

## ABSTRACT

Title of Document: ACTIVE AND PASSIVE IMMUNIZATION STRATEGIES FOR PROTECTION OF MICE AND MONKEYS AGAINST ORTHOPOXVIRUS INFECTION.

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The *Poxviridae* are large DNA viruses that replicate in the cytoplasm of vertebrates or invertebrates. The genus *Orthopoxvirus* includes variola virus, the cause of smallpox, and vaccinia virus (VACV), the prototypal family member used in the licensed smallpox vaccine. Interest in the development of an alternative smallpox vaccine emerged because of complications associated with recent vaccination efforts and the growing number of people excluded from vaccination. Antibody therapies are also of interest for *Orthopoxvirus* infection treatment instead of vaccinia immune globulin from human donors. Essential to these efforts are studies that elucidate aspects of the immune response required for protection against disease. Two infectious forms of virus exist, intracellular mature virus (IMV), which mediates spread between hosts, and extracellular virus (EV), which is required for efficient spread within a host. IMV and EV each possess an outer membrane with viral

proteins targeted by the adaptive immune response.

I have used soluble baculovirus-expressed forms of VACV proteins from the IMV and EV in order to understand the role of immunity to these particles during infection. Subcutaneous immunization of mice multiple times with the EV proteins A33 and B5 and the IMV protein L1 either individually or in combinations induced specific antibody responses and protected against weight loss and death caused by virus infection, especially following immunization with A33+B5+L1 or A33+L1. Similar patterns of protection were observed by passive immunization of mice with polyclonal or monoclonal antibodies against A33, B5, or L1 prior to or after intranasal challenge. A27 was investigated as an alternative IMV protein to L1, but proved less effective alone or in combination with A33. Potent and more rapid immune responses to the A33 and L1 proteins were stimulated by the use of the adjuvants QS-21, or alum mixed with CpG oligodeoxynucleotides. Protection against a lethal challenge was observed in a small study with monkeys that were immunized with A33, B5, and L1 and challenged with monkeypox. My data indicate protection against orthopoxviruses is seen in animal models so long as a good antibody response is made to both the IMV and EV forms.

ACTIVE AND PASSIVE IMMUNIZATION STRATEGIES FOR PROTECTION  
OF MICE AND MONKEYS AGAINST ORTHOPOXVIRUS INFECTION.

By

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## **Dedication**

I dedicate this work to my parents, Michael and Jeannette Root, for their infinite support of my education and exploration of this amazing world.

To my husband James, for sharing all of my woes and joys both in and out of the laboratory.

To my daughter Elizabeth, for inspiration and motivation for continued exploration and preservation of the world that I leave to her.

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# Table of Contents

Dedication.....	ii
Acknowledgements .....	iii
Table of Contents .....	iv
List of Tables .....	viii
List of Figures .....	ix
List of Abbreviations.....	xi
<b>Chapter 1 General Introduction and Research Goals.....</b>	<b>1</b>
<b>Chapter 2 Review of Literature .....</b>	<b>3</b>
2.1 The Poxviridae.....	3
2.1.1 Classification.....	3
2.1.2 Genome organization.....	4
2.1.3 Virion Morphology and Types of Infectious Particles.....	6
2.1.4 Viral Proteins.....	7
2.2 Viral Replicative Cycle.....	7
2.2.1 Viral Binding and Fusion.....	7
2.2.2 Temporal Expression of Viral Genes and Genome Replication.....	9
2.2.3 Maturation of Viral Particles and Viral Egress.....	12
2.3 Smallpox, Edward Jenner and the Dawn of Vaccination.....	15
2.3.1 Smallpox and Variola Virus .....	15
2.3.2 Edward Jenner and the Discovery of Vaccination .....	16
2.3.3 Smallpox Eradication .....	16
2.3.4 Smallpox as a Biological Weapon .....	17
2.4 Orthopoxvirus Pathogenesis.....	18
2.4.1 The Two Forms of Infectious Virus and Their Role in Disease.....	18
2.4.2 Primary Infection of Humans with Variola Virus.....	20
2.4.3 Ectromelia Virus as a Model for Viral Pathogenesis.....	22
2.4.4 Vaccinia Virus as a Model for Viral Pathogenesis .....	25
2.4.5 The Complement Cascade and its Role During VACV Infection .....	28
2.5 Past, Present and Futures Immunization Strategies Against Smallpox .....	31
2.5.1 Dryvax Vaccination of Humans and its Side Effects.....	31
2.5.2 Attenuated Poxviruses as Vaccines.....	32
2.5.3 DNA and Protein Subunit Vaccines.....	34
2.5.4 Vaccinia Immune Globulin and Passive Antibody Therapy .....	35
<b>Chapter 3 Protective Vaccination of Mice with Recombinant Outer Membrane Proteins from the Extracellular and Intracellular Forms of Vaccinia Virus .....</b>	<b>38</b>
3.1 Introduction .....	38
3.2 Materials and Methods.....	42
3.2.1 Cells and Viruses .....	42
3.2.2 Recombinant Proteins.....	43
3.2.3 SDS-PAGE and Western Blot Analysis.....	44

3.2.4	PNGase F Treatment of Recombinant Proteins .....	45
3.2.5	Mouse Immunization Protocol.....	45
3.2.6	Vaccinia Virus-Infected Cell Lysates .....	46
3.2.7	Enzyme-Linked Immunosorbent Assay (ELISA).....	47
3.2.8	IMV Neutralization and Comet Reduction Assays.....	48
3.2.9	Intranasal Vaccinia Virus Challenge of Mice.....	49
3.2.10	Statistical analysis .....	49
3.3	Results .....	50
3.3.1	Biochemical Analysis of Recombinant Vaccinia Virus Proteins .....	50
3.3.2	Antibody Responses Induced by Immunization with IMV and EV Proteins.....	54
3.3.3	IgG Isotypes Induced by Protein and Virus Immunization .....	55
3.3.4	Characterization of Neutralizing Antibodies Induced by Recombinant Protein Immunization .....	59
3.3.5	Protection of Immunized Mice to an Intranasal Challenge with 5 LD <sub>50</sub> of VACV WR .....	63
3.3.6	Protection of Immunized Mice to an Intranasal Challenge with 100 LD <sub>50</sub> of VACV WR.....	64
3.3.7	Multiple Immunizations are Required for Protection .....	67
3.4	Discussion .....	70
<b>Chapter 4</b>	<b>Passive Protection of Mice with Antibodies against Multiple Outer Membrane Proteins of the Extracellular and Intracellular Forms of Vaccinia Virus.....</b>	<b>73</b>
4.1	Introduction .....	73
4.2	Materials and Methods.....	75
4.2.1	Cells and Viruses.....	75
4.2.2	Hybridomas and Monoclonal Antibodies.....	75
4.2.3	Polyclonal Antibodies .....	76
4.2.4	Vaccinia Immune Globulin.....	77
4.2.5	Enzyme-Linked Immunosorbent Assays (ELISAs).....	77
4.2.6	IMV Neutralization and Comet Reduction Assays.....	78
4.2.7	Passive Immunization and Intranasal Virus Challenge .....	78
4.2.8	Statistical Analysis .....	79
4.3	Results .....	80
4.3.1	Characterization of PABs and MABs Used for Passive Immunization.....	80
4.3.2	Protection of Mice with Individual or Combinations of PABs .....	86
4.3.3	Protection of Mice with Individual or Combinations of MABs.....	92
4.3.4	Effect of Time of Passive Immunization of MABs Relative to Challenge .....	94
4.3.5	Passive Immunization of SCID Mice.....	97
4.4	Discussion .....	99

<b>Chapter 5</b>	<b>Protection of Mice Against a Lethal Challenge by Multiple Immunizations with Different Proteins of the Intracellular Mature Virus .....</b>	<b>103</b>
5.1	Introduction .....	103
5.2	Materials and Methods .....	105
5.2.1	Cells and Viruses .....	105
5.2.2	Recombinant Proteins.....	105
5.2.3	Immunization Protocol .....	106
5.2.4	ELISA.....	107
5.2.5	IMV Neutralization and Comet Reduction Assays.....	107
5.2.6	Virus Challenge.....	108
5.2.7	Statistical Analysis .....	108
5.3	Results .....	109
5.3.1	Biochemical Characterization of Recombinant A27 Protein.....	109
5.3.2	Antibody Responses Following Multiple Protein Immunizations....	112
5.3.3	Measurement of Neutralizing Antibodies Induced by Protein Immunization .....	115
5.3.4	Protection of Mice Following IN Challenge with $10^6$ pfu of VACV WR .....	117
5.3.5	Protection of Mice Following IN Challenge with $2 \times 10^7$ pfu of VACV WR .....	120
5.4	Discussion .....	123
<b>Chapter 6</b>	<b>Improved Antibody Responses to Recombinant Protein Immunization with Different Adjuvants Corresponds to Enhanced Protection of Mice and Monkeys against Orthopoxvirus Challenge.....</b>	<b>126</b>
6.1	Introduction .....	126
6.2	Materials and Methods.....	129
6.2.1	Cells and Viruses .....	129
6.2.2	Recombinant Proteins and ODNs .....	129
6.2.3	Immunization Protocol .....	130
6.2.4	Intranasal Virus Challenge of Mice .....	130
6.2.5	Monkey Immunization and Challenge Protocol .....	131
6.2.6	ELISA.....	131
6.2.7	MV Neutralization and Comet Reduction Assays .....	132
6.2.9	Statistical Analysis .....	133
6.3	Results.....	134
6.3.1	Antibody Responses to A33 and L1 Proteins Following Immunization with Different Adjuvants.....	134
6.3.2	Induction of Neutralizing and Comet-Reducing Antibodies.....	141
6.3.3	The Effect of Different Adjuvants on Protection of Mice Against Lethal Intranasal Virus Challenge.....	141
6.3.4	Induction of Binding and Neutralizing Antibodies in Cynomolgous Monkeys Immunized with Recombinant A33, B5 and L1 Proteins with QS-21.....	147

6.3.5	Protection of Monkeys Against an Intravenous MPXV Challenge Following Multiple Protein Immunizations .....	150
6.4	Discussion .....	152
<b>Chapter 7</b>	<b>Conclusion .....</b>	<b>156</b>
<b>Bibliography.....</b>		<b>166</b>

## List of Tables

- Table 3-1: Reciprocal endpoint ELISA titers.
- Table 4-1: Reciprocal endpoint ELISA titers of PAbs and MAbs.
- Table 4-2: Comparison of ELISA endpoint titers following passive or active immunization.
- Table 6-1: Individual ELISA titers against A33 and L1 after two immunizations.
- Table 6-2: *P*-values determined by pairwise comparisons of individual ELISA data using t-tests with pooled standard deviations.
- Table 6-3. Table of *p*-values calculated with area under the curve analysis followed by the Wilcoxon rank sum test using the Holm *p*-value adjustment.

## List of Figures

- Figure 2-1: Diagrammatic representations of the Vaccinia virus (VACV) genome.
- Figure 2-2: Replicative cycle of orthopoxviruses.
- Figure 3-1: Coomassie staining of recombinant baculovirus proteins.
- Figure 3-2: Western blot analysis of recombinant proteins.
- Figure 3-3: Deglycosylation of recombinant proteins.
- Figure 3-4: Antibody responses following multiple immunizations with recombinant proteins.
- Figure 3-5: Characterization of IgG isotypes.
- Figure 3-6: Induction of neutralizing antibodies against IMV and EV.
- Figure 3-7: Weight loss of mice following intranasal challenge with  $10^6$  pfu of VACV WR.
- Figure 3-8: Survival and weight loss of mice challenged IN with  $2 \times 10^7$  pfu of VACV WR.
- Figure 3-9: Multiple immunizations are required for protection against a lethal IN challenge with  $2 \times 10^7$  pfu of VACV WR.
- Figure 4-1: Comet reduction in the presence of anti-EV antibodies.
- Figure 4-2: Protection of mice by passive immunization with PAbs.
- Figure 4-3: Protection of mice by passive immunization with MAbs.
- Figure 4-4: Immunization before or after intranasal virus challenge.
- Figure 4-5: Survival of passive immunized SCID mice following virus challenge.
- Figure 5-1: Coomassie staining and western blot analysis of recombinant A27 protein.

- Figure 5-2: Analysis of binding antibodies by ELISA.
- Figure 5-3: Measurement of neutralizing antibodies following multiple protein immunizations.
- Figure 5-4: Comet reduction assay.
- Figure 5-5: Intranasal challenge with  $10^6$  pfu (5 LD<sub>50</sub>) of VACV WR.
- Figure 5-6: Intranasal challenge with  $2 \times 10^7$  pfu (100 LD<sub>50</sub>) of VACV WR.
- Figure 6-1: ELISA values of pooled mouse sera following immunizations with A33 and L1 proteins combined with different adjuvants.
- Figure 6-2: Induction of neutralizing antibody.
- Figure 6-3: Induction of comet-reducing antibody.
- Figure 6-4: Survival and weight loss in mice immunized with A33 and L1 proteins and different adjuvants followed by intranasal VACV challenge.
- Figure 6-5: Analysis of monkey sera following immunizations with A33, B5 and L1 combined with QS-21.
- Figure 6-6: Protein immunization reduces skin lesions and circulating virus in macaques.

## List of Abbreviations

A	Adenine
$A_{\lambda}$	Absorbance at specified wavelength
Ab	Antibody
ANOVA	Analysis of variance
Asn	Asparagine
bps	Base pairs
BSA	Bovine serum albumin
C	Cytosine
°C	Degrees Celcius
CD	Cluster of differentiation
CEV	Cell-associated enveloped virus
CMI	Cell-mediated immunity
CTL	Cytotoxic T-cell
DMEM	Dulbecco's modified Eagle medium
dNTP	Deoxyribonucleotide triphosphate
DNA	Deoxyribonucleic acid
dpi	days post-infection
<i>E.coli</i>	<i>Escherichia coli</i>
ECTV	Ectromelia virus
ELISA	Enzyme linked immunosorbent assay
EM	Electron microscopy
EMEM	Earle's modified Eagle medium
EV	Enveloped virus
EEV	Extracellular enveloped virus
FDA	Food and Drug Administration
G	Guanine
GFP	Green fluorescence protein
HIV	Human immunodeficiency virus
hpi	Hours post-infection

HRP	Horseradish peroxidase
IC <sub>50</sub>	50% inhibitory concentration
IDD	Immunodominant determinant
IEV	Intracellular enveloped virus
IgG	Gamma immunoglobulin
IHD-J	International Health Department strain J of VACV
IMV	Intracellular mature virus
IN	Intranasal
IP	Intraperitoneal
ITR	Inverted terminal repetition
IV	Immature virus or intravenous
kBps	Kilobase pairs
kD	Kilodalton
LD <sub>50</sub>	50% lethal dose
MAb	Monoclonal antibody
MAC	Membrane attack complex
MEM	Modified Eagle medium
μg	Microgram
mg	Milligram
MHC	Major histocompatibility complex
MPXV	Monkeypox virus
MPL+TDM	Monophosphoryl lipid A and trehalose dicorynomycolate
mRNA	Messenger ribonucleic acid
MV	Mature Virus
MVA	Modified vaccinia Ankara
N	Asparagine
NK	Natural killer cell
nm	Nanometer
nt	Nucleotide

ORF	Open reading frame
PAb	Polyclonal antibody
PBS	Phosphate-buffered saline
pfu	plaque-forming units
PRNT	Plaque reduction neutralization test
RNA	Ribonucleic acid
RT	Room temperature
SCID	Severe combined immunodeficiency
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Standard error of the mean
T	Thymine
T <sub>CD4+</sub>	CD4-specific T-cells
T <sub>CD8+</sub>	CD8-specific T-cells
TBST	Tris-buffered saline with Tween-20
Th	T-helper cell
USAMRIID	United States Army Medical Research Institute for Infectious Diseases.
VACV	Vaccinia virus
VACV WR	Western Reserve strain of VACV
VACV Wyeth	Wyeth strain of VACV
VARV	Variola virus
VIG	Vaccinia immune globulin
VIGIV	Vaccinia immune globulin for intravenous use
WHO	World Health Organization

# Chapter 1

## General Introduction and Research Goals

The *Poxviridae* are a family of large enveloped DNA viruses that encode a diverse array of structural and nonstructural gene products and infect a wide range of hosts. Variola virus (VARV), a member of the genus *Orthopoxvirus*, is the causative agent of smallpox, a horrific disease that has shaped the course of human history, including the development of the science of vaccination. Variola virus was eradicated more than 30 years ago, and the majority of the global population is not immune to this virus. The rising specter of the use of smallpox as a biological weapon has revived interest in vaccination against variola virus. The licensed live virus vaccine against smallpox is comprised of vaccinia virus (VACV), a closely related orthopoxvirus that induces cross-protective immunity but does not cause the severe morbidity and mortality associated with VARV. Despite the successful use of this vaccine during the global eradication campaign, the numerous side effects associated with vaccination have caused growing reluctance towards its use in the general population. For this reason, next generation smallpox vaccines are being developed with the hope of creating a product that induces effective protection with fewer side effects, especially for use in immunocompromised or otherwise ineligible individuals.

The main goal of my dissertation is to study new active and passive immunization strategies for protection against orthopoxvirus infection in animal models. The specific aims of these studies include:

1. Testing the efficacy of recombinant vaccinia virus proteins, individually or in combination, as immunogens in a mouse model of respiratory infection.
2. Examining protection of mice passively immunized with polyclonal or monoclonal antibodies against individual vaccinia virus proteins with the same virus challenge model.
3. Comparing the use of different immunostimulatory adjuvants for optimization of the immune responses and protection induced by recombinant vaccinia virus protein immunization.
4. Testing protection of cynomolgous monkeys immunized with recombinant vaccinia virus proteins against a lethal intravenous monkeypox virus challenge.

## Chapter 2

### Review of Literature

#### 2.1 The Poxviridae

##### 2.1.1 Classification.

The family *Poxviridae* are enveloped DNA viruses with an ovoid or brick-shaped morphology. Particles are typically 140-260 nm in diameter and 220-450 nm in length. *Poxviridae* are divided into two subfamilies, the *Chordopoxvirinae* that infect vertebrate hosts and *Entomopoxvirinae* that infect invertebrate hosts. *Chordopoxvirinae* are classified into eight genera based on sequence similarity, and members of each genus infect common hosts and are antigenically similar. The genus *Orthopoxvirus* includes members that infect a wide variety of hosts including humans, primates and rodents. Vaccinia virus (VACV) is a member of the orthopoxviruses and has no known natural host. VACV shares genetic and antigenic similarities with other members, including variola virus (VARV), which is the cause of smallpox disease, and monkeypox (MPXV), a zoonotic infection that resembles a smallpox-like disease in humans. VACV has effectively been used as the vaccine strain in the live virus vaccine for smallpox and is commonly used in the laboratory as a tool to understand the basic virological properties of the orthopoxviruses. Members of the *Chordopoxvirinae* are the only known poxviruses that have been observed to infect humans, and VARV and molluscum contagiosum virus infections (genus *Molluscipoxvirus*) are restricted to humans.

