Chang YS, Chang W (1998) A27L protein mediates vaccinia virus interaction with cell surface heparan sulfate. *J Virol* 72: 1577-1585.
27. Chiu WL, Lin CL, Yang MH, Tzou DL, Chang W (2007) Vaccinia virus 4c (A26L) protein on intracellular mature virus binds to the extracellular cellular matrix laminin. *J Virol* 81: 2149-2157.
28. Hsiao JC, Chung CS, Chang W (1999) Vaccinia virus envelope D8L protein binds to cell surface chondroitin sulfate and mediates the adsorption of intracellular mature virions to cells. *J Virol* 73: 8750-8761.
29. Carter GC, Law M, Hollinshead M, Smith GL (2005) Entry of the vaccinia virus intracellular mature virion and its interactions with glycosaminoglycans. *J Gen Virol* 86: 1279-1290.
30. Senkevich TG, Ojeda S, Townsley A, Nelson GE, Moss B (2005) Poxvirus multiprotein entry-fusion complex. *Proc Natl Acad Sci U S A* 102: 18572-18577.
31. Moss B (2006) Poxvirus entry and membrane fusion. *Virology* 344: 48-54.
32. Bisht H, Weisberg AS, Moss B (2008) Vaccinia virus I1 protein is required for cell entry and membrane fusion. *J Virol* 82: 8687-8694.
33. Condit RC, Moussatche N, Traktman P (2006) In a nutshell: structure and assembly of the vaccinia virion. *Adv Virus Res* 66: 31-124.
34. Smith GL, Vanderplasschen A, Law M (2002) The formation and function of extracellular enveloped vaccinia virus. *J Gen Virol* 83: 2915-2931.
35. Reeves PM, Bommarius B, Lebeis S, McNulty S, Christensen J, et al. (2005) Disabling poxvirus pathogenesis by inhibition of Abl-family tyrosine kinases. *Nat Med* 11: 731-739.

36. Reeves PM, Smith SK, Olson VA, Thorne SH, Bornmann W, et al. (2011) Variola and monkeypox viruses utilize conserved mechanisms of virion motility and release that depend on abl and SRC family tyrosine kinases. *J Virol* 85: 21-31.
37. Law M, Carter GC, Roberts KL, Hollinshead M, Smith GL (2006) Ligand-induced and nonfusogenic dissolution of a viral membrane. *Proc Natl Acad Sci U S A* 103: 5989-5994.
38. Roberts KL, Breiman A, Carter GC, Ewles HA, Hollinshead M, et al. (2009) Acidic residues in the membrane-proximal stalk region of vaccinia virus protein B5 are required for glycosaminoglycan-mediated disruption of the extracellular enveloped virus outer membrane. *J Gen Virol* 90: 1582-1591.
39. Wolffe EJ, Isaacs SN, Moss B (1993) Deletion of the vaccinia virus B5R gene encoding a 42-kilodalton membrane glycoprotein inhibits extracellular virus envelope formation and dissemination. *J Virol* 67: 4732-4741.
40. Tschärke DC, Reading PC, Smith GL (2002) Dermal infection with vaccinia virus reveals roles for virus proteins not seen using other inoculation routes. *J Gen Virol* 83: 1977-1986.
41. Smith GL, Vanderplasschen A (1998) Extracellular enveloped vaccinia virus. Entry, egress, and evasion. *Adv Exp Med Biol* 440: 395-414.
42. Payne LG (1980) Significance of extracellular enveloped virus in the in vitro and in vivo dissemination of vaccinia. *J Gen Virol* 50: 89-100.
43. McIntosh AA, Smith GL (1996) Vaccinia virus glycoprotein A34R is required for infectivity of extracellular enveloped virus. *J Virol* 70: 272-281.
44. Law M, Smith GL (2001) Antibody neutralization of the extracellular enveloped form of vaccinia virus. *Virology* 280: 132-142.
45. Ichihashi Y (1996) Extracellular enveloped vaccinia virus escapes neutralization. *Virology* 217: 478-485.
46. Vanderplasschen A, Mathew E, Hollinshead M, Sim RB, Smith GL (1998) Extracellular enveloped vaccinia virus is resistant to complement because of incorporation of host complement control proteins into its envelope. *Proc Natl Acad Sci U S A* 95: 7544-7549.
47. Massung RF, Esposito JJ, Liu LI, Qi J, Utterback TR, et al. (1993) Potential virulence determinants in terminal regions of variola smallpox virus genome. *Nature* 366: 748-751.
48. Gubser C, Hue S, Kellam P, Smith GL (2004) Poxvirus genomes: a phylogenetic analysis. *J Gen Virol* 85: 105-117.
49. Upton C, Slack S, Hunter AL, Ehlers A, Roper RL (2003) Poxvirus orthologous clusters: toward defining the minimum essential poxvirus genome. *J Virol* 77: 7590-7600.
50. Schramm B, Locker JK (2005) Cytoplasmic organization of POXvirus DNA replication. *Traffic* 6: 839-846.
51. Whitbeck JC, Foo CH, Ponce de Leon M, Eisenberg RJ, Cohen GH (2009) Vaccinia virus exhibits cell-type-dependent entry characteristics. *Virology* 385: 383-391.
52. Doms RW, Blumenthal R, Moss B (1990) Fusion of intra- and extracellular forms of vaccinia virus with the cell membrane. *J Virol* 64: 4884-4892.

53. Mercer J, Helenius A (2008) Vaccinia virus uses macropinocytosis and apoptotic mimicry to enter host cells. *Science* 320: 531-535.
54. Mercer J, Knebel S, Schmidt FI, Crouse J, Burkard C, et al. (2010) Vaccinia virus strains use distinct forms of macropinocytosis for host-cell entry. *Proc Natl Acad Sci U S A* 107: 9346-9351.
55. Moser TS, Jones RG, Thompson CB, Coyne CB, Cherry S (2010) A kinome RNAi screen identified AMPK as promoting poxvirus entry through the control of actin dynamics. *PLoS Pathog* 6: e1000954.
56. Townsley AC, Weisberg AS, Wagenaar TR, Moss B (2006) Vaccinia virus entry into cells via a low-pH-dependent endosomal pathway. *J Virol* 80: 8899-8908.
57. Carter GC, Rodger G, Murphy BJ, Law M, Krauss O, et al. (2003) Vaccinia virus cores are transported on microtubules. *J Gen Virol* 84: 2443-2458.
58. Katsafanas GC, Moss B (2007) Colocalization of transcription and translation within cytoplasmic poxvirus factories coordinates viral expression and subjugates host functions. *Cell Host Microbe* 2: 221-228.
59. Risco C, Rodriguez JR, Lopez-Iglesias C, Carrascosa JL, Esteban M, et al. (2002) Endoplasmic reticulum-Golgi intermediate compartment membranes and vimentin filaments participate in vaccinia virus assembly. *J Virol* 76: 1839-1855.
60. Moss B, Ahn BY, Amegadzie B, Gershon PD, Keck JG (1991) Cytoplasmic transcription system encoded by vaccinia virus. *J Biol Chem* 266: 1355-1358.
61. Seet BT, Johnston JB, Brunetti CR, Barrett JW, Everett H, et al. (2003) Poxviruses and immune evasion. *Annu Rev Immunol* 21: 377-423.
62. Keck JG, Baldick CJ, Jr., Moss B (1990) Role of DNA replication in vaccinia virus gene expression: a naked template is required for transcription of three late trans-activator genes. *Cell* 61: 801-809.
63. Vos JC, Stunnenberg HG (1988) Derepression of a novel class of vaccinia virus genes upon DNA replication. *Embo J* 7: 3487-3492.
64. Wright CF, Keck JG, Tsai MM, Moss B (1991) A transcription factor for expression of vaccinia virus late genes is encoded by an intermediate gene. *J Virol* 65: 3715-3720.
65. Wright CF, Moss B (1989) Identification of factors specific for transcription of the late class of vaccinia virus genes. *J Virol* 63: 4224-4233.
66. Gershon PD, Moss B (1990) Early transcription factor subunits are encoded by vaccinia virus late genes. *Proc Natl Acad Sci U S A* 87: 4401-4405.
67. Dales S, Siminovitch L (1961) The development of vaccinia virus in Earle's L strain cells as examined by electron microscopy. *J Biophys Biochem Cytol* 10: 475-503.
68. Szajner P, Weisberg AS, Lebowitz J, Heuser J, Moss B (2005) External scaffold of spherical immature poxvirus particles is made of protein trimers, forming a honeycomb lattice. *J Cell Biol* 170: 971-981.
69. Morgan C (1976) The insertion of DNA into vaccinia virus. *Science* 193: 591-592.
70. Bisht H, Weisberg AS, Szajner P, Moss B (2009) Assembly and disassembly of the capsid-like external scaffold of immature virions during vaccinia virus morphogenesis. *J Virol* 83: 9140-9150.