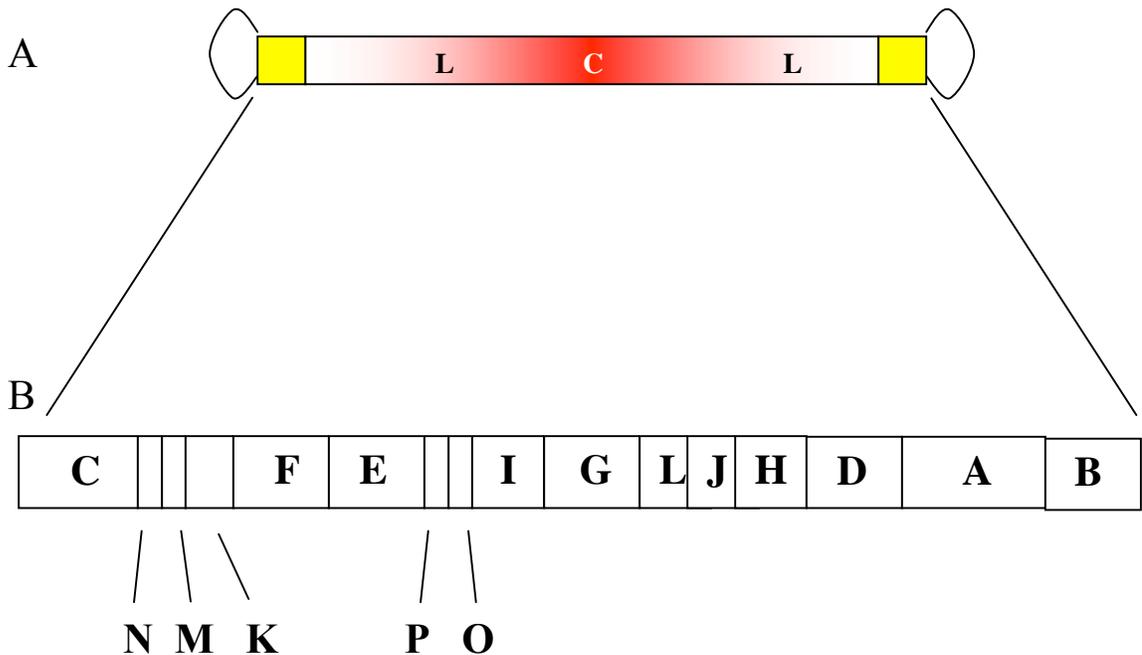
### **2.1.2 Genome organization.**

The viral core of poxviruses contains a single molecule of linear double stranded DNA (130-375 kbps) that is joined at either end by a covalently closed hairpin structure approximately 100 base pairs (bp) in length [1]. Genes that are highly conserved amongst the *Poxviridae* are located in the center of the molecule and are involved in essential functions including transcription, translation and DNA replication. Less conserved genes with host range and immune defense functions are found towards the ends of the genome. A pair of identical A+T-rich inverted terminal repetitions (ITRs) is located in opposite orientation at either end of the genome [2]. The ITRs are approximately 10,000 bps long, and each include a partially base-paired loop of approximately 100 bps that connects the two DNA strands [3]. Several open reading frames (ORFs) have also been identified within the ITRs and there is also a series of 13 to 17 tandem 70-bp repeats sequences on either side of a 435-bp intervening sequence [4]. Another feature characteristic of all ITRs is a highly conserved sequence of approximately 100 bp that is required for resolution of concatameric genomic DNA into unit length segments [5, 6]. A schematic representation of the viral genome is shown in figure 2-1A.

VACV is the most commonly studied orthopoxvirus because it is a convenient and safe alternative to more pathogenic viruses, including variola virus and monkeypox. Early studies with VACV were carried out prior to knowledge of the

**Figure 2-1. Diagrammatic representations of the Vaccinia virus (VACV) genome.**

(A) Schematic representation of double stranded DNA genome of VACV. Loops on the right and left hand sides represent covalently closed loops connecting the two DNA strands. The yellow boxes represents *inverted terminal repetitions*; **L** represents *less conserved genes* involved in host range and immunomodulatory functions; **C** represents *conserved genes* involved in transcription, translation and DNA replication. (B) Diagram of *HindIII* fragments generated by digestion of genomic DNA. Fragments are enumerated alphabetically with the largest fragment denoted by the letter A.



complete genome sequence, and the genome was defined by enumerating fragments (letters A-O, followed by number within fragment) that resulted from HindIII restriction endonuclease digestion of the genomic DNA. The largest fragment is labeled A, and smaller fragments are labeled alphabetically by decreasing size. ORFs are numbered within each fragment in order from left to right, and each gene includes an L or R letter that indicates the left or right direction of the ORF. An example of a gene name is A33R, which is the 33<sup>rd</sup> ORF in the A fragment in the right orientation. VACV has approximately 200 ORFs that have been confirmed or predicted, and other orthopoxviruses have a similar number of ORFs. A schematic diagram of the genome organization of VACV is shown in figure 2-1B. Genes will herein be described just by their letter and number (i.e., A33).

### **2.1.3 Virion Morphology and Types of Infectious Particles.**

Vaccinia virus has two types of infectious particles, intracellular mature virus (IMV) and enveloped virus (EV), which were first defined by electron microscopy (EM) and density gradient centrifugation. The intracellular mature virion (IMV) appears as a rectangular or ovoid particle surrounded by single lipid bilayer [7-9] that contains an electron-dense core with a dumbbell shape, which may be membrane-bound [10] and lateral bodies parallel to the biconcave core. IMVs are assembled within the cytoplasm of infected cells and are released upon cell lysis. Enveloped virions are IMVs surrounded by an additional lipoprotein membrane and form a similar shape as IMVs. EVs either remain associated with the plasma membrane as cell-associated enveloped virus (CEV) or are released from the cell surface as extracellular enveloped virus (EEV) [11].

#### **2.1.4 Viral Proteins.**

Orthopoxviruses have approximately 200 ORFs and encode a diverse number of proteins involved in every step of the virus life cycle [1]. Enzymes for RNA synthesis, mRNA capping and poly(A)-tail formation, DNA replication, phosphorylation and disulfide bond formation have been identified in VACV. Several immune defense molecules are also encoded by VACV, including, complement binding protein, cytokine and chemokine binding proteins, cytokine receptor homologs, protein kinase R inhibitors, caspase inhibitors and serine protease inhibitors [1, 12]. The viral core contains enzymes involved in establishing viral transcription in the cytoplasm, including the RNA polymerase, enzymes involved in mRNA capping and polyadenylation, and early transcription factors. The core also contains DNA binding proteins and proteins that may be involved in morphogenesis of immature particles into infectious mature particles [13]. VACV encodes multiple structural proteins integrated in the IMV membrane that are required for entry and fusion and virion morphogenesis. A unique complement of proteins is associated with the EV membrane and these proteins are engaged in particle morphogenesis, movement of particles to the cell surface via microtubules, and actin tail-mediated spread of EVs between cells [14].

## **2.2 Viral Replicative Cycle**

### **2.2.1 Viral Binding and Fusion.**

The *Poxviridae* are unique among DNA viruses because they replicate entirely within the cytoplasm, which is enabled by the numerous enzymes encoded by the

viral genome for transcription and replication. The first step of the virus life cycle is binding and entry into the host cell. The proteins involved in cell penetration have only recently been elucidated and the mechanism of entry is still not well defined. Our understanding of entry is also complicated by the existence of two forms of infectious virus, IMV and EV. IMV has been shown to directly fuse to the plasma membrane by EM [15, 16] and this was confirmed by biochemical studies [17, 18]. There is no evidence that the EV membrane fuses directly with the plasma membrane; rather, a recent study provides evidence that the EV membrane may be disrupted by a 'ligand-induced nonfusogenic reaction' mediated by polyanionic molecules on the plasma membrane [19]. This reaction disrupts the EV membrane and exposes the IMV membrane, thus making it available for fusion. Genetic studies with conditional lethal mutant viruses have revealed that repression of the genes A21, A28, H2, L5, or A16 causes a similar phenotype [19-24]. These viruses replicate and form normal progeny in the presence of the inducer IPTG, which drives expression by displacement of the *E.coli* lac repressor protein from the operator sequence downstream from the synthetic promoter. In the absence of IPTG, virions are able to bind to cells, but viral cores are not seen in the cytoplasm. Virions with repression of any one of these genes cannot trigger low-pH-induced cell-to-cell fusion from within or without. This unique phenotype was the first evidence of specific genes involved in poxvirus entry. Another study identified the G3, G9, and J5 proteins as possible additional members of the putative entry complex by immunoaffinity purification and mass spectrometry, although genetic studies have not yet confirmed these findings [25]. Earlier studies also showed that the IMV proteins D8, H3, and A27 bind to

glycosaminoglycans [26-28]. This interaction may be a precursor to fusion, although none of these genes are essential for virus entry. After membrane fusion is complete, the viral core is trafficked away from the cell periphery via microtubules [29].

### **2.2.2 Temporal Expression of Viral Genes and Genome Replication.**

Poxvirus gene expression occurs in three temporal stages, early, intermediate, and late, and specific promoters with unique sequence features govern the time of expression [30]. Viral cores contain a single copy of the genome and all of the proteins needed for synthesis of early viral mRNA. These proteins are synthesized late in infection and are packaged into the viral core and include the RNA polymerase, mRNA capping and polyadenylation enzymes, and early transcription factors. Roughly half of all viral genes are transcribed early [31], including the enzymes for viral DNA replication, namely, the DNA polymerase, nucleoside triphosphatase, uracil DNA glycosylase, and a DNA polymerase processivity factor. Other early proteins include viral growth factor, immune defense molecules and intermediate transcription factors. Early messenger RNA transcripts (mRNAs) are characterized by an A+T-rich consensus core promoter sequence of AAA AAA TGA AAA AA/TA and initiation occurs 12 to 17 nucleotides (nts) downstream from the promoter at an A or G [32]. Early mRNAs are unique from intermediate or late mRNAs because they also have a highly conserved termination signal sequence of TTTTNT usually found at the end of an early gene [33]. Transcript termination occurs 20 to 50 bps downstream from this signal after which transcripts are polyadenylated. Early mRNAs can be made *in vitro* inside the viral core and are

extruded via an ATP-dependent mechanism [34]. Early mRNAs are detected in vaccinia virus-infected cells at 20 minutes post-infection and peak at 100 minutes post-infection [35]. Microscopy studies have shown that early mRNAs and their translation products accumulate and form distinct granular structures at a distance from the viral core [36, 37]. These RNA structures are distinct from the site of DNA replication as well. Both early mRNAs and viral cores associate with microtubules, which may facilitate the organization of distinct subcellular compartments for translation and replication. These studies also confirmed earlier biochemical evidence that protein synthesis is required for core uncoating and subsequent DNA replication [38], and this transition is associated with termination of early transcription. Levels of early mRNAs decrease rapidly after core decondensation and this may be due to cessation of early transcription as well as the high-rate of both cellular and viral mRNA degradation during infection [35].

DNA replication occurs in discrete foci in the cytoplasm of infected cells that are called viral factories. These structures can be visualized by fluorescent microscopy through the use of fluorescent DNA labels like Hoecht's stain. One or more DNA factories are usually seen near the periphery of the cell's nucleus and may be associated with the cytoplasmic side of the ER as described above.

Orthopoxviruses encode enzymes for deoxyribonucleotide biosynthesis, which may be required to attain optimal levels of dNTP precursors for viral genome replication [1]. DNA replication begins 1 to 2 hours post-infection (hpi) through 12 hpi in cultured cells and is dependent entirely upon viral proteins including the DNA polymerase (E9), a DNA processivity factor (A20), a uracil DNA glycosylase (D4)

and a nucleoside triphosphatase (D5) [39]. Initiation of DNA replication conceivably begins with nicking of the closed double-stranded genome, most likely near one or both of hairpin termini, although an origin of replication has yet to be identified. Elongation occurs next with dNTP addition at the free 3' ends of the nicked DNA, which is followed by displacement of the template with the nascent DNA strand. Large concatamers of genomic DNA are formed during elongation and are resolved into unit-length molecules by the Holliday junction resolvase (A22) at the concatameric junctions formed at the hairpins [40, 41].

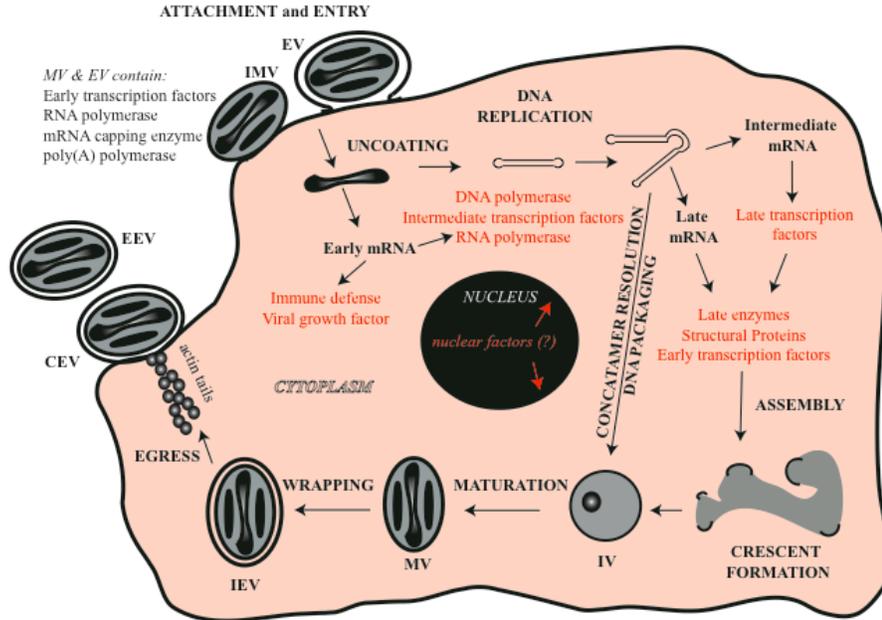
Intermediate genes are transcribed and expressed after the onset of DNA replication, usually around 100 minutes post infection. Few intermediate genes have been identified, including three late transcription factors [42]. Intermediate genes are defined by an A+T-rich sequence 14-bps upstream from a TAAA initiator element, and initiation occurs at an AAA sequence [43]. Intermediate transcription requires the viral RNA polymerase, capping enzyme, and two viral intermediate transcription factors. Intermediate transcripts cannot be synthesized *in vitro* without addition of a nuclear extract, and the mammalian proteins Ras-GTPase-activating protein SH3 domain-binding protein (G3BP) and cytoplasmic activation/proliferation-associated protein (p137) are sufficient to complement transcription and may be required for *in vivo* intermediate transcription [44, 45].

Late gene transcription commences after intermediate transcription, starting at 140 minutes post-infection and lasting until 48 hours post-infection. Late proteins include the structural proteins required for virus particle formation and components of the early transcription machinery that are packaged into the viral cores. Late

promoters are different from those of early and intermediate genes and have three defined regions: an A+T-rich sequence of about 20 bps, a 6-bp spacer region, and a TAAAT element initiation element [46]. The second T of the initiation element is usually followed by a purine, and the presence of AAA residues upstream from the translation initiation site results in formation of a poly(A) leader sequence in late gene transcripts downstream from the 5'-cap. [47, 48]. Both intermediate and late transcripts do not have defined 3'-ends and also lack poly(A) tails. Three viral late transcription factors that are expressed from intermediate transcripts are required for late transcription, in addition to a host protein, in the presence of the viral RNA polymerase and capping enzyme [49].

### **2.2.3 Maturation of Viral Particles and Viral Egress**

Virus particle formation follows late transcription, and the majority of proteins found in mature virus particles are the products of late transcripts. Several intermediate stages precede formation of the infectious mature virion as denoted in figure 2-2. The different stages of viral morphogenesis were first defined by EM studies [50]. Crescent-shaped membranes surround areas of electron-dense viroplasm and form spherical immature virions (IVs) that soon contain a single copy of the viral genome, seen as dense nucleoid matter within the IV. The source of the crescent membrane has yet to be definitively characterized. Crescents may be formed by an unidentified *de novo* mechanism [9], although recent evidence suggests that membranes may originate from the endoplasmic reticulum-Golgi intermediate compartment [51, 52]. Genetic studies have revealed a multiprotein viral



**Figure 2-2: Replicative cycle of orthopoxviruses.** A virus particle (intracellular mature virion (IMV) or enveloped virion (EV)) contains the DNA genome, replication enzymes and transcription factors. **Attachment** begins with disruption of the outer membrane of EV particles, and the exposed IMV membrane mediates fusion with a multiprotein complex. The core is released into the cytoplasm and **early mRNAs** are synthesized from the core, including genes for intermediate transcription factors, host defense and viral growth factors. **Uncoating** commences after early gene transcription, followed by **replication** of concatamers of the full-length viral genome. **Intermediate mRNAs** are transcribed, and translation of these molecules includes late transcription factors. **Late mRNAs** are transcribed and translated and include structural proteins, and the late enzymes and transcription factors that are packaged into viral particles. Discrete structures surrounded by crescent-shaped membranes are formed at the beginning of virion **assembly**. Concatameric DNA molecules are resolved into unit-length genomes and a single molecule is packaged into an immature virus particle (IV). **IMVs** are formed by further maturation steps, including proteolytic processing, and some particles are wrapped with additional membranes to form IEVs. **IEVs** are transported to the cell periphery and fuse with the plasma membrane. Particles remain associated with the cell surface as cell-associated enveloped particles (CEVs) or are propelled by actin tails to nearby cells as extracellular enveloped particles (EEVs). Bold-faced text denotes viral replication events depicted in the cartoon shown above. Figure adapted from reference 1.

complex required for IV formation, which include the proteins F10L, H5R, G5R, A14L, A17L, D13R and A11R [44, 53-62]. Proteolytic cleavage of both membrane (A17L) and core (A4L, A10L, and L4R) proteins is required for maturation of the IV into the infectious IMV particle [63-67]. Two viral proteins, I7L and G1L, have been characterized as putative proteases required for cleavage and/or IMV formation in cell culture [68, 69]. The outer membrane of mature IMVs contain several transmembrane proteins with disulfide bonds, in spite of the fact that these proteins are expressed in the cytoplasm. Typically in uninfected cells, the cellular thiol oxidation machinery resides in the highly oxidizing environment of the ER. IMV protein disulfide bond formation appears to be mediated by three viral proteins, E10, a member of the ERV1/ARL family of sulfhydryl oxidases with a characteristic CxxC motif, A2.5, which contains a similar CxxxC motif, and G4, a glutaredoxin with *in vitro* thiol transferase activity [70-74]. E10R forms a disulfide-bonded heterodimer with A2.5, followed by formation of a disulfide-bonded heterodimer between A2.5 and G4. G4 forms a similar dimer with the viral protein substrates, including L1, an essential protein that is the target of neutralizing antibodies, as well as several members of the entry-fusion complex including A16, A21, A28, and L5.

IMV particles are infectious, but are only released from the cytoplasm by cell lysis. Some IMVs are wrapped by a double membrane derived from the trans-Golgi network or early endosome to form intracellular enveloped viruses (IEVs) [75-78]. IEVs are transported to the cell surface by a microtubule-dependant mechanism, and the outermost membrane of the IEV fuses with the plasma membrane. The extracellular particles remain associated with the plasma membrane as cell-associated

extracellular viruses (CEVs) at the tips of actin-containing microvilli or are released as extracellular enveloped viruses (EEVs) [79], and both CEV and EEV particles consist of an IMV surrounded by an additional lipoprotein envelope [80]. Since CEV and EEV are identical in all respects excluding attachment to the cell, they will be collectively referred to as enveloped virus (EV).

## **2.3 Smallpox, Edward Jenner and the Dawn of Vaccination**

### **2.3.1 Smallpox and Variola Virus**

Smallpox is a disease caused by variola virus and is known only to infect humans. Variola major was the prominent strain responsible for human illness, although a milder strain, variola minor, was first described at the beginning of the 20<sup>th</sup> century [81]. Variola virus likely emerged from a rodent reservoir, which is typical of other orthopoxviruses, and may have mutated in an intermediate animal, like cattle or equids, 10,000-12,000 years ago at the time of the first agricultural revolution and the establishment of human settlements, which included the domestication of such animals [82]. Smallpox disease has been described by several ancient civilizations beginning approximately 3,000 years ago and was endemic in China, India, North Africa and Europe. Explorers colonizing the New World brought the disease which decimated native populations who had not previously been exposed to smallpox [83]. The severity of disease caused by smallpox was reduced by intentional inoculation of the skin or by inhalation with scab material or pustule fluid derived from smallpox infection. This practice of “variolation” was first seen in India 1000 years ago and soon after in China, and was later adapted in Europe [82]. This is the first time that

deliberate infection was used as a method to protect against severe disease and is evidence of the first foray into the practice of vaccination.

### **2.3.2 Edward Jenner and the Discovery of Vaccination**

Edward Jenner was an English physician and scientist living in Berkeley, Gloucestershire in the late 18<sup>th</sup> century when he published a monograph detailing use of cowpox material as a method of inoculation against smallpox [84]. The terms “vaccine” and “vaccination” from the Latin word for cow, *vacca* were coined to honor Jenner’s work. This discovery has been considered one of the most important in medicine, but the practice of vaccination with cowpox, and soon after, vaccinia, was also met with controversy. The sources of vaccine in the 18<sup>th</sup> and 19<sup>th</sup> centuries were from the arms of vaccinated humans or the flanks of vaccinated calves. This vaccine could not be stored for long periods, and its potency was somewhat variable. Immunized individuals were sometimes not completely protected against smallpox and people were reluctant to be vaccinated with material derived from a cow[81]. Long term storage and large-scale vaccine production emerged in the middle of the 20<sup>th</sup> century, at which time smallpox became practically nonexistent in North America and Europe [85].

### **2.3.3 Smallpox Eradication**

The success of modern vaccine production led to a proposal in 1958 by the World Health Organization (WHO) to carry out a global smallpox eradication program [86]. The majority of remaining smallpox outbreaks was in Sub-Saharan Africa and India, and two approaches greatly improved the success of the eradication

program. Firstly, surveillance and containment protocols were greatly improved, and a “ring” vaccination approach was adapted to contain outbreaks by rapid diagnosis of smallpox and vaccination of all contacts of newly diagnosed cases. Secondly, the use of the bifurcated needle for administration of the vaccine significantly improved the “take” rate of vaccine and enabled vaccination to be carried out in regions with limited resources and a lack of trained medical professionals [87]. The last naturally-occurring case of smallpox was diagnosed in Somalia in 1977, although a tragic infection of a laboratory worker in 1978 was the last official case of smallpox. The disease was declared eradicated by the WHO in 1980, and this success is due in part to the fact that humans are the only hosts to this virus [87]. The stunning success of eradication has led to the suspension of smallpox vaccination of the general public, and now 80% of the population is estimated to be unvaccinated [88].

### **2.3.4 Smallpox as a Biological Weapon**

The successful eradication of smallpox has left in its wake a continuing debate over whether or not to destroy the remaining stocks of variola virus. Presently, the Center for Disease Control and Prevention (CDC) in Atlanta, GA, and the State Center for the Virology and Biotechnology in Koltsovo, Russia are the only two locations presently believed to be holding these stocks. Unfortunately, since the *Bacillus anthracis* attack of October 2001 in the United States, new concern has emerged over the nefarious use of biological agents by terrorists. Smallpox is considered as a biological weapon because (1) it is easily transmitted from infected to uninfected individuals via aerosol, (2) the majority of individuals have not been vaccinated, (3) smallpox disease has a 30% mortality rate and high morbidity, (4)

most health care professionals have never seen a case of human smallpox and there would likely be a delay in initial diagnoses, and (5) no antiviral drugs have been tested for effectiveness against human smallpox [89]. The federal law called Project Bioshield was signed into law in July 2004 and facilitates the development of new strategies for protection against chemical, biological, radiological and nuclear attacks. One aim of Project Bioshield is the development of a safer second-generation smallpox vaccine—further evidence that smallpox may effectively be gone but not forgotten [90].

## **2.4 Orthopoxvirus Pathogenesis**

### **2.4.1 The Two Forms of Infectious Virus and Their Role in Disease**

As described earlier, two infectious forms of virus are formed during poxvirus infection, the IMV and EV. The discovery of these two forms was crucial to understanding the pathogenesis of poxvirus-induced infections. The different forms of virus were first defined by EM studies, but a series of animal studies demonstrated that antigenically distinct responses were generated against IMV and EV. Boulter, Appleyard and co-workers showed that rabbits were better protected against rabbitpox, another orthopoxvirus, after immunization with live VACV as compared to inactivated VACV [91-93]. Inactivated virus was produced from purified IMV, and induced an IMV-specific immune response. High levels of IMV-neutralizing antibodies were detected in antisera from inactivated virus immunization, but this antisera was unable to inhibit the formation of ‘comet-shaped’ plaques in cell monolayers that were formed by the release of EV. On the other hand, live virus vaccination resulted in an infection, and an immune response to both IMV and EV.

Antisera from live virus vaccination were able to neutralize IMV *in vitro* and inhibit comet plaque formation. These and similar studies with VACV alone [94, 95] provided the first evidence that two kinds of infectious particles existed, and immune responses were different following immunization with inactivated versus live virus vaccines. IMV is considered the primary form responsible for the spread between hosts, and EV is responsible for long range spread within a host. Payne also provided evidence from rabbit studies that higher levels of EV were released by certain VACV isolates, and this was associated with greater spread into the brain of intranasally-infected mice [96]. More importantly, this study showed for the first time that passive immunization with antisera raised against the outer EV envelope was sufficient to protect mice from a lethal VACV challenge. These studies also put to rest the likelihood that an inactivated IMV vaccine would be sufficient to replace the live virus since vaccination did not induce responses against EV.

The initial immunological characterization of two infectious forms of virus was also followed by biochemical characterization of these particles. Cesium chloride density gradient centrifugation of the two particles revealed that each had a different buoyant density; EV had a buoyant density of 1.23-1.24 g/ml, and IMV were 1.27-1.28 g/ml [93]. These buoyant density measurements agreed with EM studies showing an additional membrane around the EV [92, 97]. The EV particle was further characterized by the presence of a unique complement of polypeptides including A33 [98], A34 [99], A56 [100], B5 [101, 102], and F13 [103]. The formation of the IEV precedes the exocytosis and release of the EV during virus replication, and an additional membrane wraps the IEV as compared to EV. The IEV

membrane also contains two additional polypeptides, A36 and F12, and these proteins are involved in IEV movement towards the plasma membrane via microtubules, and, in the case of A36R, the formation of actin tails associated with CEV[104, 105]. None of the EV or IEV proteins are essential for virus replication, and viruses lacking any of these genes, excluding A56, show a similar phenotype. These deletion mutants all produce wild type levels of IMV and form small plaques. Curiously, viruses with deletions of A33 [106], A34 [107] or one of the short consensus repeats (SCR) in B5 [108-110] produced much higher levels of EV, and mice challenged with viruses containing deletions of A34, A36, B5, F12, F13, or B5 SCRs showed attenuated disease and reduced pathogenicity, in spite of the level of EV production [111]. EV produced by these deletion mutants may have abnormal outer envelopes that reduce the spread or penetration of virus *in vivo*. Another reason for attenuation is that some of these mutants produce higher levels of EEV and reduced levels of CEV may be attenuated because of reduced cell-to-cell spread of virus by CEV. The discovery of two different forms of infectious virus has been essential to understanding the pathogenesis of poxvirus disease in animal models and is central to the development of alternative smallpox vaccines.

#### **2.4.2 Primary Infection of Humans with Variola Virus**

An understanding of primary smallpox infection in humans is necessary for the development and use of appropriate animal models. Transmission of human smallpox occurs most commonly by inhalation of droplets emitted from the oral or nasopharyngeal mucosa of infected individuals. Smallpox can also be acquired from close contact to and inhalation of scab material, but this route of transmission is less

efficient [81]. An incubation period of 12 to 14 days (with a range of 7-17 days) follows during which the infected individual shows no signs of illness and does not shed any virus. Influenza-like symptoms follow the incubation period for 2 to 5 days and include high fever, malaise, and prostration with headache and backache. The fever and flu-like symptoms subside, but the characteristic macropapular rash erupts at this time, in spite of the individual feeling better. Lesions first appear on the mucus membranes of the mouth and nose, the face, hands and forearms. Lesions spread to the trunk after a few days, and this centrifugal formation of lesions is a key characteristic of smallpox disease diagnosis. The lesions in the nasal and oral cavities ulcerate several days after eruption and release large amounts of virus that can be spread to uninfected individuals by droplets dispersed from coughing or sneezing. Lesions develop from macules to papules to vesicles and finally to pustules. The pustules eventually scab 8 to 9 days after rash eruption. Scabs eventually fall off and the underlying skin heals as a pitted depigmented scar. Death usually occurs at the end of the first or the beginning of the second week of illness and is attributed to massive viremia. Two main forms of variola virus have been described, variola major and variola minor. Variola major has been typically more severe with patients bed ridden and showing higher fevers, a greater number of lesions, and more severe prostration and a mortality rate of 30%. On the other hand, the symptoms of variola minor are less severe and the mortality rate is around 1%. Hemorrhagic and malignant smallpox are two rare forms of disease. Hemorrhagic smallpox is characterized by a rash that causes hemorrhage of the mucus membranes and gastrointestinal tract and is almost always lethal. Malignant smallpox is defined by

lesions that do not progress to the papule stage and remain soft and flat and is also fatal [81, 112].

### **2.4.3 Ectromelia Virus as a Model for Viral Pathogenesis**

Mousepox, also known as ectromelia virus (ECTV), is an orthopoxvirus whose natural host is the mouse. ECTV-Hampstead was first discovered in 1930 in a laboratory mouse colony [113], and new strains were also isolated from later outbreaks in the United States and Europe, including the recently isolated ECTV strain Naval and the highly virulent ECTV strain Moscow [114, 115]. ECTV reservoirs likely exist in wild mouse populations in North America and abroad, as suggested by the emergence of ECTV Naval in laboratory mice injected with pooled sera from mice of diverse origin. The disease caused by ECTV in mice is most similar to VARV in humans, thus ECTV is a powerful tool for understanding the pathogenesis of orthopoxviruses. Mice are naturally infected through minute wounds in the skin from direct contact with infected mice or from contaminated materials (e.g., bedding), but mice can also be experimentally infected subcutaneously, intradermally, intranasally, intravenously, intracerebrally or orally [116]. The route of infection is different from VARV in humans, which enters by respiration of infectious droplets. The different entry routes also suggest that primary infection differs between ECTV and VARV since the immunological landscape differs greatly between the skin and the upper respiratory tract. Primary ECTV virus replication occurs in the dermal and epidermal skin layers and virus spreads to and replicates in nearby draining lymph nodes. Viral replication in the lymph nodes causes a primary viremia in the bloodstream, followed by replication in the liver and spleen and a

secondary viremia that leads to the eventual formation of skin lesions distal from the site of initial infection [117]. The initial entry of virus into the skin also initiates a cascade of pro-inflammatory cytokines in response to the wound. Interestingly, analysis of histological sections from primary lesions caused by ECTV inoculation show no evidence of infiltrating inflammatory cells, like macrophages and neutrophils, in spite of the presence of virus [118]. This is indirect evidence of the work of viral immunomodulators that are able to quell the typical flood of cytokines usually associated with infection. ECTV, as well as the other orthopoxviruses, expresses proteins that bind cytokines and chemokines, inhibit the complement cascade, and block apoptosis [118].

ECTV has also been used to characterize the immune responses associated with orthopoxvirus infection in a natural host. Indeed, this is one of the best models for understanding the immune responses to VARV since ECTV has coevolved with its host much like VARV in humans. Immunological studies of ECTV infection have also benefited from the use of inbred mouse strains that are resistant (C57BL/6, AKR/J) or are susceptible (BALB/c, A/J, DBA/2) to infection [119]. Interferon- $\alpha/\beta$  and interferon- $\gamma$  are required for clearance of a primary ECTV infection, and the activation of phagocytic monocytes, CD8<sup>+</sup> cytotoxic T cells (T<sub>CD8+</sub>), and NK cells is essential to controlling viral infection [120-124]. A recent study illustrated that resistant mice (C57BL/6) generate a T-helper cell type 1-polarized cytokine response, including the upregulation of IFN- $\gamma$ , TNF, and IL-2, followed by a strong T<sub>CD8+</sub> response and effective clearance of the virus. Alternatively, susceptible mice (A/J and BALB/c) generate a T-helper cell type 2-polarized response, showing an increase

in IL-4 levels, but little IFN- $\gamma$  and IL-2. These mice have a weak T<sub>CD8+</sub> response, high levels of virus replication, and succumb to infection [125]. This study is the first to show the profoundly different responses to orthopoxvirus infection in correlation to the type of T-helper cell response.

A study using CD40 knockout mice proposed that antibody and cell-mediated mechanisms are complementary to the successful clearance of ECTV infection [126]. These mice were initially able to limit virus replication, and they also had sustained ECTV-specific T<sub>CD8+</sub> cell responses [127]. In spite of a strong cell-mediated immune (CMI) response, mice eventually succumbed to mousepox. This result was most likely caused by the absence of a strong antibody response, thus hindered by a lack of T<sub>CD4+</sub> cell costimulation mediated by the interaction of CD154 and CD40.

The critical parameters for protection against a secondary response to ECTV were defined in a study that showed profound differences as compared to protection against a primary infection [128]. Neither interferons nor T<sub>CD8+</sub> are required for protection against a secondary challenge, but a potent antibody response is most important for control of infection. These findings contribute to the determination of the optimal immune responses needed for both early infection and long-term clearance of virus, and are essential to the design of an appropriate next-generation smallpox vaccine. The study of ECTV in mice has revolutionized our understanding of poxvirus immunology, but many immunological studies have also been conducted in mice with vaccinia virus.

#### **2.4.4 Vaccinia Virus as a Model for Viral Pathogenesis**

Edward Jenner used VACV in place of cowpox virus to inoculate humans against smallpox soon after the publication of his monograph on vaccination [84]. The earliest reports of complications caused by vaccination were from humans with congenital immunological defects that were immunized with VACV. These observations were the basis of our understanding of the immune response to poxviruses. Boulter observed that VACV immunization caused high morbidity or mortality in individuals with defects in both the CMI and humoral responses [91]. Individuals with a defect in the CMI response alone that generated a measurable antibody response developed a less severe disease. Progressive vaccinia is an example of an inappropriate side effect to vaccination in humans with defective CMI responses and is characterized by uncontrolled spread of the primary lesion, rapid onset of primary viremia, formation of similar secondary lesions and death [129]. These observations suggested that both arms of the adaptive immune response are needed for optimal control of infection, but the presence of anti-VACV antibodies lessened the severity of disease.

Many studies have been carried out in inbred mice with VACV to understand the nature of the immune response to both primary infection and virus challenge following immunization. Recent work by Xu and co-workers supports evidence for the importance of the humoral response [130]. Their studies used both Ab-mediated depletion of cell subsets and knockout mice to determine the essential elements for protection against an acute infection. They concluded that in the presence of a good humoral response, T<sub>CD8+</sub> cells contribute little to virus clearance. They also showed

that T<sub>CD8+</sub> cells control infection in the absence of an effective antibody response. Several recent studies have defined the essential immune response elements required for protection following immunization with both live virus vaccine and replication deficient strains of vaccinia virus (Modified vaccinia Ankara (MVA) and NYVAC). Belyakov and co-workers showed that antibody-mediated protection was most important following immunization with the live or replication deficient virus upon a lethal challenge [131]. Immunized mice that were B-cell deficient were not well protected from virus challenge, although mice depleted of T<sub>CD4+</sub> or T<sub>CD8+</sub> cells were better able to control infection. Infection was not restricted in unimmunized mice depleted of both T<sub>CD4+</sub> and T<sub>CD8+</sub> cells, but was better controlled in mice when either of these cell subsets was depleted prior to challenge. The strategies used to study the immune response in this study differed from those used by Xu and co-workers, with respect to methods by which to exclude immune cell subsets (e.g., immunization route, knockout mice, antibody-mediated depletion, and adoptive transfer), but both of these studies concluded that antibody-mediated protection is the most important arm of the adaptive immune response for control of acute infection and long-term protection. Another study with an array of MVA-immunized knockout mice showed that B-cell-deficient and  $\beta_2$ -microglobulin-deficient mice were well protected from a stringent virus challenge [132]. Protection was reduced in either T<sub>CD4+</sub> cell or MHC class II knockout mice and even more so in MHC class I and class II double knockout mice. These results indicate that T<sub>CD4+</sub> cells and MHC II are pivotal for the activation of an optimal adaptive immune response that includes both cell-mediated and humoral arms. As described above, T<sub>CD4+</sub> cell depletion immediately prior to

challenge does not reduce protection [131], but CD4 knockout mice are not protected [132]. This discrepancy can be accounted for by central role of T<sub>CD4+</sub> cells in generating the primary B cell and T<sub>CD8+</sub> cell responses rather than the effector function of T<sub>CD4+</sub> cells towards infected cells. Recently, T<sub>CD4+</sub> cells have been shown to be fundamental for promotion of an appropriate memory T<sub>CD8+</sub> cell response [133]. A study in human smallpox vaccinees supports the role of T<sub>CD4+</sub> cells such that individuals with detectable VACV-specific T<sub>CD4+</sub> cells (observed many years after primary vaccination) showed rapid and robust expansion of T<sub>CD4+</sub> and T<sub>CD8+</sub> cells after a second vaccination [134].