71. Moss B, Rosenblum EN (1973) Letter: Protein cleavage and poxvirus morphogenesis: tryptic peptide analysis of core precursors accumulated by blocking assembly with rifampicin. *J Mol Biol* 81: 267-269.
72. Tooze J, Hollinshead M, Reis B, Radsak K, Kern H (1993) Progeny vaccinia and human cytomegalovirus particles utilize early endosomal cisternae for their envelopes. *Eur J Cell Biol* 60: 163-178.
73. Schmelz M, Sodeik B, Ericsson M, Wolffe EJ, Shida H, et al. (1994) Assembly of vaccinia virus: the second wrapping cisterna is derived from the trans Golgi network. *J Virol* 68: 130-147.
74. Herrero-Martinez E, Roberts KL, Hollinshead M, Smith GL (2005) Vaccinia virus intracellular enveloped virions move to the cell periphery on microtubules in the absence of the A36R protein. *J Gen Virol* 86: 2961-2968.
75. Hollinshead M, Rodger G, Van Eijl H, Law M, Hollinshead R, et al. (2001) Vaccinia virus utilizes microtubules for movement to the cell surface. *J Cell Biol* 154: 389-402.
76. Ward BM, Moss B (2001) Vaccinia virus intracellular movement is associated with microtubules and independent of actin tails. *J Virol* 75: 11651-11663.
77. Arakawa Y, Cordeiro JV, Schleich S, Newsome TP, Way M (2007) The release of vaccinia virus from infected cells requires RhoA-mDia modulation of cortical actin. *Cell Host Microbe* 1: 227-240.
78. Blasco R, Moss B (1992) Role of cell-associated enveloped vaccinia virus in cell-to-cell spread. *J Virol* 66: 4170-4179.
79. Newsome TP, Scaplehorn N, Way M (2004) SRC mediates a switch from microtubule- to actin-based motility of vaccinia virus. *Science* 306: 124-129.
80. Newsome TP, Weisswange I, Frischknecht F, Way M (2006) Abl collaborates with Src family kinases to stimulate actin-based motility of vaccinia virus. *Cell Microbiol* 8: 233-241.
81. Smith GL, Murphy BJ, Law M (2003) Vaccinia virus motility. *Annu Rev Microbiol* 57: 323-342.
82. Xu RH, Cohen M, Tang Y, Lazear E, Whitbeck JC, et al. (2008) The orthopoxvirus type I IFN binding protein is essential for virulence and an effective target for vaccination. *J Exp Med* 205: 981-992.
83. Parker AK, Parker S, Yokoyama WM, Corbett JA, Buller RM (2007) Induction of natural killer cell responses by ectromelia virus controls infection. *J Virol* 81: 4070-4079.
84. Fang M, Lanier LL, Sigal LJ (2008) A role for NKG2D in NK cell-mediated resistance to poxvirus disease. *PLoS Pathog* 4: e30.
85. Delano ML, Brownstein DG (1995) Innate resistance to lethal mousepox is genetically linked to the NK gene complex on chromosome 6 and correlates with early restriction of virus replication by cells with an NK phenotype. *J Virol* 69: 5875-5877.
86. Moulton EA, Atkinson JP, Buller RM (2008) Surviving mousepox infection requires the complement system. *PLoS Pathog* 4: e1000249.

87. Samuelsson C, Hausmann J, Lauterbach H, Schmidt M, Akira S, et al. (2008) Survival of lethal poxvirus infection in mice depends on TLR9, and therapeutic vaccination provides protection. *J Clin Invest* 118: 1776-1784.
88. Chaudhri G, Panchanathan V, Bluethmann H, Karupiah G (2006) Obligatory requirement for antibody in recovery from a primary poxvirus infection. *J Virol* 80: 6339-6344.
89. Fang M, Sigal LJ (2005) Antibodies and CD8+ T cells are complementary and essential for natural resistance to a highly lethal cytopathic virus. *J Immunol* 175: 6829-6836.
90. Crotty S, Felgner P, Davies H, Glidewell J, Villarreal L, et al. (2003) Cutting edge: long-term B cell memory in humans after smallpox vaccination. *J Immunol* 171: 4969-4973.
91. Taub DD, Ershler WB, Janowski M, Artz A, Key ML, et al. (2008) Immunity from smallpox vaccine persists for decades: a longitudinal study. *Am J Med* 121: 1058-1064.
92. Hammarlund E, Lewis MW, Hansen SG, Strelow LI, Nelson JA, et al. (2003) Duration of antiviral immunity after smallpox vaccination. *Nat Med* 9: 1131-1137.
93. Amanna IJ, Slifka MK, Crotty S (2006) Immunity and immunological memory following smallpox vaccination. *Immunol Rev* 211: 320-337.
94. Xu R, Johnson AJ, Liggitt D, Bevan MJ (2004) Cellular and humoral immunity against vaccinia virus infection of mice. *J Immunol* 172: 6265-6271.
95. Xu RH, Fang M, Klein-Szanto A, Sigal LJ (2007) Memory CD8+ T cells are gatekeepers of the lymph node draining the site of viral infection. *Proc Natl Acad Sci U S A* 104: 10992-10997.
96. Lustig S, Fogg C, Whitbeck JC, Eisenberg RJ, Cohen GH, et al. (2005) Combinations of polyclonal or monoclonal antibodies to proteins of the outer membranes of the two infectious forms of vaccinia virus protect mice against a lethal respiratory challenge. *J Virol* 79: 13454-13462.
97. Lustig S, Fogg C, Whitbeck JC, Moss B (2004) Synergistic neutralizing activities of antibodies to outer membrane proteins of the two infectious forms of vaccinia virus in the presence of complement. *Virology* 328: 30-35.
98. Fogg C, Lustig S, Whitbeck JC, Eisenberg RJ, Cohen GH, et al. (2004) Protective immunity to vaccinia virus induced by vaccination with multiple recombinant outer membrane proteins of intracellular and extracellular virions. *J Virol* 78: 10230-10237.
99. Davies DH, McCausland MM, Valdez C, Huynh D, Hernandez JE, et al. (2005) Vaccinia virus H3L envelope protein is a major target of neutralizing antibodies in humans and elicits protection against lethal challenge in mice. *J Virol* 79: 11724-11733.
100. Belyakov IM, Earl P, Dzutsev A, Kuznetsov VA, Lemon M, et al. (2003) Shared modes of protection against poxvirus infection by attenuated and conventional smallpox vaccine viruses. *Proc Natl Acad Sci U S A* 100: 9458-9463.
101. Fang M, Cheng H, Dai Z, Bu Z, Sigal LJ (2006) Immunization with a single extracellular enveloped virus protein produced in bacteria provides partial