T<sub>CD4+</sub> cell-dependent antibody responses are of great importance for control of primary and secondary poxvirus infections, but studies with both ECTV and VACV also define a role for T<sub>CD8+</sub> cells for effective virus clearance. To this end, human and murine T<sub>CD8+</sub> cell peptide determinants have recently been defined [135-139]. A study carried out by Tschärke and co-workers showed that the hierarchy of T<sub>CD8+</sub> cell responses to 5 different peptide determinants varied based on the route of infection and the virus strain, but the majority of stimulated T cells were specific to the immunodominant determinant (IDD) from the B8 gene of VACV [139]. These peptide determinants were conserved among orthopoxviruses, but the B8 peptide was conserved in a fragment of a gene in MVA, suggesting that methods used for discovery of immunogenic peptides within ORFs may overlook other determinants. Interestingly, mice immunized with the B8 peptide were partially protected from ECTV, thus the IDD is not only cross-reactive, but also cross-protective. This discovery of a diverse array of peptide determinants restricted to MHC class I

molecules reveals the diverse T<sub>CD8+</sub> cell responses generated within a population and indicates that attenuated vaccine strains with deletions in a number of ORFs may be less effective at inducing a protective T<sub>CD8+</sub> response.

#### **2.4.5 The Complement Cascade and its Role During VACV Infection**

The complement system is collection of over 30 proteins that participate in both innate and antibody-mediated mechanisms targeting infected cells, bacteria and viruses. Complement was first characterized many years ago as a heat-labile fraction in plasma that promoted antibody-mediated killing of bacteria [140]. Complement is also a part of the innate immune response and does not require antibody for this activity. Complement proteins participate in opsonization, which aids in phagocytosis of bacteria or infected cells, neutralization of virus particles, stimulation of the inflammatory response, and clearance of antigen-antibody complexes. Activation of complement occurs through a “triggered-enzyme cascade” in which a pro-enzyme or zymogen proteolytically cleaves itself and then cleaves its substrate. Many of the complement proteins are found in the blood and tissues but do not cause inflammation until specifically activated by self-cleavage in the presence of antibody or bacteria. An advantage of a triggered-enzyme cascade is that relatively few molecules are involved in initiating the cascade, but a huge effector response is generated.

Three different pathways can initiate complement activation, and these pathways eventually converge to form an effector complex. The classical complement pathway involves both the innate and adaptive immune responses and is initiated by direct binding of C1q to the cell surface or to antigen-antibody complexes

and subsequent formation of the C1q<sub>r</sub>2s<sub>2</sub> complement protein complex. The alternative pathway is part of the innate immune response and typically targets bacteria. Spontaneously cleaved C3 protein binds to the surface of cell membranes or bacteria to initiate the alternative pathway, although further damage to host cells is blocked by complement regulatory proteins. The mannan-binding lectin pathway is the third route of complement initiation and is activated by the serum protein complex, mannan-binding lectin, binding to mannose residues from carbohydrates on the surface of bacteria or viruses. A series of complement proteins are cleaved in each pathway that all lead to deposition of proteins in the target membrane. These proteins form the membrane attack complex (MAC) and are comprised of the C5b, C6, C7, C8 and C9. Initially, C5b, C6 and C7 come together as a hydrophobic complex that binds to the membrane and a small pore is formed by C8 interacting with this complex. C9 interacts with the C5b678 complex and expands the pore, thus allowing it to function as an ionic channel that destroys the cell or bacteria by osmotic lysis [141].

The lysis mechanism described above has typically been observed in infected cells or bacteria, but the mechanism of complement-mediated lysis of viral particles may be different. Lesions on the viral membrane of avian infectious bronchitis virus were observed in the presence of complement by electron microscopy [142]. Lysis mediated by pore formation was hypothesized as the mechanism by which complement destroyed viral particles, but studies with the pore forming drug nystatin did not cause lysis of rat leukemia virus [143]. Complement, presumed to be the MAC, lysed these viral particles and reduced infectivity, and the drug melittin also

reduced infectivity. The results of this study suggested that while pore formation was not required for lysis, complement proteins may cause lysis by destabilizing the viral membrane. Human immunodeficiency virus (HIV) is also lysed by complement and is dependent on the classical pathway and formation of the MAC [144]. Many studies have been conducted with HIV and complement as well as both HIV-specific and non-specific antibodies, but the mechanism of lysis and how it relates to prototypical MAC-driven membrane disruption remains elusive [145, 146].

Complement is a highly effective first line of defense against invading pathogens and also augments the effectiveness of antibodies. Viruses have evolved with several different strategies to elude or quell complement activity. Orthopoxviruses, specifically VACV, express a regulator of complement activity called the VACV complement control protein (VCP) [147, 148]. VCP inhibits both the classical and alternative pathways by interacting with C4b and C3b, respectively [149]. Deletion of VCP from VACV attenuates infection in both guinea pig and rabbit models, suggesting that complement can indeed limit infection [149]. EV particles have also been described as innately resistant to complement in the absence of EV-specific antibodies and this has been attributed to the presence of regulators of complement activity derived from the host cell and present in the EV membrane [150]. The strategies used by VACV do not completely extinguish complement activity and leave open the possibility of other complement-dependent mechanisms to inhibit infection, including those involving antibody.

## **2.5 Past, Present and Futures Immunization Strategies Against Smallpox**

### **2.5.1 Dryvax Vaccination of Humans and its Side Effects**

The present method for vaccinating humans against smallpox has changed little since the time of Edward Jenner. Vaccinia virus is the only approved and licensed vaccine strain and was successfully administered during the global eradication of smallpox. Several different seed strains have been used as sources for vaccine production, including the Lister strain, which originated in England and was used by the WHO for its eradication program, and the New York City Board of Health (Dryvax) strain, which was produced by Wyeth Laboratories and is presently in use in the United States [81]. VACV designated for vaccine use was grown on the skin of calves by dermal scarification and the resulting lesions were harvested by scraping. The virus was purified from the skin lesions using fluorocarbon and differential centrifugation and lyophilized for indefinite storage at  $-20^{\circ}\text{C}$  [81, 151]. Small amounts of antibiotics were added during vaccine production to reduce the level of contamination typically associated with virus grown in this manner. Virus is reconstituted for inoculation in a diluent comprised of 50% glycerin, and 0.25% phenol in sterile water [152]. A bifurcated needle is dipped into the reconstituted inoculum and holds approximately 0.0025ml (equal to  $2.5 \times 10^5$  pfu), and the vaccine is administered percutaneously on the arm over the insertion of the deltoid muscle by a rapid multiple-puncture technique. A “robust take” is defined as formation of a papule 2 to 5 days after vaccination, which progresses to a vesicle and then an umbilicated, indurated pustule and accompanied by an area of erythema 8 to 12 days post vaccination. Regional lymphadenopathy, fever, and malaise typically manifest

at this point and subside as the pustule forms a scab [152]. A single dose of smallpox vaccine is sufficient for vaccination of the general population, although it is recommended that laboratory workers who are regularly exposed to orthopoxviruses be revaccinated every ten years. As described earlier, several adverse reactions are associated with this live vaccine and a growing segment of the population is contraindicated for vaccination because of health conditions including eczema, a diagnosed heart condition, or immunocompromised status from HIV infection, organ transplantation, or cancer treatment [153]. The recent smallpox vaccine campaign in 2002 and 2003 targeting health care workers was largely unsuccessful because of individuals fearing unforeseen side effects and adverse reactions caused by vaccination against a disease that exists only as a threat [154]. The federal government has set aside funding for the research and development of safer smallpox vaccines, in part, due to the failure of this vaccine campaign.

### **2.5.2 Attenuated Poxviruses as Vaccines**

A need for safer smallpox vaccines has been apparent since the global eradication effort in the 1960's. Serial passage of VACV was used as a strategy to make attenuated strains for vaccines. One such strain is modified vaccinia Ankara (MVA) that was attenuated by >570 passages in chick embryo fibroblasts. The genome of MVA is 15% smaller than its parental strain and has several deletions in genes required for host range and evasion of the host immune response [155-157]. Its replication is markedly diminished or inhibited in most mammalian cell lines, which is due to a blockage late in virus assembly caused by several different deletions or mutations [158-160]. Nonetheless, MVA has been tested in animal models, including

immune-suppressed non-human primates [161, 162], as well as in humans without causing any adverse effects [163, 164]. MVA was intended for use as an attenuated smallpox vaccine in non-endemic regions and was used to vaccinate >100,000 individuals in Germany and Turkey, but its efficacy was never tested during a smallpox outbreak [164].

Interest has re-emerged in MVA as a smallpox vaccine in light of concern over the use of smallpox as a biological weapon [165]. The development of new vaccines is hampered by the fact that they cannot be field-tested against smallpox. Therefore, the FDA has put forth a policy to approve new vaccines and antiviral drugs against smallpox based on animal efficacy studies [166]. In light of this policy, murine and primate models have recently been employed to better understand immune responses to MVA and to compare MVA with Dryvax. A mouse study by Wyatt and co-workers showed a dose-dependent response to MVA vaccination with respect to induction of T<sub>CD8+</sub> cell and antibody responses [132]. Mice immunized with a single dose of MVA were protected equally as well as mice immunized with Dryvax following a lethal respiratory challenge. Studies in non-human primates have also shown significant protection against lethal intravenous or respiratory monkeypox challenge following immunization with two doses of MVA [167, 168]. These results lend support to the use of MVA as an alternative vaccine in immunocompromised individuals. MVA could also be administered as a primary vaccine to be followed by Dryvax in order to reduce the side effects of Dryvax vaccination in otherwise healthy individuals.

### 2.5.3 DNA and Protein Subunit Vaccines

An important goal in the development of a new vaccine is to create a product that is efficacious and safe. The next generation smallpox vaccines must be as effective as Dryvax but with fewer negative side effects, and one strategy is the development of a subunit vaccine. Subunit vaccines are comprised of immunogenic components of the pathogen, such that immunization generates an appropriate and lasting immunological response should the host be infected. Subunit vaccines are comprised of recombinant DNA or proteins, and multiple antigens can be combined for induction of a broad immune response. Characterization of the immune responses that are protective against orthopoxvirus infection has been described above. Optimal protection against disease is associated with immune responses against both types of infectious particles, the IMV and EV. This knowledge has led to the design of subunit vaccines targeting responses to these particles. Selection of appropriate immunogens has been in part driven by the discovery of the targets of neutralizing antibodies. *In vitro* detection of IMV-neutralizing antibodies is straightforward, but the delicate nature of the outer EV envelope has made *in vitro* EV neutralization assays less reliable [169-171]. Several individual recombinant proteins have been tested as immunogens, including the IMV protein A27, which is a known target of neutralizing antibodies and was shown to protect mice from a lethal intraperitoneal vaccinia virus challenge [172, 173]. The immune responses to recombinant EV proteins was investigated by Galmiche and co-workers, and they observed that mice immunized with B5 produced antisera that neutralized EV *in vitro*, and these mice were protected against a lethal respiratory challenge with vaccinia virus [174].

Surprisingly, mice immunized with A33, another EV protein, were protected against a lethal challenge but did not have any detectable EV-neutralizing antibodies detected by *in vitro* assay (refer to page 40 for additional information).

Hooper and co-workers studied the immunogenicity of and protection induced by a multi-subunit DNA vaccine in both mouse and monkey lethal challenge models [175-177]. They included the IMV genes A27 and L1, both known targets of neutralizing antibodies [172, 178, 179], and the EV proteins A33 and B5. Mice and monkeys immunized with all four genes generated specific antibodies to these targets. Antisera from immunized animals was also able neutralize IMV, presumably targeting both A27 and L1. Anti-EV antibodies were also detected *in vitro* by the comet reduction assay, as shown by a reduction in the formation of satellite plaques in infected cell monolayers. Mice and monkeys were best protected when immunized with both IMV and EV genes, and diminished protection was observed in animals given IMV or EV genes alone.

#### **2.5.4 Vaccinia Immune Globulin and Passive Antibody Therapy**

Vaccinia immune globulin (VIG) was developed as a therapeutic drug nearly 60 years ago for the treatment of adverse reactions from vaccination or the early stages of smallpox. Dr. Henry Kempe was the first to suggest the use of VIG, upon observation that vaccinated infants with poor “takes” had high levels of circulating maternal antibodies [180]. Kempe’s efforts to develop VIG for the treatment of individuals with complications following vaccination was based on the belief that these problems were related to a deficient or absent IgG response. Unfortunately, the early use of VIG was not through a placebo-control clinical trial, but a retrospective

review of the clinical efficacy of VIG indicates that it is effective for the reduction of mortality and morbidity associated with progressive vaccinia and eczema vaccinatum, which are otherwise lethal complications [181]. Presently, indications for VIG therapy include, aberrant infections caused by VACV, including inoculation of the eye or mouth, eczema vaccinatum, progressive vaccinia, severe generalized vaccinia, and VACV infections in individuals with pre-existing skin lesions (due to burns, eczema, impetigo, poison ivy or varicella-zoster virus) [182].

VIG has also been recommended as a therapy for smallpox infection and as a prophylactic treatment of contacts of smallpox-infected individuals. These recommendations are based on both anecdotal evidence showing reduced severity of disease in infected individuals and accelerated responses in vaccines following coadministration of VIG [183]. The potential value of VIG as a therapeutic drug in light of a biological attack or as a tool for managing complications caused by vaccination has led to the renewed production of this product as an intravenous preparation [184].

Regrettably, VIG is derived from the serum of vaccinated humans and the production and use of large quantities of this human-derived blood product is both difficult and inherently risky. As a result, alternative VIG products are being developed that include antibodies targeting specific antigens of the IMV and EV. Several groups have studied the specificity of antibodies to VACV in VIG and have defined the both IMV and EV antigens [185, 186]. Alternatively, passive transfer studies have shown partial protection of mice with antibodies or antisera against the IMV proteins A27 or H3 [28, 173] and EV proteins A33 and B5 [174, 187].

Chimeric chimpanzee/human mAbs against B5 have also been able to protect mice against a lethal respiratory challenge [188]. A defined VIG product comprised of MAbs will be a nearly unlimited source of product that will hopefully be both safer and equivalently therapeutic as the present VIG.

## Chapter 3

### **Protective Vaccination of Mice with Recombinant Outer Membrane Proteins from the Extracellular and Intracellular Forms of Vaccinia Virus**

#### **3.1 Introduction**

Growing interest surrounds the development of an alternative smallpox vaccine in light of concern over the use of smallpox as a biological weapon. The licensed live vaccinia virus vaccine provided sufficient protection against variola virus, the causative agent of smallpox, and was successfully used to eradicate this disease [87]. The use of this vaccine has been met with some resistance during the recent vaccine campaign in 2002-2003 because of the considerable range of side effects associated with vaccination at a time of smallpox existing only as a threat rather than an epidemic [154]. A well-accepted alternative vaccine would be able to provide sufficient immunity without the adverse events associated with live virus vaccination, including generalized vaccinia, progressive vaccinia, eczema vaccinatum, encephalitis, or heart attack [189]. The absence of endemic smallpox makes it difficult to field test a new vaccine, but the expanding body of knowledge regarding poxvirus replication and immunology aid in the design of new kinds of vaccines, including attenuated viruses, recombinant DNA or proteins [167, 174, 177].

Orthopoxvirus replication produces two infectious forms of virus, the IMV and EV. The majority of infectious particles produced during infection are IMVs,

which are highly stable membrane-bound virions found in the cytoplasm and only released by cell lysis, and are considered responsible for spread between hosts. EVs are formed by wrapping of IMVs by a double membrane followed by exocytosis and fusion of the outermost membrane with the plasma membrane [111]. Thus the EV particle is an IMV particle enveloped by one additional membrane. EVs remain cell-associated and mediate cell-to-cell spread or are released from the cell surface and disseminate throughout a host [96, 190].

Studies in animal models show that superior protection against a lethal challenge is associated with immune responses to both IMV and EV. Live virus vaccination induces antibody responses against both types of particles and provide better protection against a challenge compared to inactivated virus vaccination which only induces anti-IMV immunity [94, 95]. The outstanding protection associated with immunity to IMV and EV may be due to the fact that these two kinds of particles have unique outer membranes and may bind to and enter cells by different mechanisms [191]. IMV entry is mediated through direct fusion of the outer viral membrane with the plasma membrane [17, 18, 25], and this mechanism is dependant on a multi-protein complex of integral and peripheral proteins associated with IMV membrane [20-25]. On the other hand, the outer membrane of EVs is disrupted by interactions with polyanionic molecules on the plasma membrane surface, consequently exposing the IMV membrane and enabling IMV-mediated fusion [192].

Orthopoxvirus infection induces antibodies (Abs) capable of *in vitro* neutralization, and some of the proteins with neutralizing epitopes have been identified, including A27, D8, H3, and L1 [28, 172, 178, 179, 193]. Of these

proteins, mice immunized with an *E. coli*-expressed recombinant A27 formed neutralizing antibodies and were partially protected against a lethal intraperitoneal challenge [173]. Similarly, a monoclonal antibody against A27 provided passive protection when administered before or after a lethal challenge [173]. The identification of EV proteins important for protection has been hindered by the lack of a reliable *in vitro* EV-neutralization assay [169, 194, 195]. Interestingly, mice immunized with recombinant forms of the EV proteins A33 or B5 were protected from a lethal intranasal challenge, but only antiserum from B5-immunized mice neutralized EV *in vitro* [174]. These results suggest that EV-specific antibodies that are protective *in vivo* do not necessarily neutralize virus *in vitro*. These studies also demonstrate that recombinant proteins can induce a protective immune response in a mouse model. Hooper and co-workers carried out similar studies in mice immunized with recombinant DNA plasmids encoding the IMV genes A27 and L1 and EV genes A33 and B5 [175, 176]. IMV-neutralizing antibodies were detected in sera of mice immunized with plasmids expressing IMV proteins. Antibodies able to inhibit the spread of EV *in vitro*, as measured by the comet inhibition assay, were detected in mice immunized with A33 and B5. Most importantly, mice immunized with plasmids that induced expression of a combination of IMV and EV proteins were better protected from a lethal challenge than mice immunized with individual plasmids. The results of these DNA immunization studies reiterate the importance of generating immune responses against both EV and IMV, and this observation should continue to guide the design of future smallpox vaccines.

We have expressed recombinant forms of two EV proteins, A33 and B5 and the IMV protein L1 using the baculovirus expression system. A33 is a 23-38 kDa type II membrane protein that forms homodimers and also associates with A36R, another EV-associated membrane protein [196-198]. A33 is expressed at both early and late times during infection and includes several post-translational modifications, including N- and O-linked glycosylation [196, 197], phosphorylation of serine and threonine residues [198] and acylation [196, 199]. A33 is not required for virus replication, as shown by construction of an A33 deletion mutant virus that formed infectious IMV and EV, but showed a small plaque phenotype in tissue culture, indicative of a role for A33 in cell-to-cell spread [200].

B5 is another non-essential EV protein expressed at early and late times and is expressed as a 42 kDa membrane-bound monomer with a type I topology or as a 35 kDa secreted protein [201-203]. A deletion mutant virus lacking B5 forms small plaques in tissue culture, and produces significantly less EV, unlike the A33 deletion mutant [203-206]. The B5 deletion mutant is significantly attenuated *in vivo* based on studies with intranasally-infected mice [205]. B5 also has several post-translational modifications, including N-glycosylation [196, 201, 202] and acylation [196, 199] and associates with the EV protein F13L during IMV wrapping [196], and with A33 and A34R to mediate extracellular release of cell-associated EV [198]. Interestingly, B5 contains four domains with sequence similarity to the short consensus repeats (SCRs) of complement control proteins [201].