- protection from a lethal orthopoxvirus infection in a natural host. *Virology* 345: 231-243.
102. Cornberg M, Sheridan BS, Saccoccio FM, Brehm MA, Selin LK (2007) Protection against vaccinia virus challenge by CD8 memory T cells resolved by molecular mimicry. *J Virol* 81: 934-944.
 103. Panchanathan V, Chaudhri G, Karupiah G (2006) Protective immunity against secondary poxvirus infection is dependent on antibody but not on CD4 or CD8 T-cell function. *J Virol* 80: 6333-6338.
 104. Earl PL, Americo JL, Wyatt LS, Eller LA, Whitbeck JC, et al. (2004) Immunogenicity of a highly attenuated MVA smallpox vaccine and protection against monkeypox. *Nature* 428: 182-185.
 105. Fogg CN, Americo JL, Lustig S, Huggins JW, Smith SK, et al. (2007) Adjuvant-enhanced antibody responses to recombinant proteins correlates with protection of mice and monkeys to orthopoxvirus challenges. *Vaccine* 25: 2787-2799.
 106. Hooper JW, Thompson E, Wilhelmsen C, Zimmerman M, Ichou MA, et al. (2004) Smallpox DNA vaccine protects nonhuman primates against lethal monkeypox. *J Virol* 78: 4433-4443.
 107. Edghill-Smith Y, Golding H, Manischewitz J, King LR, Scott D, et al. (2005) Smallpox vaccine-induced antibodies are necessary and sufficient for protection against monkeypox virus. *Nat Med* 11: 740-747.
 108. Buchman GW, Cohen ME, Xiao Y, Richardson-Harman N, Silvera P, et al. (2010) A protein-based smallpox vaccine protects non-human primates from a lethal monkeypox virus challenge. *Vaccine* 28: 6627-6636.
 109. Galmiche MC, Goenaga J, Wittek R, Rindisbacher L (1999) Neutralizing and protective antibodies directed against vaccinia virus envelope antigens. *Virology* 254: 71-80.
 110. Xiao Y, Aldaz-Carroll L, Ortiz AM, Whitbeck JC, Alexander E, et al. (2007) A protein-based smallpox vaccine protects mice from vaccinia and ectromelia virus challenges when given as a prime and single boost. *Vaccine* 25: 1214-1224.
 111. Viner KM, Isaacs SN (2005) Activity of vaccinia virus-neutralizing antibody in the sera of smallpox vaccinees. *Microbes Infect* 7: 579-583.
 112. Lawrence SJ, Lottenbach KR, Newman FK, Buller RM, Bellone CJ, et al. (2007) Antibody responses to vaccinia membrane proteins after smallpox vaccination. *J Infect Dis* 196: 220-229.
 113. Bell E, Shamim M, Whitbeck JC, Sfyroera G, Lambris JD, et al. (2004) Antibodies against the extracellular enveloped virus B5R protein are mainly responsible for the EEV neutralizing capacity of vaccinia immune globulin. *Virology* 325: 425-431.
 114. Davies DH, Molina DM, Wrammert J, Miller J, Hirst S, et al. (2007) Proteome-wide analysis of the serological response to vaccinia and smallpox. *Proteomics* 7: 1678-1686.
 115. Jones-Trower A, Garcia A, Meseda CA, He Y, Weiss C, et al. (2005) Identification and preliminary characterization of vaccinia virus (Dryvax) antigens recognized by vaccinia immune globulin. *Virology* 343: 128-140.

116. Appleyard G, Andrews C (1974) Neutralizing activities of antisera to poxvirus soluble antigens. *J Gen Virol* 23: 197-200.
117. Meseda CA, Garcia AD, Kumar A, Mayer AE, Manischewitz J, et al. (2005) Enhanced immunogenicity and protective effect conferred by vaccination with combinations of modified vaccinia virus Ankara and licensed smallpox vaccine Dryvax in a mouse model. *Virology* 339: 164-175.
118. Meseda CA, Mayer AE, Kumar A, Garcia AD, Campbell J, et al. (2009) Comparative evaluation of the immune responses and protection engendered by LC16m8 and Dryvax smallpox vaccines in a mouse model. *Clin Vaccine Immunol* 16: 1261-1271.
119. Mehlhop E, Fuchs A, Engle M, Diamond MS (2009) Complement modulates pathogenesis and antibody-dependent neutralization of West Nile virus infection through a C5-independent mechanism. *Virology* 393: 11-15.
120. Mozdzanowska K, Feng J, Eid M, Zharikova D, Gerhard W (2006) Enhancement of neutralizing activity of influenza virus-specific antibodies by serum components. *Virology* 352: 418-426.
121. Beebe DP, Schreiber RD, Cooper NR (1983) Neutralization of influenza virus by normal human sera: mechanisms involving antibody and complement. *J Immunol* 130: 1317-1322.
122. Wallis C, Melnick JL (1971) Herpesvirus neutralization: the role of complement. *J Immunol* 107: 1235-1242.
123. Lerner AM, Shippey MJ, Crane LR (1974) Serologic responses to herpes simplex virus in rabbits: complement-requiring neutralizing, conventional neutralizing, and passive hemagglutinating antibodies. *J Infect Dis* 129: 623-636.
124. Benhnia MR, McCausland MM, Laudenslager J, Granger SW, Rickert S, et al. (2009) Heavily isotype-dependent protective activities of human antibodies against vaccinia virus extracellular virion antigen B5. *J Virol* 83: 12355-12367.
125. Benhnia MR, McCausland MM, Moyron J, Laudenslager J, Granger S, et al. (2009) Vaccinia virus extracellular enveloped virion neutralization in vitro and protection in vivo depend on complement. *J Virol* 83: 1201-1215.
126. Isaacs SN, Kotwal GJ, Moss B (1992) Vaccinia virus complement-control protein prevents antibody-dependent complement-enhanced neutralization of infectivity and contributes to virulence. *Proc Natl Acad Sci U S A* 89: 628-632.
127. Davies DH, Liang X, Hernandez JE, Randall A, Hirst S, et al. (2005) Profiling the humoral immune response to infection by using proteome microarrays: high-throughput vaccine and diagnostic antigen discovery. *Proc Natl Acad Sci U S A* 102: 547-552.
128. He Y, Manischewitz J, Meseda CA, Merchlinsky M, Vassell RA, et al. (2007) Antibodies to the A27 protein of vaccinia virus neutralize and protect against infection but represent a minor component of Dryvax vaccine-induced immunity. *J Infect Dis* 196: 1026-1032.
129. Putz MM, Midgley CM, Law M, Smith GL (2006) Quantification of antibody responses against multiple antigens of the two infectious forms of Vaccinia virus provides a benchmark for smallpox vaccination. *Nat Med* 12: 1310-1315.

130. Davies DH, Wyatt LS, Newman FK, Earl PL, Chun S, et al. (2008) Antibody profiling by proteome microarray reveals the immunogenicity of the attenuated smallpox vaccine modified vaccinia virus ankara is comparable to that of Dryvax. *J Virol* 82: 652-663.
131. Schmid K, Keasey SL, Pittman P, Emerson GL, Meegan J, et al. (2008) Analysis of the human immune response to vaccinia by use of a novel protein microarray suggests that antibodies recognize less than 10% of the total viral proteome. *Proteomics Clin Appl* 2: 1528-1538.
132. Benhnia MR, McCausland MM, Su HP, Singh K, Hoffmann J, et al. (2008) Redundancy and plasticity of neutralizing antibody responses are cornerstone attributes of the human immune response to the smallpox vaccine. *J Virol* 82: 3751-3768.
133. Duke-Cohan JS, Wollenick K, Witten EA, Seaman MS, Baden LR, et al. (2009) The heterogeneity of human antibody responses to vaccinia virus revealed through use of focused protein arrays. *Vaccine* 27: 1154-1165.
134. Rodriguez JF, Paez E, Esteban M (1987) A 14,000-Mr envelope protein of vaccinia virus is involved in cell fusion and forms covalently linked trimers. *J Virol* 61: 395-404.
135. Sanderson CM, Hollinshead M, Smith GL (2000) The vaccinia virus A27L protein is needed for the microtubule-dependent transport of intracellular mature virus particles. *J Gen Virol* 81: 47-58.
136. Ramirez JC, Tapia E, Esteban M (2002) Administration to mice of a monoclonal antibody that neutralizes the intracellular mature virus form of vaccinia virus limits virus replication efficiently under prophylactic and therapeutic conditions. *J Gen Virol* 83: 1059-1067.
137. Fogg CN, Americo JL, Earl PL, Resch W, Aldaz-Carroll L, et al. (2008) Disparity between levels of in vitro neutralization of vaccinia virus by antibody to the A27 protein and protection of mice against intranasal challenge. *J Virol* 82: 8022-8029.
138. Hooper JW, Custer DM, Thompson E (2003) Four-gene-combination DNA vaccine protects mice against a lethal vaccinia virus challenge and elicits appropriate antibody responses in nonhuman primates. *Virology* 306: 181-195.
139. Lai CF, Gong SC, Esteban M (1991) The purified 14-kilodalton envelope protein of vaccinia virus produced in *Escherichia coli* induces virus immunity in animals. *Journal of Virology* 65: 5631-5635.
140. Senkevich TG, Ward BM, Moss B (2004) Vaccinia virus entry into cells is dependent on a virion surface protein encoded by the A28L gene. *J Virol* 78: 2357-2366.
141. Nelson GE, Wagenaar TR, Moss B (2008) A conserved sequence within the H2 subunit of the vaccinia virus entry/fusion complex is important for interaction with the A28 subunit and infectivity. *J Virol* 82: 6244-6250.
142. Senkevich TG, Moss B (2005) Vaccinia virus H2 protein is an essential component of a complex involved in virus entry and cell-cell fusion. *J Virol* 79: 4744-4754.

143. Nelson GE, Sisler JR, Chandran D, Moss B (2008) Vaccinia virus entry/fusion complex subunit A28 is a target of neutralizing and protective antibodies. *Virology* 380: 394-401.
144. Shinoda K, Wyatt LS, Moss B (2010) The neutralizing antibody response to the vaccinia virus A28 protein is specifically enhanced by its association with the H2 protein. *Virology* 405: 41-49.
145. Aldaz-Carroll L, Whitbeck JC, Ponce de Leon M, Lou H, Pannell LK, et al. (2005) Physical and immunological characterization of a recombinant secreted form of the membrane protein encoded by the vaccinia virus L1R gene. *Virology* 341: 59-71.
146. Ichihashi Y, Oie M (1996) Neutralizing epitope on penetration protein of vaccinia virus. *Virology* 220: 491-494.
147. Wolffe EJ, Vijaya S, Moss B (1995) A myristylated membrane protein encoded by the vaccinia virus L1R open reading frame is the target of potent neutralizing monoclonal antibodies. *Virology* 211: 53-63.
148. Foo CH, Lou H, Whitbeck JC, Ponce-de-Leon M, Atanasiu D, et al. (2009) Vaccinia virus L1 binds to cell surfaces and blocks virus entry independently of glycosaminoglycans. *Virology* 385: 368-382.
149. Heraud JM, Edghill-Smith Y, Ayala V, Kalisz I, Parrino J, et al. (2006) Subunit recombinant vaccine protects against monkeypox. *J Immunol* 177: 2552-2564.
150. Maa JS, Rodriguez JF, Esteban M (1990) Structural and functional characterization of a cell surface binding protein of vaccinia virus. *J Biol Chem* 265: 1569-1577.
151. Chernos VI, Vovk TS, Ivanova ON, Antonova TP, Loparev VN (1993) Insertion mutants of the vaccinia virus. The effect of inactivating E7R and D8L genes on the biological properties of the virus. *Mol Gen Mikrobiol Virusol*: 30-34.
152. Demkowicz WE, Maa JS, Esteban M (1992) Identification and characterization of vaccinia virus genes encoding proteins that are highly antigenic in animals and are immunodominant in vaccinated humans. *J Virol* 66: 386-398.
153. Sakhatskyy P, Wang S, Chou TH, Lu S (2006) Immunogenicity and protection efficacy of monovalent and polyvalent poxvirus vaccines that include the D8 antigen. *Virology* 355: 164-174.
154. Lin CL, Chung CS, Heine HG, Chang W (2000) Vaccinia virus envelope H3L protein binds to cell surface heparan sulfate and is important for intracellular mature virion morphogenesis and virus infection in vitro and in vivo. *J Virol* 74: 3353-3365.
155. Vanderplasschen A, Hollinshead M, Smith GL (1997) Antibodies against vaccinia virus do not neutralize extracellular enveloped virus but prevent virus release from infected cells and comet formation. *J Gen Virol* 78 (Pt 8): 2041-2048.
156. Roper RL, Payne LG, Moss B (1996) Extracellular vaccinia virus envelope glycoprotein encoded by the A33R gene. *J Virol* 70: 3753-3762.
157. Wolffe EJ, Weisberg AS, Moss B (2001) The vaccinia virus A33R protein provides a chaperone function for viral membrane localization and tyrosine phosphorylation of the A36R protein. *J Virol* 75: 303-310.

158. Law M, Hollinshead R, Smith GL (2002) Antibody-sensitive and antibody-resistant cell-to-cell spread by vaccinia virus: role of the A33R protein in antibody-resistant spread. *J Gen Virol* 83: 209-222.
159. Chen Z, Earl P, Americo J, Damon I, Smith SK, et al. (2006) Chimpanzee/human mAbs to vaccinia virus B5 protein neutralize vaccinia and smallpox viruses and protect mice against vaccinia virus. *Proc Natl Acad Sci U S A* 103: 1882-1887.
160. Hooper JW, Custer DM, Schmaljohn CS, Schmaljohn AL (2000) DNA vaccination with vaccinia virus L1R and A33R genes protects mice against a lethal poxvirus challenge. *Virology* 266: 329-339.
161. Hooper JW, Golden JW, Ferro AM, King AD (2007) Smallpox DNA vaccine delivered by novel skin electroporation device protects mice against intranasal poxvirus challenge. *Vaccine* 25: 1814-1823.
162. Sakhatskyy P, Wang S, Zhang C, Chou TH, Kishko M, et al. (2008) Immunogenicity and protection efficacy of subunit-based smallpox vaccines using variola major antigens. *Virology* 371: 98-107.
163. Isaacs SN, Wolffe EJ, Payne LG, Moss B (1992) Characterization of a vaccinia virus-encoded 42-kilodalton class I membrane glycoprotein component of the extracellular virus envelope. *J Virol* 66: 7217-7224.
164. Engelstad M, Howard ST, Smith GL (1992) A constitutively expressed vaccinia gene encodes a 42-kDa glycoprotein related to complement control factors that forms part of the extracellular virus envelope. *Virology* 188: 801-810.
165. Engelstad M, Smith GL (1993) The vaccinia virus 42-kDa envelope protein is required for the envelopment and egress of extracellular virus and for virus virulence. *Virology* 194: 627-637.
166. Aldaz-Carroll L, Whitbeck JC, Ponce de Leon M, Lou H, Hirao L, et al. (2005) Epitope-mapping studies define two major neutralization sites on the vaccinia virus extracellular enveloped virus glycoprotein B5R. *J Virol* 79: 6260-6271.
167. Aldaz-Carroll L, Xiao Y, Whitbeck JC, Ponce de Leon M, Lou H, et al. (2007) Major neutralizing sites on vaccinia virus glycoprotein B5 are exposed differently on variola virus ortholog B6. *J Virol* 23: 23.
168. Golovkin M, Spitsin S, Andrianov V, Smirnov Y, Xiao Y, et al. (2007) Smallpox subunit vaccine produced in *Planta* confers protection in mice. *Proc Natl Acad Sci U S A* 104: 6864-6869.
169. Pulford DJ, Gates A, Bridge SH, Robinson JH, Ulaeto D (2004) Differential efficacy of vaccinia virus envelope proteins administered by DNA immunisation in protection of BALB/c mice from a lethal intranasal poxvirus challenge. *Vaccine* 22: 3358-3366.
170. Presta LG (2008) Molecular engineering and design of therapeutic antibodies. *Curr Opin Immunol* 20: 460-470.
171. Nimmerjahn F, Ravetch JV (2006) Fcγ receptors: old friends and new family members. *Immunity* 24: 19-28.
172. Kojouharova M, Reid K, Gadjeva M (2010) New insights into the molecular mechanisms of classical complement activation. *Mol Immunol* 47: 2154-2160.