L1 is a myristylated IMV protein expressed late in infection [178, 207-209]. L1 is an essential viral protein and is required for virion morphogenesis [210]. L1 has

three disulfide bonds that are formed in the cytoplasm of infected cells by a virally expressed redox system [73]. L1 is the target of neutralizing antibodies, and as such, it has been suggested that it may be involved in binding to target cells, although it has not been found in the multiprotein fusion complex [25, 178, 179].

The study described in this chapter investigates the protective immunity induced by immunization of mice with recombinant forms of A33, B5, and L1. The goals of this study include characterization of the antibody responses following immunization and determination of the protection provided by immunization with these proteins individually or in various combinations. The hypothesis guiding the design of this study is that immunization with proteins of both the IMV and EV provides superior protection from a lethal challenge than immunization with solely IMV or EV proteins. This study has been previously described in reference 222.

## **3.2 Materials and Methods**

### **3.2.1 Cells and Viruses**

BS-C-1 (ATCC CCL-26) monolayer cells were maintained in Earle's modified Eagle medium (EMEM) (Quality Biologicals, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), 10 U/ml penicillin (Invitrogen), and 10 µg/ml streptomycin (Invitrogen) at 37°C and 5% CO<sub>2</sub>. HeLa S3 (ATCC CCL-2.2) were maintained in suspension at 37°C in modified Eagle medium for spinner cells (Quality Biologicals) supplemented with 5% heat-inactivated equine serum (Hyclone), 2 mM L-glutamine, 10 U/ml penicillin and 10 µg/ml streptomycin. Serum

was heat-inactivated by incubation at 56°C for 30 minutes to destroy complement activity.

The vaccinia virus (VACV) strains used were Western Reserve (VACV-WR) (ATCC VR-1354), International Health Department-J (IHD-J) (from S. Dales, Rockefeller University), recombinant VV-WR-NP-SIINFEKL-EGFP [211, 212], and Wyeth smallpox vaccine seed (Wyeth Ayerst Laboratories, Marietta, PA). Viral stocks were grown in HeLa S3 suspension cells and purified by sucrose gradient centrifugation [213]. Viral titers were determined by plaque assay in confluent BS-C-1 monolayers grown in six-well cluster plates. Briefly, 10-fold serial dilutions of viral stocks were prepared in EMEM supplemented with 2.5% heat-inactivated fetal bovine serum, L-glutamine, penicillin, and streptomycin (infection medium). Cells were washed with infection medium, overlaid with 0.5 ml of serially-diluted virus inoculum and incubated for two hours at 37°C and 5% CO<sub>2</sub>, after which virus inoculum was removed and cells were washed once again. Cells were overlaid with semi-solid medium (EMEM with 0.5% methylcellulose) and incubated for 48 hours, and resultant plaques were visualized by staining monolayers with a solution of 0.1% crystal violet (w/v) and 20% ethanol in deionized water.

### **3.2.2 Recombinant Proteins**

Recombinant proteins were prepared by collaborators in the laboratory Drs. Gary Cohen and Roselyn Eisenberg at the University of Pennsylvania, Philadelphia, PA. Recombinant forms of A33, B5, and L1 were expressed using recombinant baculoviruses as described by Aldaz-Carroll et al [214]. Secreted and soluble forms of each protein were made by polymerase chain reaction (PCR) amplification of each

ORF excluding their predicted transmembrane domains. Each PCR product was cloned into a baculovirus transfer vector (pVT-Bac) to allow for recombination into the baculovirus genome at the polyhedrin locus. Cloned PCR products were inserted in frame into pVT-Bac downstream from the mellitin signal sequence in order to target recombinant proteins into the secretory pathway of baculovirus-infected insect cells. A six-histidine residue tag was included at the NH<sub>2</sub>-terminus of B5 and the COOH-termini of A33 and L1 for purification of secreted protein by nickel-nitrilotriacetic acid affinity chromatography. Protein concentrations were measured with the bichinchoninic acid assay (BCA, Pierce, Rockford, IL) using bovine serum albumin as a standard. These recombinant proteins were used for both immunization and enzyme-linked immunosorbent assays.

### **3.2.3 SDS-PAGE and Western Blot Analysis**

Protein purity was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein samples were diluted in NuPage LDS sample buffer (Invitrogen) and heated for 10 minutes at 70°C. Samples were resolved by electrophoresis through 12% NuPage gel with 3-(N-morpholino)propanesulfonic acid, 4-morpholinepropanesulfonic acid running buffer (Invitrogen). Proteins were visualized directly in gels fixed with 50% methanol and 10% acetic acid in deionized water and stained with GelCode Blue (Pierce), a colloidal Coomassie stain. Western blotting was carried out by transferring proteins to NitroPure supported nitrocellulose (Osmonics, Westborough, MA) followed by overnight blocking at 4°C with 10% milk in tris-buffered saline with 0.1% Tween-20 (TBST). Membranes were probed with specific monoclonal or polyclonal primary antibodies against A33, B5 or L1 for

1 hour at room temperature (RT). Membranes were washed with TBST and probed with secondary horseradish peroxidase-conjugated (HRP) anti-rabbit (polyclonal Abs), anti-mouse (against A33 and L1 MAbs) (Amersham Biosciences) or anti-rat (against B5 MAb) (Jackson Laboratories) for 1 hour at RT. Following several washes with TBST, bands were visualized by chemiluminescence (Pierce).

### **3.2.4 PNGase F Treatment of Recombinant Proteins**

Protein samples were denatured and reduced with 0.5% SDS and 1%  $\beta$ -ME, respectively, at 100°C for 10 minutes. Samples were then diluted in 50 mM sodium phosphate pH 7.5 and 1% NP-40 in the absence or presence of 1500 units of PNGase F (New England Biolabs, Beverly, MA) and incubated for 1 hour at 37°C. Samples were analyzed by SDS-PAGE and western blotting with an anti-tetra-His antibody (Qiagen). Anti-mouse-HRP was used as a secondary antibody followed by visualization with chemiluminescence.

### **3.2.5 Mouse Immunization Protocol**

5- to 6-week old female BALB/c mice were purchased from Taconic (Germantown, NY) and housed at the National Institutes of Health in sterile microisolators. Recombinant proteins were diluted in a stable emulsion of phosphate buffered saline (PBS) with a Ribi adjuvant system comprised of monophosphoryl-lipid A and trehalose dicorynomycolate (MPL+TDM; Sigma-Aldrich, St. Louis, MO). Alternatively, proteins were diluted in PBS with the saponin adjuvant QS-21 (Antigenics, Inc., New York, NY). Proteins were diluted such that mice were immunized 10  $\mu$ g of each protein in a total injection volume of 0.1 ml. MPL+TDM

adjuvant was freshly prepared prior to each immunization according to manufacturer's instructions. QS-21 aliquots (2 mg/ml in sterile water) were thawed from storage at -20°C and were diluted to a final injection concentration of 15 µg in 0.1 ml. Protein immunizations were administered subcutaneously at the nape of the neck with a 25-gauge hypodermic needle. Purified VACV strain Wyeth was used for live virus vaccination by tail scarification. Aliquots of purified Wyeth diluted to 10<sup>9</sup> plaque forming units (pfu) in PBS were thawed from storage at -80°C and briefly sonicated to disrupt virus aggregates. 10 µl of virus was pipetted at the base of the tail and introduced into the skin by a series of 25-30 scratches with a 25-gauge needle at the site of the inoculum. Pustules or scabs were observed at the site of inoculation 3 to 4 days after scarification and this was characterized as a "robust response." Tail vein bleeds were performed one day prior to each immunization and serum was separated from clotted blood samples following centrifugation. Serum pools were prepared from groups of mice immunized with like immunogens and these samples were heat-inactivated at 56°C for 30 minutes.

### **3.2.6 Vaccinia Virus-Infected Cell Lysates**

An infected cell lysate was prepared for use in antibody binding assays. HeLa S3 cell monolayers were prepared in T-150 flasks and infected with VACV-WR at a multiplicity of 2. Infected cells were harvested 72 hours post infection and pelleted at 208 x g for 5 minutes. Cell pellets were resuspended in 10 mM Tris-HCl pH 8.0, homogenized with a Dounce homogenizer and centrifuged for 5 minutes at 208 x g to remove nuclei. Supernatants were decanted and pelleted by high-speed centrifugation

at 112,845 x g for 30 minutes. Lysate pellets were resuspended in 10 mM Tris-HCl pH 8.0, aliquoted and stored at -80°C.

### **3.2.7 Enzyme-Linked Immunosorbent Assay (ELISA)**

ELISAs were used to measure antibody binding to VACV and recombinant protein antigens. 96-well round bottom polystyrene cluster plates (Corning, Inc., Acton, MA) were coated with 0.1 ml/well of recombinant protein antigens diluted in PBS by overnight incubation at 4°C. Optimal concentrations of A33, B5, and L1 were determined to be 90 ng, 100 ng and 40 ng per well, respectively. VACV-infected cell lysate was diluted 1:500 in Universal Coating Buffer (Immunochemistry Technologies, Bloomington, MN) for coating plates with 0.1 ml/well overnight at 37°C. VACV-infected cell lysate-coated plates were fixed for 10 minutes at RT with 2% paraformaldehyde in PBS following overnight incubation, after which both lysate and protein coated plates were washed in a solution of 27 g NaCl and 3 ml Tween-20 in 60 ml of deionized water (ELISA wash). Plates were incubated with 0.2 ml/well blocking buffer (5% nonfat dry milk with 0.2% Tween-20 in PBS) for 1 hour at 37°C followed by washing with ELISA wash. Serial twofold dilutions of mouse sera were made in duplicate in plates using blocking buffer as a diluent, followed by incubation at 37°C for 1 hour. Plates were washed and mouse antibodies were detected by addition of 0.1 ml/well of HRP-conjugated anti-mouse IgG ( $\gamma$ -chain) (Roche Diagnostics GmbH, Mannheim, Germany) diluted 1:5000 in PBS/0.2% Tween-20 and incubation for 1 hour at 37°C. Isotype-specific ELISAs were carried out with HRP-conjugated anti-mouse IgG1 or IgG2a secondary antibodies (BD Pharmingen, San Diego, CA) diluted 1:1000 in PBS/0.2% Tween-20. Plates were washed again

and incubated with 0.1 mL/well of a ready-to-use solution of 3,3', 5,5'-tetramethylbenzidine (BM Blue, POD substrate; Roche) for 30 minutes at RT. A Spectra MAX Plus plate reader (Molecular Devices, Sunnyvale, CA) was used to measure absorbance at  $A_{370}$  and  $A_{492}$ , and endpoint titers were determined to be the serum dilution with an absorbance value more than two standard deviations greater than absorbance measured in wells with no mouse sera.

### **3.2.8 IMV Neutralization and Comet Reduction Assays**

IMV neutralization was determined using a flow cytometry-based assay described in detail elsewhere [215]. Briefly, twofold serial dilutions of mouse sera were made a 96-well cluster plate in MEM spinner/2% FBS and mixed with a recombinant vaccinia virus expressing green fluorescent protein (VV-WR-NP-SIINFEKL-EGFP) and incubated for 1 hour at 37°C. HeLa S3 suspension cells were then added to the antibody-virus mixture in the presence of cytosine arabinoside (to inhibit virus replication) and cells were incubated 16 to 18 hours at 37°C. Single color flow cytometry was used to determine the percentage of infected cells in the absence and presence of serially diluted mouse sera, and the 50% inhibitory concentration for each sera sample was calculated from these measurements.

The comet reduction assay was used to observe reduction of the size and shape of comet-shaped plaques in the presence of EV-specific antibodies. BS-C-1 cells were grown to confluency in 12-well cluster plates (Corning) and were infected for 2 hours at at 37°C with 40 plaques/well of VACV strain IHD-J diluted in infection medium. The virus inoculum was subsequently removed and cells were washed with infection medium and treated with mouse sera diluted to different concentrations in

the same medium. The plates were incubated for 48 hours at 37°C and stained with crystal violet.

### **3.2.9 Intranasal Vaccinia Virus Challenge of Mice**

An aliquot of purified VACV-WR was thawed and sonicated immediately prior to challenge. The virus was diluted in sterile PBS to a final challenge concentration of  $10^6$  pfu or  $2 \times 10^7$  pfu per 20  $\mu$ l. These virus doses correspond to 5 and 100 times the 50% lethal dose ( $LD_{50}$ ), respectively in mice of a similar age. Mice were sedated with inhaled isoflurane and inoculated intranasally with 20  $\mu$ l of virus (approximately 10  $\mu$ l/nostril). Daily weights were measured as weight loss is an indirect sign of vaccinia-induced disease and mice were euthanized if their weight loss was greater than 30% of their initial weight. Mice were bled from the tail vein one day prior to challenge for serological analysis. All mouse experiments were carried out in accordance to protocols approved by the Animal Care and Use Committee (ACUC) of the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD.

### **3.2.10 Statistical analysis**

The StatView statistical software package (SAS Institute, Inc., Cary, NC) was used for analysis of mouse weight data. Multiple pairwise comparisons were made to determine the effect of different kinds of immunizations on weight loss following virus challenge using analysis of variance (ANOVA). ANOVA showed that some differences between groups were significant, thus a *post hoc* test, The Fisher's

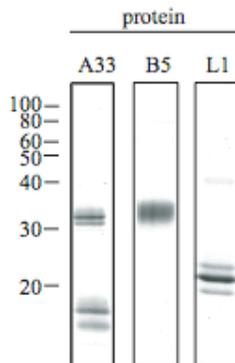
protected least significance difference test, was used to quantitate the differences between groups. Significance levels were set at a *p*-value of 0.05.

### **3.3 Results**

#### **3.3.1 Biochemical Analysis of Recombinant Vaccinia Virus Proteins**

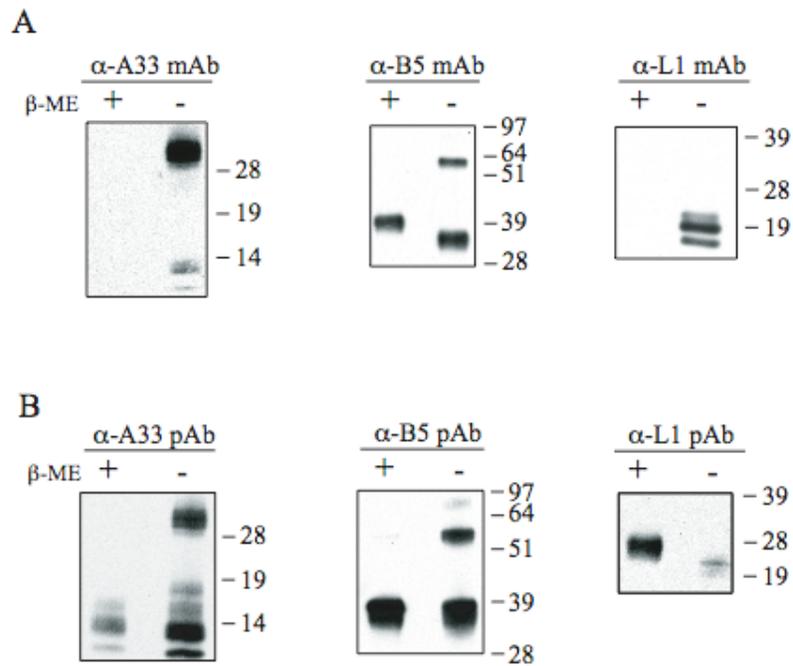
Recombinant forms of the VACV EV proteins A33 and B5 and the IMV protein L1 were expressed as secreted, polyhistidine-tagged forms in insect cells infected with recombinant baculoviruses. These proteins were purified from supernatants by nickel-nitrilotriacetic acid affinity chromatography. This work was carried out by collaborators in the laboratory of Drs. Gary Cohen and Roselyn Eisenberg at the University of Pennsylvania, Philadelphia, PA. The recombinant proteins were truncated compared to their viral counterparts because transmembrane domains were excluded to permit secretion from insect cells. A melittin signal sequence was also included to target proteins to the secretory pathway of infected insect cells. SDS-PAGE and Coomassie staining was used to assess the purity of each batch of protein as shown in figure 3-1. The mobility of recombinant A33 was 16 kDa for the monomer and 32 kDa for the dimer, 34 kDa for B5 and 22 kDa for L1. B5 and L1 samples also had faint bands that were larger than the primary band. B5- and L1-specific antibodies did not recognize these bands by western blot analysis, suggesting that these bands may be minor contaminants that co-purified with these proteins. Western blot analysis of recombinant proteins (figure 3-2) confirmed the identity of the non-reduced proteins shown in figure 3-1. The anti-A33 and anti-L1 mAbs in figure 3-2A recognize only native forms of the viral proteins, therefore

recognition of recombinant proteins by these mAbs suggested that they were in the correct structural conformation. Protein samples reduced with  $\beta$ -mercaptoethanol ( $\beta$ -ME) showed a shift in mobility, as seen in the middle panel of figure 3-2A, and in all three panels of figure 3-2B. This mobility shift can be attributed to a change in tertiary structure caused by disruption of disulfide bonds. Multiple bands or a single broad band were observed for each protein, and this may be attributed to co- and post-translational modifications, such as glycosylation. Asparagine-linked (*N*-linked) glycosylation occurs co-translationally on asparagine residues within the sequence Asn-X-Ser or Asn-X-Thr during translocation into the endoplasmic reticulum. The presence of such a modification can cause a change in mobility. Treatment of recombinant proteins with PNGase F glycosidase removes the polysaccharides added by *N*-linked glycosylation and figure 3-3 shows the effect of PNGase F treatment on recombinant A33, B5, and L1. A33 has 2 predicted *N*-linked glycosylation sites, and B5 and L1 each have 3, and unsurprisingly, PNGase F treatment resulted in a single, faster-migrating band for each of these proteins. Viral EV proteins, including A33 and B5, are normally glycosylated. On the other hand, IMV proteins, like L1, are translated and processed in the cytoplasm and disulfide bonds are formed by poxvirus-encoded thiol oxidoreductase. IMV proteins do not undergo further processing in the secretory pathway, but the mobility shift in L1 seen in figure 3-3 suggests that the recombinant form is glycosylated and is different from viral L1. Proper folding of recombinant L1 was indicated by recognition with a conformation-dependant anti-L1 mAb (figure 3-2A), but immunogenicity may be affected by glycosylation.



**Figure 3-1: Coomassie staining of recombinant baculovirus proteins.**

Protein samples were diluted in NuPage LDS sample buffer, heated for ten minutes at 70°C and resolved on 12% NuPage Bis-Tris SDS-polyacrylamide gels with MOPS buffer. Gels were fixed with 50% methanol and 10% acetic acid and stained with GelCode Blue, a colloidal Coomassie stain. Approximately 1  $\mu\text{g}$  of protein was loaded for each sample. The molecular weight of each protein was approximated according to the migration of protein bands from a molecular weight marker represented by dash marks on the left side of the panel.