173. Lubinski JM, Lazear HM, Awasthi S, Wang F, Friedman HM (2011) The herpes simplex virus 1 IgG fc receptor blocks antibody-mediated complement activation and antibody-dependent cellular cytotoxicity in vivo. *J Virol* 85: 3239-3249.
174. Hessel AJ, Hangartner L, Hunter M, Havenith CE, Beurskens FJ, et al. (2007) Fc receptor but not complement binding is important in antibody protection against HIV. *Nature* 449: 101-104.
175. Holl V, Hemmerter S, Burrer R, Schmidt S, Bohbot A, et al. (2004) Involvement of Fc gamma RI (CD64) in the mechanism of HIV-1 inhibition by polyclonal IgG purified from infected patients in cultured monocyte-derived macrophages. *J Immunol* 173: 6274-6283.
176. Holl V, Peressin M, Decoville T, Schmidt S, Zolla-Pazner S, et al. (2006) Nonneutralizing antibodies are able to inhibit human immunodeficiency virus type 1 replication in macrophages and immature dendritic cells. *J Virol* 80: 6177-6181.
177. Peressin M, Holl V, Schmidt S, Decoville T, Mirisky D, et al. (2011) HIV-1 replication in Langerhans and interstitial dendritic cells is inhibited by neutralizing and Fc-mediated inhibitory antibodies. *J Virol* 85: 1077-1085.
178. Huber VC, Lynch JM, Bucher DJ, Le J, Metzger DW (2001) Fc receptor-mediated phagocytosis makes a significant contribution to clearance of influenza virus infections. *J Immunol* 166: 7381-7388.
179. Chung KM, Nybakken GE, Thompson BS, Engle MJ, Marri A, et al. (2006) Antibodies against West Nile Virus nonstructural protein NS1 prevent lethal infection through Fc gamma receptor-dependent and -independent mechanisms. *J Virol* 80: 1340-1351.
180. Chung KM, Thompson BS, Fremont DH, Diamond MS (2007) Antibody recognition of cell surface-associated NS1 triggers Fc-gamma receptor-mediated phagocytosis and clearance of West Nile Virus-infected cells. *J Virol* 81: 9551-9555.
181. Chu CF, Meador MG, Young CG, Strasser JE, Bourne N, et al. (2008) Antibody-mediated protection against genital herpes simplex virus type 2 disease in mice by Fc gamma receptor-dependent and -independent mechanisms. *J Reprod Immunol* 78: 58-67.
182. Schlesinger JJ, Foltzer M, Chapman S (1993) The Fc portion of antibody to yellow fever virus NS1 is a determinant of protection against YF encephalitis in mice. *Virology* 192: 132-141.
183. Harris SL, Frank I, Yee A, Cohen GH, Eisenberg RJ, et al. (1990) Glycoprotein C of herpes simplex virus type 1 prevents complement-mediated cell lysis and virus neutralization. *J Infect Dis* 162: 331-337.
184. Walport MJ (2001) Complement. Second of two parts. *N Engl J Med* 344: 1140-1144.
185. Walport MJ (2001) Complement. First of two parts. *N Engl J Med* 344: 1058-1066.
186. Azeredo da Silveira S, Kikuchi S, Fossati-Jimack L, Moll T, Saito T, et al. (2002) Complement activation selectively potentiates the pathogenicity of the IgG2b and IgG3 isotypes of a high affinity anti-erythrocyte autoantibody. *J Exp Med* 195: 665-672.

187. Ey PL, Russell-Jones GJ, Jenkin CR (1980) Isotypes of mouse IgG-I. Evidence for 'non-complement-fixing' IgG1 antibodies and characterization of their capacity to interfere with IgG2 sensitization of target red blood cells for lysis by complement. *Mol Immunol* 17: 699-710.
188. Neuberger MS, Rajewsky K (1981) Activation of mouse complement by monoclonal mouse antibodies. *Eur J Immunol* 11: 1012-1016.
189. Ward ES, Ghetie V (1995) The effector functions of immunoglobulins: implications for therapy. *Ther Immunol* 2: 77-94.
190. Bindon CI, Hale G, Bruggemann M, Waldmann H (1988) Human monoclonal IgG isotypes differ in complement activating function at the level of C4 as well as C1q. *J Exp Med* 168: 127-142.
191. Bernet J, Mullick J, Singh AK, Sahu A (2003) Viral mimicry of the complement system. *J Biosci* 28: 249-264.
192. Lambris JD, Ricklin D, Geisbrecht BV (2008) Complement evasion by human pathogens. *Nat Rev Microbiol* 6: 132-142.
193. Girgis NM, Dehaven BC, Fan X, Viner KM, Shamim M, et al. (2008) Cell surface expression of the vaccinia virus complement control protein is mediated by interaction with the viral A56 protein and protects infected cells from complement attack. *J Virol* 82: 4205-4214.
194. Friedman HM, Wang L, Pangburn MK, Lambris JD, Lubinski J (2000) Novel mechanism of antibody-independent complement neutralization of herpes simplex virus type 1. *J Immunol* 165: 4528-4536.
195. Perrin LH, Joseph BS, Cooper NR, Oldstone MB (1976) Mechanism of injury of virus-infected cells by antiviral antibody and complement: participation of IgG, F(ab')₂, and the alternative complement pathway. *J Exp Med* 143: 1027-1041.
196. Dunkelberger JR, Song WC (2010) Complement and its role in innate and adaptive immune responses. *Cell Res* 20: 34-50.
197. Marschang P, Sodroski J, Wurzner R, Dierich MP (1995) Decay-accelerating factor (CD55) protects human immunodeficiency virus type 1 from inactivation by human complement. *Eur J Immunol* 25: 285-290.
198. Johnson JB, Grant K, Parks GD (2009) The paramyxoviruses simian virus 5 and mumps virus recruit host cell CD46 to evade complement-mediated neutralization. *J Virol* 83: 7602-7611.
199. Zipfel PF, Skerka C (2009) Complement regulators and inhibitory proteins. *Nat Rev Immunol* 9: 729-740.
200. Kotwal GJ, Isaacs SN, McKenzie R, Frank MM, Moss B (1990) Inhibition of the complement cascade by the major secretory protein of vaccinia virus. *Science* 250: 827-830.
201. McKenzie R, Kotwal GJ, Moss B, Hammer CH, Frank MM (1992) Regulation of complement activity by vaccinia virus complement-control protein. *J Infect Dis* 166: 1245-1250.
202. Friedman HM, Cohen GH, Eisenberg RJ, Seidel CA, Cines DB (1984) Glycoprotein C of herpes simplex virus 1 acts as a receptor for the C3b complement component on infected cells. *Nature* 309: 633-635.