**Figure 3-2: Western blot analysis of recombinant proteins.** Recombinant proteins (200 mg/lane) were denatured and reduced and resolved by SDS-PAGE on 12% Bis-Tris NuPage gels with MOPS running buffer. Proteins were transferred to reinforced nitrocellulose for Western blot analysis and the molecular weight of each protein was approximated according to the migration of a molecular weight marker. (A) Western blots shown here include reduced or non-reduced samples (+ or - b-ME) probed with primary mAbs specific to A33 (left), B5 (middle), or L1 (right) and HRP-conjugated secondary Abs for visualization by chemiluminescence. A33 and L1 mAbs only recognized non-reduced forms of recombinant proteins, while the anti-B5 mAb recognized both reduced and non-reduced forms. (B) Western blots probed with PABs raised in rabbits immunized with the same recombinant proteins showed recognition of both reduced and non-reduced forms of A33 (left), B5 (middle), and L1 (right). Abbreviations: b-ME, b-mercaptoethanol; Ab, antibody; mAb, monoclonal Ab; pAb, polyclonal Ab.

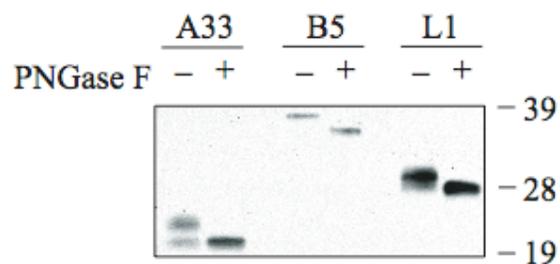
### **3.3.2 Antibody Responses Induced by Immunization with IMV and EV Proteins.**

Mice were immunized subcutaneously four times at three-week intervals with recombinant proteins and MPL+TDM adjuvant in order to characterize the immune response to each protein. The enzyme-linked immunosorbent assay (ELISA) was used to determine the endpoint antibody titers of pooled mouse sera, and antibodies against A33, B5, and L1 were detected by this assay using plates coated with the respective recombinant protein. Figure 3-4 shows antibody titers generated in mice immunized multiple times with each protein. Little or no antibodies were detected after one immunization, but increased most remarkably after two or three immunizations and very slightly, if at all, after a fourth immunization. Table 3-1 includes ELISA endpoints from mice immunized 4 times with individual or multiple proteins. Mice immunized with individual proteins showed little cross-reactivity with other antigens, suggesting that very low levels of insect cell or baculovirus contaminants were present. Mice immunized with protein combinations had measurable antibodies to each immunogen, but titers were slightly lower than in mice immunized with a single protein. Table 3-1 also shows that antibodies from protein-immunized mice also reacted to in VACV-infected cell lysates and these results suggest that mice may be protected from a virus challenge. Variation between titers measured on purified proteins or lysates was most likely due to differences in the abundance of specific viral proteins in the lysate as well as limitations in the amount of lysate used on coated plates. A33 immunization induced the highest titers, as measured with both purified protein and lysate ELISAs.

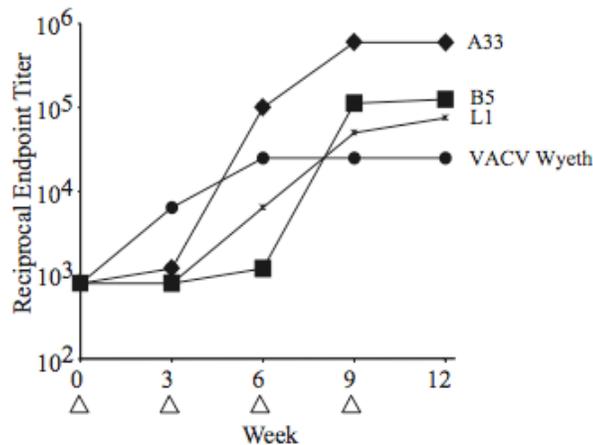
Another group of mice was immunized once with live virus ( $10^7$  pfu of VACV Wyeth) by tail scarification to recapitulate human smallpox immunization. This immunization strategy reproducibly induces the formation of a pustular lesion at the site of immunization on the base of the mouse tail without causing any other signs of illness. Figure 3-4 shows antibody titers measured with an infected cell lysate ELISA in mice immunized at week 0 with Wyeth virus. Antibodies were first detected 3 weeks after immunization, peaked at 6 weeks post-immunization, and were maintained at peak levels until challenge (12 weeks). Wyeth-immunized mice also produced antibodies that were detected with protein ELISAs, but were lower than titers measured in protein-immunized mice (Table 3-1). Mice immunized with multiple proteins had lower antibody titers compared to mice given individual proteins (Table 3-1). The lower antibody titers may be due to the activation of multiple B-cell subsets by T-helper cells. Crosstalk between different T-helper cells may inhibit the different B-cell populations in order to modulate overall antibody response.

### **3.3.3 IgG Isotypes Induced by Protein and Virus Immunization**

Unstimulated B-cells express IgM molecules with transmembrane domains, and antigen stimulation induces differentiation of B-cells into plasma cells that express secreted IgM. Stimulated B-cells may also undergo isotype switching, which results in expression of different IgG isotypes depending on the cytokine microenvironment [140]. Induction of IgG2a is characteristic of a Th1-polarized T-helper cell response, often induced during viral infection. Th2-polarized responses



**Figure 3-3: Deglycosylation of recombinant proteins.** Denatured and reduced proteins (200  $\mu\text{g}/\text{lane}$ ) were treated with PNGase F glycosidase and analyzed by SDS-PAGE and Western blotting with anti-His antibody. Bands were visualized by chemiluminescence. Molecular weight markers were used to approximate the molecular weight of each protein.



**Figure 3-4: Antibody responses following multiple immunizations with recombinant proteins.** Female BALB/c mice (n=12-14/group) were subcutaneously inoculated four times at three-week intervals with 10 µg of purified recombinant A33, B5, or L1 with MPL+TDM adjuvant. An additional group of mice was immunized once at week 0 with VACV Wyeth (10<sup>7</sup> pfu) by tail scarification. Mice were bled from the tail vein prior to immunization at week 0 and one day prior to each protein immunization. The reciprocal endpoint titers in pooled sera were determined by ELISA with the respective recombinant protein serving as the antigen for protein-immunized mice, or a VACV-infected cell lysate for mice immunized with VACV Wyeth. The lowest dilution of serum was 1:800. Δ, day of immunization.

**Table 3-1: Reciprocal endpoint ELISA titers.**

Immunization	Titer <sup>a</sup> with respective ELISA plate antigen				IC <sub>50</sub> <sup>b</sup>
	A33	B5	L1	VV Lysate	
Wyeth	<b>12,800</b>	<b>3200</b>	<b>12,800</b>	<b>25,000</b>	6684
A33	<b>600,000</b>	<800	<800	<b>150,000</b>	<100
B5	<800	<b>125,000</b>	<800	<b>2400</b>	<100
L1	<800	<800	<b>75,000</b>	<b>6400</b>	13,890
A33+B5	<b>400,000</b>	<b>62,500</b>	<800	<b>100,000</b>	<100
A33+L1	<b>400,000</b>	<800	<b>75,000</b>	<b>100,000</b>	9765
B5+L1	<800	<b>75,000</b>	<b>75,000</b>	<b>6400</b>	6927
A33+B5+L1	<b>400,000</b>	<b>50,000</b>	<b>100,000</b>	<b>87,500</b>	10,992

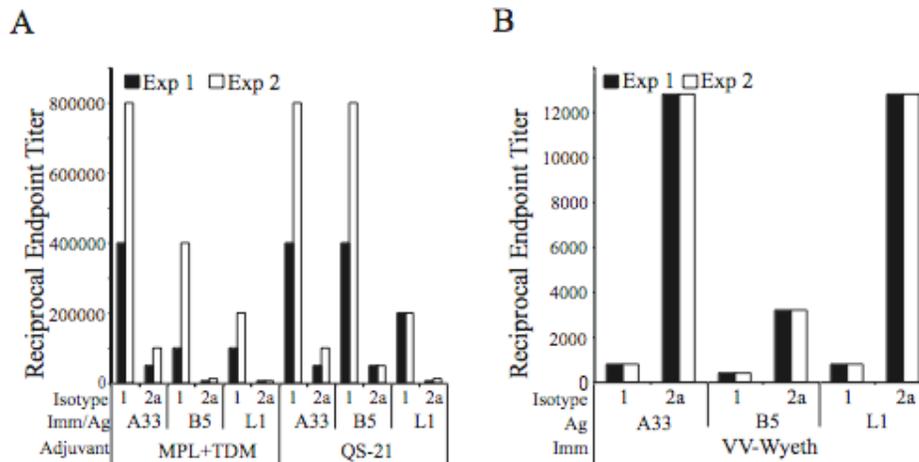
<sup>a</sup> Results for each immunization group represent the averages of two independent experiments that included 10 to 12 mice per group.

<sup>b</sup> IC<sub>50</sub>, 50% inhibitory concentration; reciprocal serum dilution that inhibits virus infection by 50% in the GFP-based neutralization assay

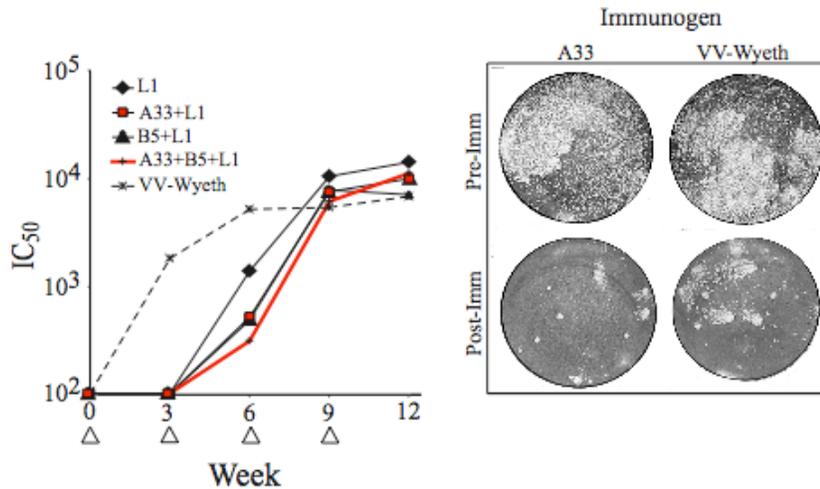
are associated with IgG1 production and are typically induced by bacterial and protein immunogens. Different adjuvants can sometimes cause a shift in the type of T-helper response induced, especially with respect to promoting production of IgG2a antibodies against protein antigens. IgG isotypes were characterized in mice immunized with recombinant proteins or virus using isotype-specific ELISAs. As expected, protein immunization induced a primarily IgG1 response, regardless of the kind of protein (figure 3-5A). The use of the saponin adjuvant QS-21 with A33, B5 and L1 proteins, which has been observed to promote Th1-type responses, did not induce significant production of IgG2a against these antigens (figure 3-5A). Not surprisingly, IgG2a antibodies were primarily induced following Wyeth immunization (figure 3-5B). Both protein and virus immunizations induced both IgG1 and IgG2a isotypes, but in each case, a dominant isotype was readily apparent.

### **3.3.4 Characterization of Neutralizing Antibodies Induced by Recombinant Protein Immunization**

Neutralizing antibodies against poxviruses have typically been measured using purified IMV particles in a cytopathic effect or plaque reduction assay. Initial observations showed that antisera from L1-immunized mice caused plaque reduction. Neutralization was quantified using a flow cytometric assay with a recombinant VACV expressing green fluorescence protein (GFP) reporter gene for measurement of infectivity [215]. Mice immunized with L1 alone or in combination with one or two EV proteins showed an increase in neutralizing Abs following 2 or 3 immunizations, and these titers were represented by the reciprocal serum dilution that



**Figure 3-5: Characterization of IgG isotypes.** (A) Mice groups (n=12-14 mice/group) were immunized 4 times with protein and MPL+TDM or QS-21 adjuvant, as described in figure 3-4. Sera collected three weeks following the fourth immunization was pooled and analyzed using an isotype-specific ELISA for IgG1 or IgG2a (1 or 2a). (B) A group of mice (n=12-14 mice) was immunized once with  $10^7$  pfu of VACV Wyeth virus by tail scarification, and isotype responses were analyzed 12 weeks after immunization. Two independent experiments (Exp 1 and Exp 2) were analyzed and the results are represented in both panels A and B. Abbreviations: 1, IgG1; 2a, IgG2a; Imm, immunization; Ag, antigen.



**Figure 3-6: Induction of neutralizing antibodies against IMV and EV. (A)**

Groups of mice (n=8-14) were immunized with recombinant L1, L1 and A33 or B5, or all three proteins as described in figure 3-4. An additional group of mice (n=12-14) was immunized with 10<sup>7</sup> pfu of VACV strain Wyeth one time by tail scarification. IMV neutralization was quantified with a quantitative flow cytometry assay using a recombinant VACV expressing green fluorescence protein. The serum dilution that inhibited the number of infected (fluorescent) cells by 50% (IC<sub>50</sub>) is plotted above.

(B) The comet inhibition assay was used to observe the presence of anti-EV antibodies that were able to inhibit the spread of EV *in vitro*. BS-C-1 cells were infected with IHD-J virus and then incubated with a liquid overlay contain a 1:50 dilution of pooled serum from 12 mice prior to immunization (Pre-Imm) or 3 weeks after 4 protein immunizations with A33 or 12 weeks after immunization with Wyeth virus (Post-Imm). Infected cell monolayers were fixed and stained with crystal violet following a 2 day incubation.

inhibited GFP expression by 50% compared to GFP expression in cells not treated with serum (figure 3-6A). The increase in neutralizing antibody titers was similar to trends observed with ELISA titers in figure 3-4 (figure 3-6A). Immunization of L1 with additional proteins resulted in IC<sub>50</sub> neutralization values within twofold of values obtained from L1 alone as shown in figure 3-6 and table 3-1. Wyeth virus immunization resulted in a steady increase in neutralizing antibodies 3 and 6 weeks following immunization, but titers remained level between 6 and 12 weeks (figure 3-6A). Immunization with live virus induces neutralizing antibodies against multiple targets, although it is likely that a subset of antibodies is L1-specific. Animals immunized with EV proteins did not have any measurable neutralizing antibodies, which is an expected result for this IMV-specific assay (Table 3-1).

The comet reduction assay was used to detect antibodies that inhibit or reduce the formation of secondary satellite plaques via EV in cell culture. The IHD-J strain of VACV was used to infect BS-C-1 cell monolayers because this strain characteristically produces high levels of EV due to a mutation the A34R gene [190], and as a result, forms comet shaped plaques under liquid medium. IHD-J-infected cells were treated with diluted antisera and these monolayers were fixed and stained with crystal violet after a 48-hour incubation for visualization of plaque morphology. Figure 3-6B shows that sera collected from mice prior to immunization with A33 or Wyeth did not inhibit comet formation and virus spread occurred throughout the monolayer. Sera from mice immunized A33 or Wyeth did cause a striking reduction in comet size (figure 3-6B), although B5 antisera did not significantly reduce plaques (data not shown), which may be attributed to lower levels of antibody (Table 3-1).

Sera from L1-immunized mice also did not inhibit comet formation since IMV antibodies are not expected to inhibit EV-mediated virus spread.

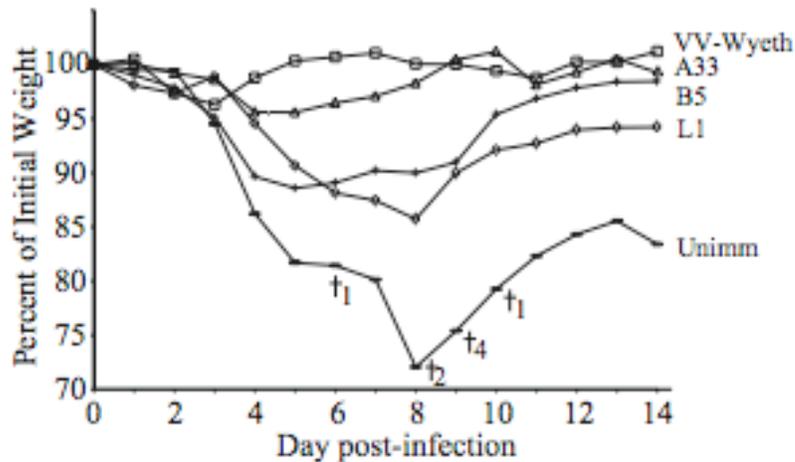
### **3.3.5 Protection of Immunized Mice to an Intranasal Challenge with 5 LD<sub>50</sub> of VACV WR**

Groups mice (n=6) were immunized with 10 µg of A33, B5, or L1 mixed with MPL+TDM adjuvant 4 times at three-week intervals. An additional group of mice was immunized a single time by tail scarification with 10<sup>7</sup> pfu of VACV Wyeth during the time of the first protein immunization. Three weeks after the final protein immunization, all groups were challenged intranasally (IN) with 10<sup>6</sup> pfu of VACV WR, which was determined to be equivalent to 5 LD<sub>50</sub> in age-matched mice. Mice were weighed daily as an indirect measure of virus replication because others have demonstrated a correlation between weight loss and viral replication in the lungs, a target organ of IN challenge [216]. Mice were sacrificed if they lost greater than 30% of their initial weight, and this percentage of weight loss has been shown to be naturally lethal in 98% of infected mice [217]. Data from two independent experiments with identical designs were averaged and shown in figure 3-7. The majority of unimmunized mice died naturally or were euthanized by 10 days post-challenge, while all mice immunized with protein or VACV Wyeth survived (figure 3-7). Differences between immunizations were discernable by analysis of weight loss, and A33 immunization provided the best protection against weight loss throughout the course of challenge. Mice immunized with A33 had statistically significantly less weight loss compared to those given B5 or L1 between 6 and 8 days

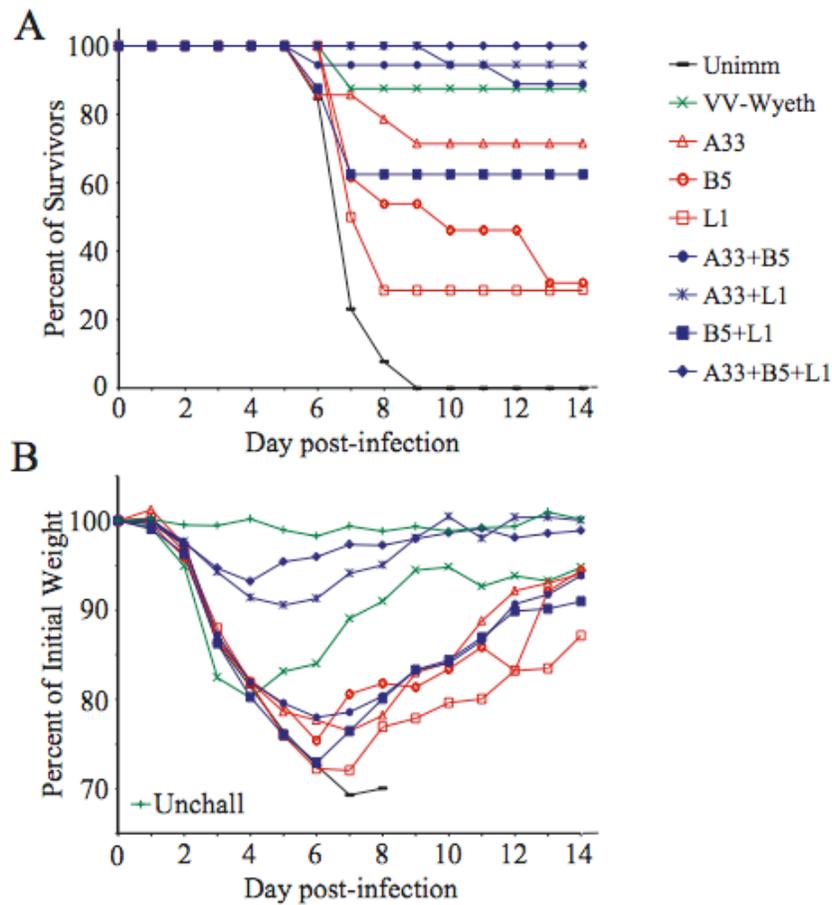
after challenge, which is typically the time of greatest weight loss. Surprisingly, mice immunized with A33 alone showed no statistically significant differences in weight loss compared to VACV Wyeth. The superior protection seen in A33-immunized mice may be attributable to the higher antibody responses observed with this immunogen (Figure 3-4, Table 3-1).

### **3.3.6 Protection of Immunized Mice to an Intranasal Challenge with 100 LD<sub>50</sub> of VACV WR**

The successful protection of mice immunized with individual recombinant proteins with a low challenge dose led us to investigate if mice could be successfully protected against a high challenge dose. We immunized mice with individual proteins as well as combinations of proteins in order to induce a more broadly protective immune response. As described for the previous challenge experiment, groups of mice (n=6-10 mice/group) were immunized with 10 µg of A33, B5 or L1 alone, or with all possible pairings or with all three proteins. Once again, a group of mice was immunized with VACV Wyeth and an additional group was unimmunized. All mice were challenged IN with  $2 \times 10^7$  pfu of VACV WR which is equivalent to 100 LD<sub>50</sub> and two identically designed independent experiments were carried out. Following challenge, all unimmunized mice died by 9 days after challenge, as well as more than two-thirds of mice immunized with B5 or L1 alone (figure 3-8A). Fewer mice died with A33 or B5+L1 immunization, and survival was further enhanced by immunization with A33+L1 or A33+B5 (figure 3-8A). Impressively, all mice given



**Figure 3-7: Weight loss of mice following intranasal challenge with 10<sup>6</sup> pfu of VACV WR.** Mice were immunized subcutaneously four times with 10 µg of recombinant A33, B5, or L1 and MPL+TDM adjuvant or a single time with VACV Wyeth by tail scarification. 3 weeks following the fourth protein immunization and 12 weeks after VACV Wyeth immunization, mice were challenged intranasally with 10<sup>6</sup> pfu of VACV WR. Mice were weighed daily and were euthanized if weight fell below 30% of initial weight. The data shown is the average percentage of initial weight for each surviving mouse in each group from two independent experiments with 12 mice total per group. Abbreviations: Unimm, unimmunized; †, number of mice died or sacrificed on given day.



**Figure 3-8: Survival and weight loss of mice challenged IN with  $2 \times 10^7$  pfu of VACV WR.** (A) Mice (groups of 6-10) were immunized with 10  $\mu$ g of each recombinant protein or possible combinations thereof as described for figure 3-7. An additional group of mice was immunized with VACV Wyeth and another group was unimmunized. Mice were challenged IN with  $2 \times 10^7$  pfu of VACV WR, were weighed daily and were euthanized if weight decreased more than 30% of initial weight. The percentage of surviving mice is shown for each group and was the average of two independent experiments. (B) Similar to figure 3-7, the percentage of initial weight was determined for each mouse daily and the average percentage of initial weight was averaged from two independent experiments. Abbreviations: Unimm, unimmunized; Unchall, unchallenged

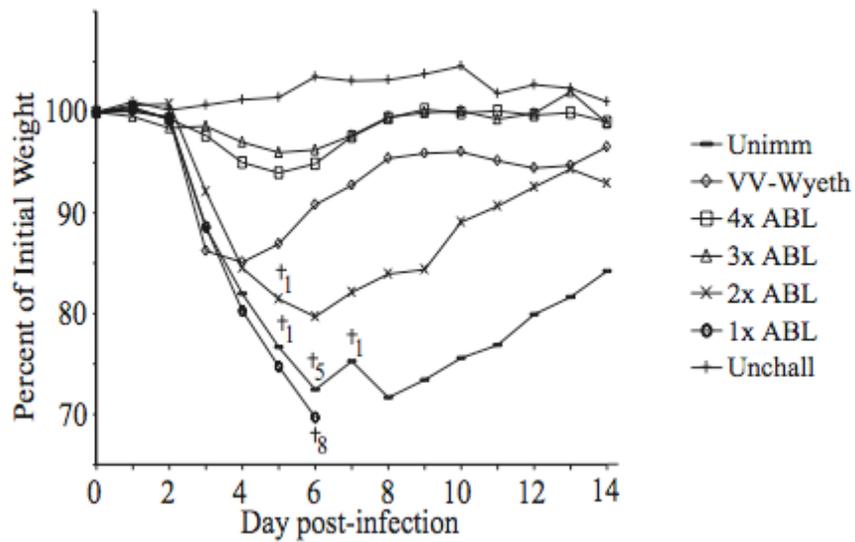
all three proteins survived challenge, which was better than observed in mice given VACV Wyeth (figure 3-8A).

Weight loss after challenge was used as another measure of protection (figure 3-8B). Significant weight loss was observed in mice immunized with individual proteins or with B5+L1 or A33+B5. VACV Wyeth immunization resulted in approximately a 20% maximal weight loss, while mice immunized with A33+L1 or all three proteins suffered less than a 10% weight. In fact a significant difference of relative weight loss was seen between VACV Wyeth and A33+L1 or A33+B5+L1 during days 4 through 6 (VV-Wyeth versus A33+L1,  $p < 0.008$ ; VACV Wyeth versus A33+B5+L1,  $p < 0.001$ ). An untreated and unchallenged (Unchall) group of mice was also included in this study as an additional control following challenge and as a way to observe normal daily fluctuations in weight. These results support the hypothesis that superior protection is attained with immunity against both IMV and EV, but the superior protection seen with A33+L1 compared to B5+L1 may be caused by differences in antibody responses to individual proteins.

### **3.3.7 Multiple Immunizations are Required for Protection**

Four immunizations were given to mice used in the challenge experiments described above, as this was a similar strategy successfully used by Galmiche and co-workers [174]. We wanted to determine the minimal number of immunizations sufficient for protection against the high dose of challenge ( $2 \times 10^7$  pfu of VACV WR). Groups of 8 mice were immunized 1, 2, 3, or 4 times with A33, B5, and L1 proteins and MPL + TDM adjuvant as described for previous experiments. An additional group was given VACV Wyeth one time as previously explained. The

study was designed such that all groups of mice were given their final immunization at the same time and all groups were intranasally challenged three weeks later with  $2 \times 10^7$  pfu of VACV WR. None of the mice immunized once survived, while 7 of 8 mice given two immunizations survived, as did all of the mice given three or four immunizations (figure 3-9). Weight loss was significant in unimmunized and singly immunized mice, but was less so in mice given 2 immunizations (figure 3-9). On the other hand, mice given 3 or 4 immunizations showed equivalently limited weight loss and fared better than mice given VACV Wyeth (figure 3-9). The results of this experiment indicate that 3 or 4 immunizations supply an equivalent degree of protection against a highly lethal challenge in mice.



**Figure 3-9: Multiple immunizations are required for protection against a lethal IN challenge with  $2 \times 10^7$  pfu of VACV WR.** Mice were immunized 1, 2, 3, or 4 times with A33, B5, and L1 proteins mixed with MPL+TDM adjuvant as previously described. All mice received final immunization at the same time and were challenged three weeks later with  $2 \times 10^7$  pfu of VACV WR (100 LD<sub>50</sub>). Abbreviations: Unimm, unimmunized; Unchall, unchallenged; †, number died on specified day; ABL, A33+B5+L1; 1x one immunization; 2x, two immunizations; 3x, three immunizations; 4x, four immunizations.

### 3.4 Discussion

This chapter describes the use of recombinant VACV proteins that were successfully used immunogens to protect mice against infection in a lethal respiratory challenge model. We were reassured that the recombinant forms of A33, B5, and L1 would be good vaccine candidates by biochemical evidence showing their resemblance to their viral counterparts. The viral proteins contain transmembrane domains, but baculovirus expression of recombinant versions required exclusion of these domains and inclusion of an insect signal peptide to allow secretion into the media of baculovirus-infected cells. A polyhistidine tag was also included for purification of proteins by affinity chromatography. Recombinant L1 contained intramolecular disulfide bonds, and A33 formed a disulfide-bonded dimer and both of these properties are characteristic of the viral proteins. All three proteins reacted with monoclonal antibodies that are also known to react with their respective viral proteins and are also protective against virus challenge when used as a passive immunization (to be described in chapter 4). Recombinant L1 also induced IMV-neutralizing antibodies, further evidence that the recombinant protein resembles its viral counterpart, in spite of the fact that unlike viral L1, the recombinant form is glycosylated. Indeed, these recombinant proteins are potentially cross-protective against variola virus since A33, B5, and L1 are highly conserved orthopoxvirus proteins with an amino acid identity of 94%, 93%, and 99%, respectively between variola and vaccinia orthologs. On the other hand, the relative ease of expressing these proteins using recombinant baculoviruses suggests that similar variola versions could also be expressed in such a manner.

This recombinant multiprotein vaccine induced specific antibody responses as well as IMV-neutralizing antibodies and antibodies that inhibited EV-mediated cell-to-cell spread *in vitro*. In fact, administration of four protein immunizations resulted in higher antibody titers than induced by live virus vaccination. The coadministration of these recombinant proteins with a Ribi adjuvant system, MPL+TDM, or QS-21, a saponin adjuvant, resulted in predominantly IgG1 antibodies, which was characteristic of a Th2-polarized response. On the other hand, live virus vaccination resulted in a Th1-polarized response, indicated by the predominance of IgG2a antibodies. T<sub>CD8+</sub> responses were not measured in this study because of the Th1-skewed response induced by and typical of protein immunization. T<sub>CD8+</sub> responses have been shown to play a role in the optimal clearance of a primary poxvirus infection [120, 124, 130] and may be especially critical for controlling infection prior to a full-scale antibody response. Then again, antibodies alone are sufficient for protection [131, 132], and T<sub>CD8+</sub> cells contribute little to the overall immune response in the presence of a strong humoral response [130].

Our results showing effective protection following immunization with a combination IMV and EV proteins confirm the results obtained by Hooper and co-workers using DNA vaccination and an intraperitoneal virus challenge [175, 176]. We have used a well-characterized intranasal challenge model instead for our experiments because it better recapitulates a respiratory illness seen with smallpox, it requires a lower LD<sub>50</sub> for challenge, which is also similar to smallpox infection, and it has been used extensively to test vaccines [131, 218] and study viral pathogenesis. Nonetheless, both of these studies provide compelling evidence that individual IMV

and EV targets can induce a protective immune response and may serve as a safe alternative to live virus vaccination. If antibodies are primarily responsible for controlling infection upon virus challenge, how may they be functioning? Intranasal challenge requires inoculation of purified IMV into the nostrils of mice, therefore anti-IMV proteins may be important for neutralizing the inoculum. Anti-EV proteins may inhibit virus dissemination during viral replication, and this is indirectly evident by inhibition of comet-shaped plaques by anti-EV sera *in vitro*. Anti-B5 antibodies have been shown by others to neutralize EV particles directly in addition to inhibiting comet formation [174]. Antibodies have other well-defined functions, including antibody-dependent cell-mediated cytotoxicity, formation of antigen-antibody complexes, and activation of complement. Vanderplasschen et al. have shown that anti-EV antibodies can cause aggregation of cell-associated EV on the plasma membrane, further evidence of the multiple means employed by antibodies to inhibit or control infection [194].

The confirmation of the hypothesis that multiple recombinant IMV and EV proteins can induce protective immunity in a lethal murine challenge model promotes further exploration of the mechanisms behind antibody-mediated protection. In addition, further testing and optimization of this and similar candidate vaccines should be considered.

## **Chapter 4**

### **Passive Protection of Mice with Antibodies against Multiple Outer Membrane Proteins of the Extracellular and Intracellular Forms of Vaccinia Virus**

#### **4.1 Introduction**

The study in the preceding chapter shows that mice immunized with recombinant vaccinia virus proteins are protected from a lethal respiratory virus challenge. Antibody responses were detected against both the IMV and EV forms of VACV, and the antibody response is required for effective control of both primary and secondary poxvirus infections [126, 131, 132, 219]. Vaccinia immune globulin (VIG) has been used to treat adverse reactions to smallpox vaccination and to reduce the severity of smallpox disease, and can also be used for immediate protection of naïve individuals in the event of a smallpox outbreak [220, 221]. In fact, anecdotal evidence suggested that prophylactic VIG treatment reduced the incidence of smallpox by one-quarter compared to no treatment during an epidemic [222]. Unfortunately, VIG is derived from the serum of recently vaccinated humans, making it both an inherently hazardous and scarce product. In this regard, there is interest in the development of a well-defined anti-vaccinia antibody product to replace VIG, preferably by using hybridoma cells, which are relatively limitless source of antibodies.

Several potential antibody targets have been defined in recent work towards the development of alternative smallpox vaccines comprised of recombinant DNA or

proteins [175-177, 223]. The results of these studies support the design of a vaccine or therapeutic antibody mixture that targets both the IMV and EV forms of infectious virus. Immunization of rabbits with inactivated IMV resulted in poor protection against a lethal orthopoxvirus challenge, and passive immunization with anti-IMV serum also protected poorly against challenge, thus affirming the importance of antibodies against EV as well as IMV [224, 225]. Passive immunization experiments with individual antibodies partially protected mice against virus challenge, but they have targeted either IMV [226, 227] or EV [174]. The goal of the study described in this chapter is to determine if passive immunization with individual or combinations of antibodies against defined IMV and EV proteins will protect mice against a lethal respiratory challenge. BALB/c or severe combined immunodeficiency disease (SCID) mice were passively immunized with rabbit polyclonal (PAbs) or rodent monoclonal (MAbs) antibodies against the IMV protein L1 or the EV proteins A33 and B5. Mice were challenged intranasally with a lethal dose of VACV WR prior to or following passive immunization and were monitored daily for signs of illness and weight loss. Our results indicate that passive immunization with the individual antibodies partially protects against challenge, but protection was enhanced when mice were given a combination of antibodies targeting both the IMV and EV. These results support the development of human monoclonal antibody-based replacement for VIG. This study was previously described in reference 273.

## **4.2 Materials and Methods**

### **4.2.1 Cells and Viruses.**

HeLa S3 suspension cells and BS-C-1 monolayers were maintained as described in chapter 3, section 3.2.1. Stocks of the viruses VACV WR, IHD-J, and VV-NP-SIINFEKL-EGFP were grown in HeLa S3 suspension cells and purified by sucrose density centrifugation as previously described in chapter 3, section 3.2.1. Viral titers were determined by plaque assay as described in chapter 3, section 3.2.1.

### **4.2.2 Hybridomas and Monoclonal Antibodies.**

Anti-A33 MAb (IgG3), anti-L1 MAb (IgG2a), and anti-B5 MAb were harvested from the supernatants of mouse hybridoma 1G10, mouse hybridoma, 7D11 (both provided by Dr. Alan Schmaljohn, USAMRIID), and rat hybridoma 19C2 [78], respectively. The 1G10 and 19C2 hybridomas were maintained in Dulbecco's modified Eagle medium (Quality Biologicals) containing 5% heat-inactivated fetal bovine serum, l-glutamine and antibiotics as described in chapter 3, section 3.2.1. The 7D11 hybridoma was maintained in RPMI 1640 (Quality Biologicals) that was supplemented in the same manner as described above. Gamma immunoglobulin (IgG) antibodies were purified from the supernatants of each hybridoma cell line with the Montage antibody purification-Prosep A kit (Millipore, Billerica, MA). The Montage kit is an affinity chromatography system for purification of antibodies from cell supernatants by centrifugation through a matrix made of protein A immobilized on porous glass. Antibodies were eluted from the Prosep-A matrix with a 0.2 M glycine/HCl buffer, pH 2.5, and the eluate was dialyzed against PBS and concentrated

using an Amicon Ultra-15 centrifugal filtration unit (Millipore). Antibodies used in the experiment described in section 4.3.4 were purified by affinity chromatography on protein A sepharose from ascites fluid made in ICR SCID mice. Purified 7D11 and 19C2 MAbs were aliquoted and stored at -20°C, while aliquots of 1G10 were stored at 4°C to prevent precipitation typically resulting from freezing and thawing of an antibody of the IgG3 isotype. SDS-PAGE and Coomassie blue stain was used to ascertain the purity of purified antibodies. Protein concentrations were determined by measurement of absorbance at 280 nm prior to each passive immunization experiment.

### **4.2.3 Polyclonal Antibodies**

Soluble forms of the VACV proteins A33, B5 and L1 were expressed by and secreted from insect cells infected with recombinant baculoviruses as described in chapter 3, section 3.2.2. Proteins were affinity purified and used for preparation of polyclonal sera in rabbits. Different rabbits were immunized with 100 mg of each purified protein diluted in PBS with an equal volume of Freund's complete adjuvant by subcutaneous and intramuscular routes. Rabbits were boosted in the same manner four times at 2-week intervals with 50 mg of the same protein diluted in PBS with an equal volume of Freund's incomplete adjuvant. Blood was collected by terminal exsanguinations two weeks after the final immunizations and serum was separated by centrifugation. IgG was purified from serum by affinity chromatography with protein A sepharose as described in section 4.2.2 of this chapter. Polyclonal serum was prepared by collaborators in the laboratories of Drs. Gary Cohen and Roselyn Eisenberg at the University of Pennsylvania, Philadelphia, PA.

#### **4.2.4 Vaccinia Immune Globulin**

Vaccinia immune globulin intravenous (VIGIV; Dynport Vaccine Company LLC) lot 1 was kindly provided by Dr. Dorothy Scott (Center for Biologics Evaluation and Research, Food and Drug Administration, Bethesda, MD). VIGIV is a sterile liquid preparation of human immunoglobulin stabilized with 1% human serum albumin and 5% sucrose [182]. The source of immunoglobulin for VIGIV is plasma from human donors who have been boosted with Dryvax smallpox vaccine. The product is vialled at a final concentration of 50 mg of immunoglobulin per ml, and is primarily IgG with trace amounts of IgM and IgA.

#### **4.2.5 Enzyme-Linked Immunosorbent Assays (ELISAs)**

The presence of passively administered antibodies was detected in mouse serum using the enzyme-linked immunosorbent assay (ELISA) as described in Chapter 3, section 3.2.7. 96-well cluster plates were coated with antigens that included recombinant A33, B5, or L1 proteins, or a VACV-infected cell lysate. Twofold serial dilutions of purified antibodies, mouse serum, or human vaccinia immune globulin (VIG) was added to antigen-coated plates and antibodies were detected with HRP-conjugated anti-rat, anti-mouse, anti-rabbit, or anti-human secondary antibodies and a ready-to-use solution of 3,3',5,5'-tetramethylbenzidine (BM Blue, POD substrate, Roche). A spectrophotometer was used to measure  $A_{370}$  and  $A_{492}$  values following incubation at room temperature for 30 minutes. Endpoint titers were established as the serum dilution with an absorbance measurement two standard deviations above the absorbance measured in wells not incubated with

primary antibody or serum but treated with HRP-conjugated secondary antibody and BM Blue.