203. Chung KM, Liszewski MK, Nybakken G, Davis AE, Townsend RR, et al. (2006) West Nile virus nonstructural protein NS1 inhibits complement activation by binding the regulatory protein factor H. *Proc Natl Acad Sci U S A* 103: 19111-19116.
204. Perrin LH, Zinkernagel RM, Oldstone MB (1977) Immune response in humans after vaccination with vaccinia virus: generation of a virus-specific cytotoxic activity by human peripheral lymphocytes. *J Exp Med* 146: 949-969.
205. Ravetch JV, Lanier LL (2000) Immune inhibitory receptors. *Science* 290: 84-89.
206. Nimmerjahn F, Bruhns P, Horiuchi K, Ravetch JV (2005) FcγRIIV: a novel FcR with distinct IgG subclass specificity. *Immunity* 23: 41-51.
207. Takai T, Li M, Sylvestre D, Clynes R, Ravetch JV (1994) FcR γ chain deletion results in pleiotropic effector cell defects. *Cell* 76: 519-529.
208. Nimmerjahn F, Ravetch JV (2005) Divergent immunoglobulin g subclass activity through selective Fc receptor binding. *Science* 310: 1510-1512.
209. Kawakami Y, Tomimori Y, Yumoto K, Hasegawa S, Ando T, et al. (2009) Inhibition of NK cell activity by IL-17 allows vaccinia virus to induce severe skin lesions in a mouse model of eczema vaccinatum. *J Exp Med* 206: 1219-1225.
210. Martinez J, Huang X, Yang Y (2010) Direct TLR2 signaling is critical for NK cell activation and function in response to vaccinia viral infection. *PLoS Pathog* 6: e1000811.
211. Karupiah G, Buller RM, Van Rooijen N, Duarte CJ, Chen J (1996) Different roles for CD4+ and CD8+ T lymphocytes and macrophage subsets in the control of a generalized virus infection. *J Virol* 70: 8301-8309.
212. Lauterbach H, Kassub R, Patzold J, Korner J, Bruckel M, et al. (2010) Immune requirements of post-exposure immunization with modified vaccinia Ankara of lethally infected mice. *PLoS One* 5: e9659.
213. Breman JG, Henderson DA (1998) Poxvirus dilemmas--monkeypox, smallpox. *N Engl J Med* 339: 556-559.
214. Atlas RM (1998) The threat of bioterrorism returns the fear of smallpox. *Curr Opin Microbiol* 1: 719-721.
215. Nalca A, Rimoin AW, Bavari S, Whitehouse CA (2005) Reemergence of monkeypox: prevalence, diagnostics, and countermeasures. *Clin Infect Dis* 41: 1765-1771.
216. Rosenthal SR, Merchlinsky M, Kleppinger C, Goldenthal KL (2001) Developing new smallpox vaccines. *Emerg Infect Dis* 7: 920-926.
217. Poland GA (2005) Smallpox vaccines: from first to second to third generation. *Lancet* 365: 362-363.
218. Wiser I, Balicer RD, Cohen D (2007) An update on smallpox vaccine candidates and their role in bioterrorism related vaccination strategies. *Vaccine* 25: 976-984.
219. Artenstein AW (2008) New generation smallpox vaccines: a review of preclinical and clinical data. *Rev Med Virol* 18: 217-231.
220. Weltzin R, Liu J, Pugachev KV, Myers GA, Coughlin B, et al. (2003) Clonal vaccinia virus grown in cell culture as a new smallpox vaccine. *Nat Med* 9: 1125-1130. Epub 2003 Aug 1117.

221. Monath TP, Caldwell JR, Mundt W, Fusco J, Johnson CS, et al. (2004) ACAM2000 clonal Vero cell culture vaccinia virus (New York City Board of Health strain) - a second-generation smallpox vaccine for biological defense. *Int J Infect Dis* 8: 31-44.
222. Marriott KA, Parkinson CV, Morefield SI, Davenport R, Nichols R, et al. (2008) Clonal vaccinia virus grown in cell culture fully protects monkeys from lethal monkeypox challenge. *Vaccine* 26: 581-588.
223. Greenberg RN, Kennedy JS (2008) ACAM2000: a newly licensed cell culture-based live vaccinia smallpox vaccine. *Expert Opin Investig Drugs* 17: 555-564.
224. CDC (2008) Notice to Readers: Newly licensed smallpox vaccine to replace old smallpox vaccine. *MMWR Morb Mortal Wkly Rep* 57: 207-208.
225. Weiner DB (2003) Keeping the lock on smallpox. *Nat Med* 9: 1115-1116.
226. Poland GA, Grabenstein JD, Neff JM (2005) The US smallpox vaccination program: a review of a large modern era smallpox vaccination implementation program. *Vaccine* 23: 2078-2081.
227. CDC (2003) Recommendations for using smallpox vaccine in a pre-event vaccination program. Supplemental recommendations of the Advisory Committee on Immunization Practices (ACIP) and the Healthcare Infection Control Practices Advisory Committee (HICPAC). *MMWR Recomm Rep* 52: 1-16.
228. Hashizume S (1975) A new attenuated strain Lc16m8 of vaccini virus for safer smallpox vaccination. *Clin Virol* 3: 229-235.
229. Hashizume S, Yoshizawa H, Morita M, Suzuki K (1985) Properties of attenuated mutant of vaccinia virus, LC16m8 derived from Lister strain. In: Quinnen J, editor. *Vaccinia viurses as vecors for vaccine antigens*. New York: Elsevier. pp. 87-100.
230. Kenner J, Cameron F, Empig C, Jobes DV, Gurwith M (2006) LC16m8: an attenuated smallpox vaccine. *Vaccine* 24: 7009-7022.
231. Takahashi-Nishimaki F, Funahashi S, Miki K, Hashizume S, Sugimoto M (1991) Regulation of plaque size and host range by a vaccinia virus gene related to complement system proteins. *Virology* 181: 158-164.
232. Empig C, Kenner JR, Perret-Gentil M, Youree BE, Bell E, et al. (2006) Highly attenuated smallpox vaccine protects rabbits and mice against pathogenic orthopoxvirus challenge. *Vaccine* 24: 3686-3694.
233. Saijo M, Ami Y, Suzaki Y, Nagata N, Iwata N, et al. (2006) LC16m8, a highly attenuated vaccinia virus vaccine lacking expression of the membrane protein B5R, protects monkeys from monkeypox. *J Virol* 80: 5179-5188.
234. Kidokoro M, Tashiro M, Shida H (2005) Genetically stable and fully effective smallpox vaccine strain constructed from highly attenuated vaccinia LC16m8. *Proc Natl Acad Sci U S A* 102: 4152-4157.
235. Mayr A (2003) Smallpox vaccination and bioterrorism with pox viruses. *Comp Immunol Microbiol Infect Dis* 26: 423-430.
236. Slifka MK (2005) The Future of Smallpox Vaccination: is MVA the key? *Med Immunol* 4: 2.

237. Wyatt LS, Earl PL, Eller LA, Moss B (2004) Highly attenuated smallpox vaccine protects mice with and without immune deficiencies against pathogenic vaccinia virus challenge. *Proc Natl Acad Sci U S A* 101: 4590-4595.
238. Phelps AL, Gates AJ, Hillier M, Eastaugh L, Ulaeto DO (2007) Comparative efficacy of modified vaccinia Ankara (MVA) as a potential replacement smallpox vaccine. *Vaccine* 25: 34-42.
239. Stittelaar KJ, van Amerongen G, Kondova I, Kuiken T, van Lavieren RF, et al. (2005) Modified vaccinia virus Ankara protects macaques against respiratory challenge with monkeypox virus. *J Virol* 79: 7845-7851.
240. Earl PL, Americo JL, Wyatt LS, Anne Eller L, Montefiori DC, et al. (2007) Recombinant modified vaccinia virus Ankara provides durable protection against disease caused by an immunodeficiency virus as well as long-term immunity to an orthopoxvirus in a non-human primate. *Virology* 10: 10.
241. Earl PL, Americo JL, Wyatt LS, Espenshade O, Bassler J, et al. (2008) Rapid protection in a monkeypox model by a single injection of a replication-deficient vaccinia virus. *Proc Natl Acad Sci U S A* 105: 10889-10894.
242. Staib C, Suezter Y, Kisling S, Kalinke U, Sutter G (2006) Short-term, but not post-exposure, protection against lethal orthopoxvirus challenge after immunization with modified vaccinia virus Ankara. *J Gen Virol* 87: 2917-2921.
243. Bielinska AU, Chepurnov AA, Landers JJ, Janczak KW, Chepurnova TS, et al. (2008) A novel, killed-virus nasal vaccinia virus vaccine. *Clin Vaccine Immunol* 15: 348-358.
244. Boulter EA, Appleyard G (1973) Differences between extracellular and intracellular forms of poxvirus and their implications. *Prog Med Virol* 16: 86-108.
245. Panchanathan V, Chaudhri G, Karupiah G (2005) Interferon function is not required for recovery from a secondary poxvirus infection. *Proc Natl Acad Sci U S A* 102: 12921-12926.
246. Kaufman DR, Goudsmit J, Holterman L, Ewald BA, Denholtz M, et al. (2008) Differential antigen requirements for protection against systemic and intranasal vaccinia virus challenges in mice. *J Virol* 82: 6829-6837.
247. Payne L (1978) Polypeptide composition of extracellular enveloped vaccinia virus. *J Virol* 27: 28-37.
248. Payne LG, Kristensson K (1982) Effect of glycosylation inhibitors on the release of enveloped vaccinia virus. *J Virol* 41: 367—375.
249. Payne LG, Kristensson K (1990) The polypeptide composition of vaccinia-infected cell membranes and rifampicin bodies. *Virus Res* 17: 15-30.
250. Payne LG (1992) Characterization of vaccinia virus glycoproteins by monoclonal antibody preparations. *Virology* 187: 251-260.
251. Rodriguez JF, Janeczko R, Esteban M (1985) Isolation and characterization of neutralizing monoclonal antibodies to vaccinia virus. *J Virol* 56: 482-488.
252. Czerny CP, Mahnel H (1990) Structural and functional analysis of orthopoxvirus epitopes with neutralizing monoclonal antibodies. *J Gen Virol* 71: 2341-2352.
253. Gordon J, Mohandas A, Wilton S, Dales S (1991) A prominent antigenic surface polypeptide involved in the biogenesis and function of the vaccinia virus envelope. *Virology* 181: 671-686.

254. Golden JW, Hooper JW (2008) Heterogeneity in the A33 protein impacts the cross-protective efficacy of a candidate smallpox DNA vaccine. *Virology* 377: 19-29.
255. Berhanu A, Wilson RL, Kirkwood-Watts DL, King DS, Warren TK, et al. (2008) Vaccination of BALB/c mice with Escherichia coli-expressed vaccinia virus proteins A27L, B5R, and D8L protects mice from lethal vaccinia virus challenge. *J Virol* 82: 3517-3529.
256. Thornburg NJ, Ray CA, Collier ML, Liao HX, Pickup DJ, et al. (2007) Vaccination with Venezuelan equine encephalitis replicons encoding cowpox virus structural proteins protects mice from intranasal cowpox virus challenge. *Virology* 362: 441-452.
257. Barefoot B, Thornburg NJ, Barouch DH, Yu JS, Sample C, et al. (2008) Comparison of multiple vaccine vectors in a single heterologous prime-boost trial. *Vaccine* 20: 20.
258. Johnston JB, McFadden G (2003) Poxvirus immunomodulatory strategies: current perspectives. *J Virol* 77: 6093-6100.
259. Rock MT, Yoder SM, Talbot TR, Edwards KM, Crowe JE, Jr. (2004) Adverse events after smallpox immunizations are associated with alterations in systemic cytokine levels. *J Infect Dis* 189: 1401-1410.
260. Laddy DJ, Weiner DB (2006) From plasmids to protection: a review of DNA vaccines against infectious diseases. *Int Rev Immunol* 25: 99-123.
261. Snyder JT, Belyakov IM, Dzutsev A, Lemonnier F, Berzofsky JA (2004) Protection against lethal vaccinia virus challenge in HLA-A2 transgenic mice by immunization with a single CD8+ T-cell peptide epitope of vaccinia and variola viruses. *J Virol* 78: 7052-7060.
262. Tschärke DC, Karupiah G, Zhou J, Palmore T, Irvine KR, et al. (2005) Identification of poxvirus CD8+ T cell determinants to enable rational design and characterization of smallpox vaccines. *J Exp Med* 201: 95-104.
263. Hanna W, Baxby D (2002) Studies in smallpox and vaccination. 1913. *Rev Med Virol* 12: 201-209.
264. Otero M, Calarota SA, Dai A, De Groot AS, Boyer JD, et al. (2006) Efficacy of novel plasmid DNA encoding vaccinia antigens in improving current smallpox vaccination strategy. *Vaccine* 24: 4461-4470.
265. Beachkofsky TM, Carrizales SC, Bidinger JJ, Hrnčir DE, Whittemore DE, et al. (2010) Adverse events following smallpox vaccination with ACAM2000 in a military population. *Arch Dermatol* 146: 656-661.
266. Paran N, Sutter G (2009) Smallpox vaccines: New formulations and revised strategies for vaccination. *Hum Vaccin* 5: 824-831.
267. Kennedy RB, Ovsyannikova I, Poland GA (2009) Smallpox vaccines for biodefense. *Vaccine* 27 Suppl 4: D73-79.
268. Kennedy JS, Greenberg RN (2009) IMVAMUNE: modified vaccinia Ankara strain as an attenuated smallpox vaccine. *Expert Rev Vaccines* 8: 13-24.
269. Riedmann EM (2010) FDA Fast Track status for IMVAMUNE. *Hum Vaccin* 6: 368 - 372.
270. Payne LG, Kristensson K (1985) Extracellular release of enveloped vaccinia virus from mouse nasal epithelial cells in vivo. *J Gen Virol* 66 (Pt 3): 643-646.

271. Roberts KL, Smith GL (2008) Vaccinia virus morphogenesis and dissemination. *Trends Microbiol* 16: 472-479.
272. Moyron-Quiroz JE, McCausland MM, Kageyama R, Sette A, Crotty S (2009) The smallpox vaccine induces an early neutralizing IgM response. *Vaccine* 28: 140-147.
273. Mehlhop E, Whitby K, Oliphant T, Marri A, Engle M, et al. (2005) Complement activation is required for induction of a protective antibody response against West Nile virus infection. *J Virol* 79: 7466-7477.
274. Wang SY, Veeramani S, Racila E, Cagley J, Fritzing DC, et al. (2009) Depletion of the C3 component of complement enhances the ability of rituximab-coated target cells to activate human NK cells and improves the efficacy of monoclonal antibody therapy in an in vivo model. *Blood* 114: 5322-5330.
275. Vogel CW, Muller-Eberhard HJ (1984) Cobra venom factor: improved method for purification and biochemical characterization. *J Immunol Methods* 73: 203-220.
276. Bengali Z, Townsley AC, Moss B (2009) Vaccinia virus strain differences in cell attachment and entry. *Virology* 389: 132-140.
277. Ichihashi Y, Takahashi T, Oie M (1994) Identification of a vaccinia virus penetration protein. *Virology* 202: 834-843.
278. Charreau B, Cassard A, Tesson L, Le Mauff B, Navenot JM, et al. (1994) Protection of rat endothelial cells from primate complement-mediated lysis by expression of human CD59 and/or decay-accelerating factor. *Transplantation* 58: 1222-1229.
279. Brazolot Millan CL, Weeratna R, Krieg AM, Siegrist CA, Davis HL (1998) CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. *Proc Natl Acad Sci U S A* 95: 15553-15558.
280. Chu RS, Targoni OS, Krieg AM, Lehmann PV, Harding CV (1997) CpG oligodeoxynucleotides act as adjuvants that switch on T helper 1 (Th1) immunity. *J Exp Med* 186: 1623-1631.
281. Zimmermann S, Egeter O, Hausmann S, Lipford GB, Rocken M, et al. (1998) CpG oligodeoxynucleotides trigger protective and curative Th1 responses in lethal murine leishmaniasis. *J Immunol* 160: 3627-3630.
282. Weeratna RD, Brazolot Millan CL, McCluskie MJ, Davis HL (2001) CpG ODN can re-direct the Th bias of established Th2 immune responses in adult and young mice. *FEMS Immunol Med Microbiol* 32: 65-71.
283. Da Costa XJ, Brockman MA, Alicot E, Ma M, Fischer MB, et al. (1999) Humoral response to herpes simplex virus is complement-dependent. *Proc Natl Acad Sci U S A* 96: 12708-12712.
284. Pozdnyakova O, Guttormsen HK, Lalani FN, Carroll MC, Kasper DL (2003) Impaired antibody response to group B streptococcal type III capsular polysaccharide in C3- and complement receptor 2-deficient mice. *J Immunol* 170: 84-90.
285. Ochsenbein AF, Pinschewer DD, Odermatt B, Carroll MC, Hengartner H, et al. (1999) Protective T cell-independent antiviral antibody responses are dependent on complement. *J Exp Med* 190: 1165-1174.

286. Jouvin-Marche E, Morgado MG, Leguern C, Voegtle D, Bonhomme F, et al. (1989) The mouse Igh-1a and Igh-1b H chain constant regions are derived from two distinct isotypic genes. *Immunogenetics* 29: 92-97.
287. Martin RM, Brady JL, Lew AM (1998) The need for IgG2c specific antiserum when isotyping antibodies from C57BL/6 and NOD mice. *J Immunol Methods* 212: 187-192.
288. Panchanathan V, Chaudhri G, Karupiah G (2010) Antiviral protection following immunization correlates with humoral but not cell-mediated immunity. *Immunol Cell Biol* 88: 461-467.
289. Delaney KN, Phipps JP, Johnson JB, Mizel SB (2010) A recombinant flagellin-poxvirus fusion protein vaccine elicits complement-dependent protection against respiratory challenge with vaccinia virus in mice. *Viral Immunol* 23: 201-210.
290. Carter RH, Fearon DT (1992) CD19: lowering the threshold for antigen receptor stimulation of B lymphocytes. *Science* 256: 105-107.
291. Fischer MB, Goerg S, Shen L, Prodeus AP, Goodnow CC, et al. (1998) Dependence of germinal center B cells on expression of CD21/CD35 for survival. *Science* 280: 582-585.
292. Klinman DM, Klaschik S, Sato T, Tross D (2009) CpG oligonucleotides as adjuvants for vaccines targeting infectious diseases. *Adv Drug Deliv Rev* 61: 248-255.
293. Klinman DM (2006) Adjuvant activity of CpG oligodeoxynucleotides. *Int Rev Immunol* 25: 135-154.
294. Hooper JW, Ferro AM, Golden JW, Silvera P, Dudek J, et al. (2009) Molecular smallpox vaccine delivered by alphavirus replicons elicits protective immunity in mice and non-human primates. *Vaccine* 28: 494-511.
295. O'Connell KP, Kovaleva E, Campbell JH, Anderson PE, Brown SG, et al. (2007) Production of a recombinant antibody fragment in whole insect larvae. *Mol Biotechnol* 36: 44-51.
296. Kovaleva ES, O'Connell KP, Buckley P, Liu Z, Davis DC (2009) Recombinant protein production in insect larvae: host choice, tissue distribution, and heterologous gene instability. *Biotechnol Lett* 31: 381-386.
297. Frey SE, Newman FK, Kennedy JS, Ennis F, Abate G, et al. (2009) Comparison of the safety and immunogenicity of ACAM1000, ACAM2000 and Dryvax in healthy vaccinia-naive adults. *Vaccine* 27: 1637-1644.
298. CDC (2007) Household transmission of vaccinia virus from contact with a military smallpox vaccinee--Illinois and Indiana, 2007. *MMWR Morb Mortal Wkly Rep* 56: 478-481.
299. CDC (2009) Progressive vaccinia in a military smallpox vaccinee -- United States, 2009. *MMWR Morb Mortal Wkly Rep* 58: 532-536.
300. Cooper CL, Davis HL, Morris ML, Efler SM, Adhami MA, et al. (2004) CPG 7909, an immunostimulatory TLR9 agonist oligodeoxynucleotide, as adjuvant to Engerix-B HBV vaccine in healthy adults: a double-blind phase I/II study. *J Clin Immunol* 24: 693-701.

301. Mullen GE, Ellis RD, Miura K, Malkin E, Nolan C, et al. (2008) Phase 1 trial of AMA1-C1/Alhydrogel plus CPG 7909: an asexual blood-stage vaccine for *Plasmodium falciparum* malaria. *PLoS One* 3: e2940.
302. Chen N, Li G, Liszewski MK, Atkinson JP, Jahrling PB, et al. (2005) Virulence differences between monkeypox virus isolates from West Africa and the Congo basin. *Virology* 340: 46-63.
303. Nigam P, Earl PL, Americo JL, Sharma S, Wyatt LS, et al. (2007) DNA/MVA HIV-1/AIDS vaccine elicits long-lived vaccinia virus-specific immunity and confers protection against a lethal monkeypox challenge. *Virology* 14: 14.
304. Huggins J, Goff A, Hensley L, Mucker E, Shamblin J, et al. (2009) Nonhuman primates are protected from smallpox virus or monkeypox virus challenges by the antiviral drug ST-246. *Antimicrob Agents Chemother* 53: 2620-2625.
305. Jordan R, Goff A, Frimm A, Corrado ML, Hensley LE, et al. (2009) ST-246 antiviral efficacy in a nonhuman primate monkeypox model: determination of the minimal effective dose and human dose justification. *Antimicrob Agents Chemother* 53: 1817-1822.
306. Zaucha GM, Jahrling PB, Geisbert TW, Swearingen JR, Hensley L (2001) The pathology of experimental aerosolized monkeypox virus infection in cynomolgus monkeys (*Macaca fascicularis*). *Lab Invest* 81: 1581-1600.
307. Saijo M, Ami Y, Suzuki Y, Nagata N, Iwata N, et al. (2009) Virulence and pathophysiology of the Congo Basin and West African strains of monkeypox virus in non-human primates. *J Gen Virol* 90: 2266-2271.
308. Law M, Smith GL (2004) Studying the Binding and Entry of the Intracellular and Extracellular Enveloped Forms of Vaccinia Virus In: Isaacs SN, editor. *Vaccinia Virus and Poxvirology: Methods and Protocols*. Totowa, NJ: Humana Press. pp. 187-203.
309. Vanderplasschen A, Hollinshead M, Smith GL (1998) Intracellular and extracellular vaccinia virions enter cells by different mechanisms. *J Gen Virol* 79 (Pt 4): 877-887.
310. Cavrois M, De Noronha C, Greene WC (2002) A sensitive and specific enzyme-based assay detecting HIV-1 virion fusion in primary T lymphocytes. *Nat Biotechnol* 20: 1151-1154.
311. Yonezawa A, Cavrois M, Greene WC (2005) Studies of ebola virus glycoprotein-mediated entry and fusion by using pseudotyped human immunodeficiency virus type 1 virions: involvement of cytoskeletal proteins and enhancement by tumor necrosis factor alpha. *J Virol* 79: 918-926.
312. Lavillette D, Bartosch B, Nourrisson D, Verney G, Cosset FL, et al. (2006) Hepatitis C virus glycoproteins mediate low pH-dependent membrane fusion with liposomes. *J Biol Chem* 281: 3909-3917.
313. Wolf MC, Wang Y, Freiberg AN, Aguilar HC, Holbrook MR, et al. (2009) A catalytically and genetically optimized beta-lactamase-matrix based assay for sensitive, specific, and higher throughput analysis of native henipavirus entry characteristics. *Virol J* 6: 119.
314. Tscherne DM, Manicassamy B, Garcia-Sastre A An enzymatic virus-like particle assay for sensitive detection of virus entry. *J Virol Methods* 163: 336-343.

315. Yoder JD, Chen TS, Gagnier CR, Vemulapalli S, Maier CS, et al. (2006) Pox proteomics: mass spectrometry analysis and identification of Vaccinia virion proteins. *Virol J* 3: 10.
316. Chung CS, Chen CH, Ho MY, Huang CY, Liao CL, et al. (2006) Vaccinia virus proteome: identification of proteins in vaccinia virus intracellular mature virion particles. *J Virol* 80: 2127-2140.
317. Falkner FG, Moss B (1988) Escherichia coli gpt gene provides dominant selection for vaccinia virus open reading frame expression vectors. *J Virol* 62: 1849-1854.
318. Isaacs SN, Kotwal GJ, Moss B (1990) Reverse guanine phosphoribosyltransferase selection of recombinant vaccinia viruses. *Virology* 178: 626-630.