#### **4.2.6 IMV Neutralization and Comet Reduction Assays**

IMV neutralization was measured using recombinant VACV expressing EGFP (VV-NP-SIINFEKL-EGFP) treated with different concentrations of antibody. HeLa S3 cells were infected with antibody-treated virus and EGFP expression was measured as an indicator of virus replication by flow cytometry. Neutralization titers were reported as the antibody concentration that inhibited infection by 50% compared to infection in the absence of antibody. The assay is described in detail in chapter 3, section 3.2.8 and reference 214.

The comet reduction assay was used to detect the presence of antibodies that inhibited the formation of secondary satellite plaques in cell culture by blocking EV-mediated infection. 6-well cluster plates containing confluent BS-C-1 monolayers were infected with 50 pfu/well of VACV strain IHD-J for 2 hours at 37°C. The virus inoculum was removed and monolayers were treated with different concentrations of antibody diluted in infection medium as defined in chapter 3, section 3.2.8. Plates were incubated at 37°C for 36 hours and plaques were visualized by staining with crystal violet.

#### **4.2.7 Passive Immunization and Intranasal Virus Challenge**

Female BALB/c mice (7- or 14-weeks old) were purchased from Taconic. Female BALB/c SCID (14-weeks old, strain C3S<sub>mn</sub>.CB17-*Prkdc scid* /J) were purchased from the Jackson Laboratory (Bar Harbor, ME). Purified antibodies at

different concentrations were diluted in sterile PBS and administered for passive immunization by intraperitoneal (IP) injection. Unimmunized were given an equal volume of sterile PBS by IP injection in parallel to passive antibody immunization.

Mice were challenged either before or after passive immunization by intranasal inoculation with VACV WR as previously illustrated in chapter 3, section 3.2.9. The LD<sub>50</sub> of IN-inoculated VACV WR in female BALB/c mice was calculated to be  $1.5 \times 10^5$  pfu for 7-week old mice and  $3 \times 10^5$  pfu in 14-week old mice. Mice were weighed daily for 2 to 12 weeks as an indirect measure of viral replication and were euthanized if their weight fell below 70% of their initial weight. The NIH Animal Care and Use Committee approved this mouse protocol.

#### **4.2.8 Statistical Analysis**

The percentage of weight change observed in mice immunized with different antibodies caused by virus challenge was analyzed statistically by analysis of variance (ANOVA) with the Statview statistical software package (SAS Institute Inc.). ANOVA showed a significant difference between at least two groups of immunized mice, thus the Fisher protected least-significance-difference test was applied *post hoc* to determine which groups showed significant differences between each other. A *p*-value less than 0.05 was considered significant.

## **4.3 Results**

### **4.3.1 Characterization of PAbs and MAbs Used for Passive Immunization.**

The principle goal of this chapter is to determine if mice can be protected by passive immunization with antibodies against IMV and EV proteins, therefore PAbs were generated as experimental reagents by multiple immunization of rabbits with recombinant forms of A33, B5, and L1. Secreted forms of these proteins were expressed by infection of insect cells with recombinant baculoviruses. The preceding chapter included biochemical characterization of the recombinant proteins and a demonstration of protective immunity induced in mice. PAbs were used as affinity-purified IgG and were characterized for antigen specificity with ELISAs with recombinant proteins or VACV-infected cell lysate as antigens as summarized in table 4-1. Each PAb reacted specifically with its respective antigen and also showed reactivity with the VACV lysate. A limited amount of viral A33, B5, or L1 may be the cause of the lower titers observed with lysate ELISAs compared to titers obtained with purified protein antigens. Vaccinia immune globulin (VIG), a purified IgG preparation derived from the plasma of Dryvax-immunized humans, was also analyzed by ELISA. Indeed, antibodies against each recombinant protein antigen were detected in VIG, as well as antibodies reactive to the VACV-infected cell lysate (table 4-1). Antibody titers were lower to specific antigens compared to monospecific PAbs, but this result is anticipated from VIG since it contains antibodies against numerous viral proteins (table 4-1).

**Table 4-1: Reciprocal endpoint ELISA titers of PAbs and MAb.**

Antibody <sup>a</sup>	Antigen			
	A33	B5	L1	Lysate
A33 PAb	330,000	-- <sup>b</sup>	--	25,600
B5 PAb	--	300,000	--	12,800
L1 PAb	--	--	61,000	1,600
VIG	1,600	6,400	800	6,400
A33 MAb	800,000	--	--	--
B5 MAb	--	800,000	--	--
L1 MAb	--	--	1,000,000	--

<sup>a</sup> IgG (1 mg/ml).

<sup>b</sup> --, not determined.

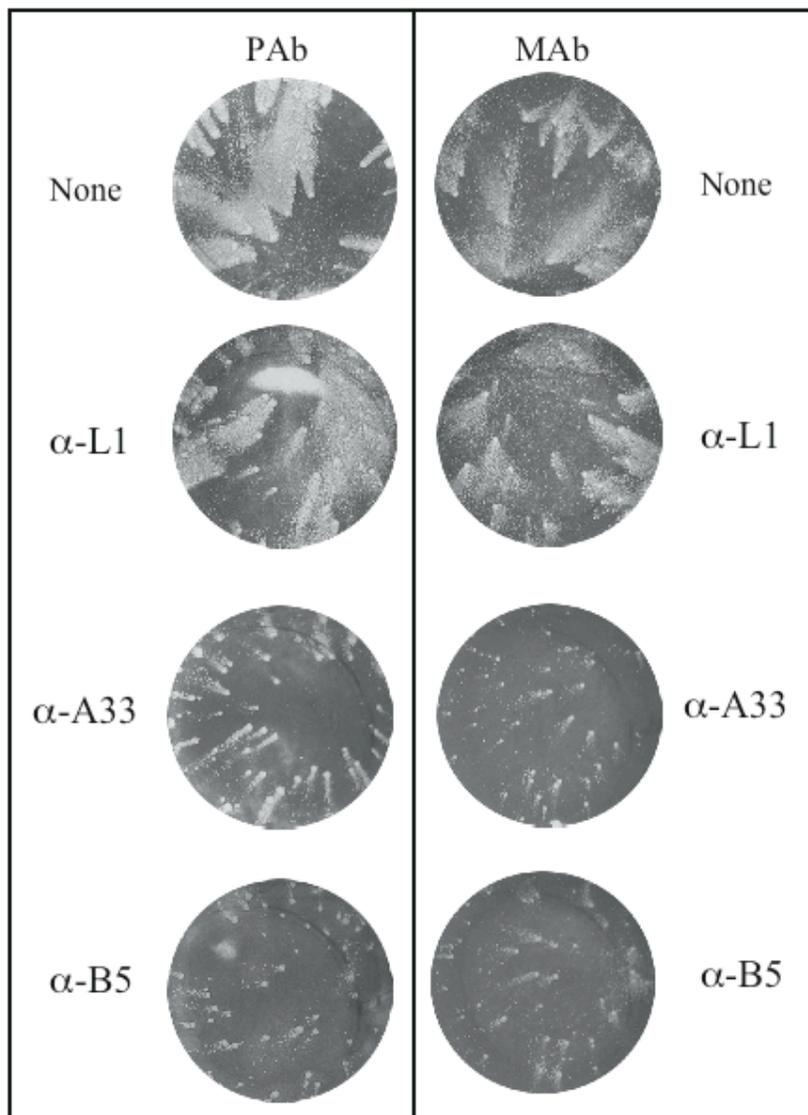
IMV neutralization was measured using a flow cytometric assay with a recombinant VACV that expresses GFP as described in reference 214 and in chapter 3, section 3.2.8. Both anti-L1 PAb IgG and VIG showed measurable IMV neutralization, and anti-A33 and anti-B5 had no detectable neutralization activity. VIG had threefold greater neutralizing activity than anti-L1 PAb IgG ( $IC_{50}$  of 6.7  $\mu$ g and 2.3  $\mu$ g, respectively), in spite of anti-L1 having significantly greater L1 binding activity compared to VIG (table 4-1). This supports data from a study by Aldaz-Carroll and co-workers showing that L1 is not the main target of neutralizing antibodies in VIG [228].

The ability of PABs to inhibit EV infection was observed using the comet reduction assay. The comet reduction assay is preferred over an EV neutralization assay because earlier studies have shown that antibodies that are protective *in vivo* also effectively reduce comets, while not all protective antibodies neutralize EV *in vitro*, including those against A33 [92, 229, 230]. In fact, a recent study showed that B5 was the major *in vitro* EV-neutralizing antibody in VIG, but removal of anti-B5 antibodies did not significantly reduce the ability of VIG to reduce comet formation. As expected, anti-A33 and anti-B5 PABs effectively reduced comet formation upon treatment of infected cells with 20  $\mu$ g of IgG per ml (figure 4-1). No comet reduction was observed with anti-L1 PAb with 20  $\mu$ g of IgG per ml (figure 4-1).

MAbs against A33, B5, and L1, which have previously been described and characterized in Chapter 3, section 3.3.1 and in references 230 and 231, were also used for passive immunization. Anti-EV antibodies include 1G10, an anti-A33 MAb that does not neutralize EV *in vitro* but binds specifically to the viral protein [175],

and 19C2, a MAb specific to B5 [78]. 7D11, a MAb specific to the IMV protein L1, was also used. All three MAbs had higher endpoint titers compared to the respective PAb when measured with specific protein ELISAs (table 4-1). This may be due to the accessibility of the epitope bound by each MAb, as well as the likelihood that PAb preparations probably contain contaminating non-specific rabbit IgG. Anti-EV MAbs reduced comet formation similarly to PAb, and, likewise, anti-L1 MAb did not cause any comet reduction (figure 4-1). 7D11 MAb had 16-fold higher binding to L1 than L1 PAb as based on ELISA endpoint titers, but its relative neutralizing activity was much greater ( $IC_{50}$  equals 3.1 ng for MAb 7D11 versus 2.3  $\mu$ g for L1 Pab).

**Figure 4-1: Comet reduction in the presence of anti-EV antibodies.** BS-C-1 cell monolayers were infected with VACV strain IHD-J for 2 hours, followed by incubation with no antibody (None; top two wells) or with 20 mg per ml of purified PAbs or MAbs against A33, B5, or L1 diluted in infection medium. Plaques were visualized by staining of cell monolayers with crystal violet 36 hours post infection.



### **4.3.2 Protection of Mice with Individual or Combinations of PABs**

The ability of passively administered antibodies to protect mice was tested with an intranasal virus challenge model. This model recreates infection of the respiratory tract similar to that seen in humans during smallpox infection and the rationale for its use is discussed in chapter 3, section 3.3.5. 14-week old female BALB/c mice were used for passive immunization to correspond with the age of mice at the time of virus challenge following multiple protein immunizations as described in chapter 3. Mice were immunized intraperitoneally (IP) with purified PAb IgG and challenged one day later IN with  $10^6$  PFU (3 LD<sub>50</sub>) of VACV WR. A preliminary experiment in which mice were immunized with 1 mg each of A33, B5 or L1 PAb resulted in no survival after virus challenge. Therefore, the following experiments used 5 mg of PAb. Mice immunized with 5 mg of A33, B5, or L1 PAb had circulating antibody titers measured prior to challenge that were similar to those observed after multiple protein immunizations (table 4-2). Figure 4-2A shows that none of the unimmunized mice, and the majority of mice given VIG did not survive challenge. On the other hand, at least 3 of the 4 mice given PAb against A33, B5, or L1 did survive challenge but lost weight to varying degrees (figure 4-2A and B). Weight loss was less severe than observed in unimmunized mice, and this difference was statistically significant on days 3 and 4 post-infection for mice given anti-L1 ( $p=0.0114$  and  $p=0.0235$ , respectively for anti-L1 versus unimmunized).

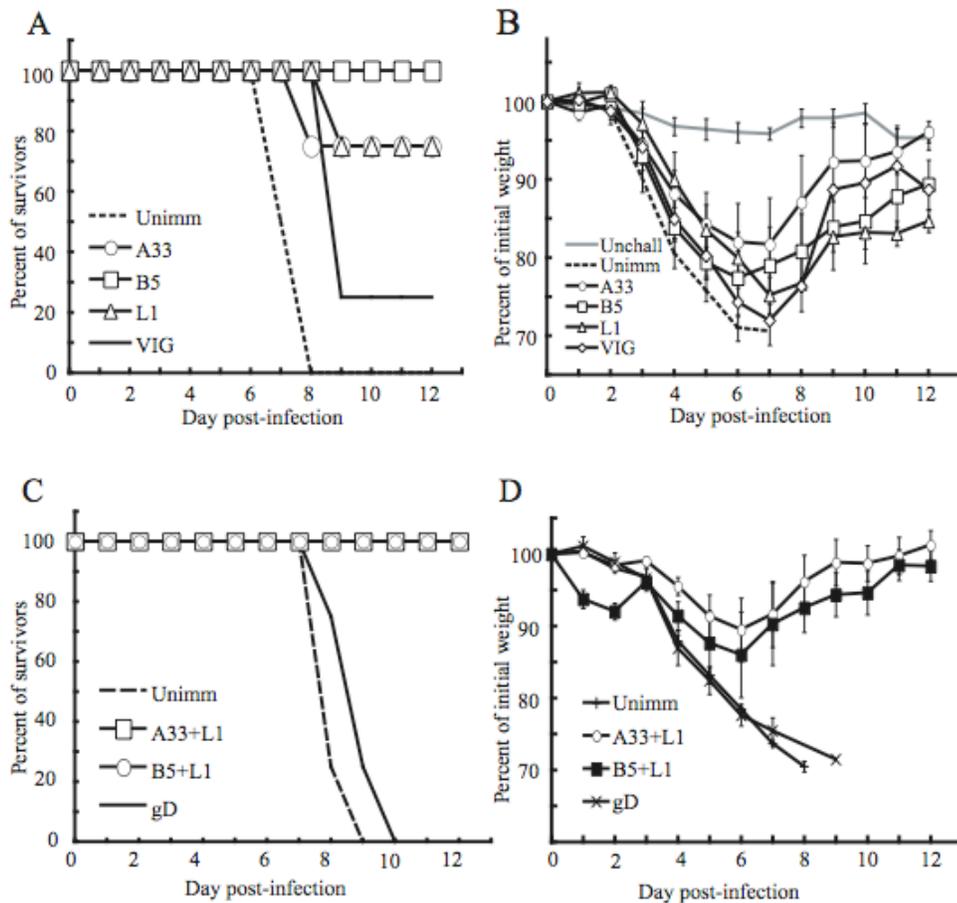
**Table 4-2: Comparison of ELISA endpoint titers following passive or active immunization.**

	Antigen		
	A33	B5	L1
A33 passive <sup>a</sup>	304,437	-- <sup>c</sup>	--
A33 active <sup>b</sup>	565,685	--	--
B5 passive	--	304,437	--
B5 active	--	100,00	--
L1 passive	--	--	59,460
L1 active	--	--	70,711

<sup>a</sup> Serum titers 24 hours after IP injection with 5 mg PAb.

<sup>b</sup> Serum titers after three protein immunizations (geometric mean titers; chapter 3 and reference 222)

<sup>c</sup> --; not determined.



**Figure 4-2: Protection of mice by passive immunization with PAbs.** (A,B) 14-week old female BALB/c mice (n=4 mice/group) were injected IP with 5 mg of purified rabbit polyclonal antibodies against A33, B5, or L1, or 5 mg of VIG. Unimmunized mice were injected in parallel with PBS and untreated mice were not immunized or challenged. One day later, mice were challenged IN with  $10^6$  pfu of VACV WR and weighed daily for 12 days. Mice were euthanized if their weight fell below 70% of initial weight. Panel A shows the percent of survivors and the mean percentage of weight change in all surviving mice +/- standard error of the mean (SEM) is shown in panel B. (C,D) Mice were immunized with 2.5 mg each of anti-A33 plus anti-L1 and 2.5 mg each of anti-B5 plus anti-L1 such that all mice received a total of 5 mg of PAb. Control mice were unimmunized as described above or received 5 mg of an anti-herpes simplex virus gD PAb. Virus challenge was carried out as described in panel A and B. Abbreviations: Unimm, unimmunized; Untr, untreated.

Similarly, mice receiving anti-A33 showed a statistically significant less weight loss 5 and 6 days after challenge compared to unimmunized mice ( $p=0.0477$  and  $p=0.0320$ , respectively), but no statistical differences were seen in mice given B5 PAb in spite of an observable difference in weight loss. VIG was given as a positive control to protect mice from virus challenge at a dose 2.5 times greater than the recommended human dose based on weight. This human polyclonal antibody mixture was less protective against death and weight loss compared to any of the rabbit PAbs (figure 4-2A and B), and the weight recovery shown in figure 4-2B after 7 days post-infection represents the one surviving mouse.

The limited protection of mice with individual PAbs suggested that a combination of PAbs may better protect against weight loss following challenge. Mice were immunized IP with combinations of PAbs so the total dose was 5 mg of IgG. Serum titers were proportionate to the amount of PAb administered and were typically less than or similar to titers observed after 3 immunizations with multiple proteins (table 4-3). We immunized an additional group of mice with rabbit PAb against an unrelated protein, gD, from herpes simplex virus. PAb against gD was also raised in rabbits by multiple immunizations with a recombinant protein expressed in baculovirus-infected insect cells and purified by nickel affinity chromatography. Once again, mice were challenged IN with  $10^6$  pfu of VACV WR one day after passive immunization. None of the unimmunized or gD PAb-immunized mice survived challenge and showed dramatic weight loss (figure 4-2C and D). Both groups of mice immunized with combinations of PAbs survived challenge, although mice receiving A33 and L1 PAbs showed less weight loss than

those given B5 and L1 (figure 4-2C and D). In fact, the mice immunized with A33 and L1 showed significant less weight loss than unimmunized mice or mice given gD PAb on 4 days post-infection ( $p=0.0281$  versus unimmunized and  $p=0.0150$  versus gD). We concluded from these challenge experiments that passive immunization with rabbit PAbs against VACV targets protected mice better than human VIG, and the best protection was observed by immunization with a PAbs against both IMV and EV targets, in this case anti-L1 with anti-A33 or anti-B5.

**Table 4-3: Comparison of reciprocal ELISA endpoint titers following immunization with combinations of PAb or proteins**

	Antigen		
	A33	B5	L1
A33+L1 pAb <sup>a</sup> 2.5 mg/Ab	1:152,218	-- <sup>c</sup>	1:75,000
A33+L1 active <sup>b</sup>	1:400,000	--	1:70,711
B5+L1 pAb 2.5 mg/Ab	--	1:29,730	1:29,730
B5+L1 active	--	1:70,711	1:70,711

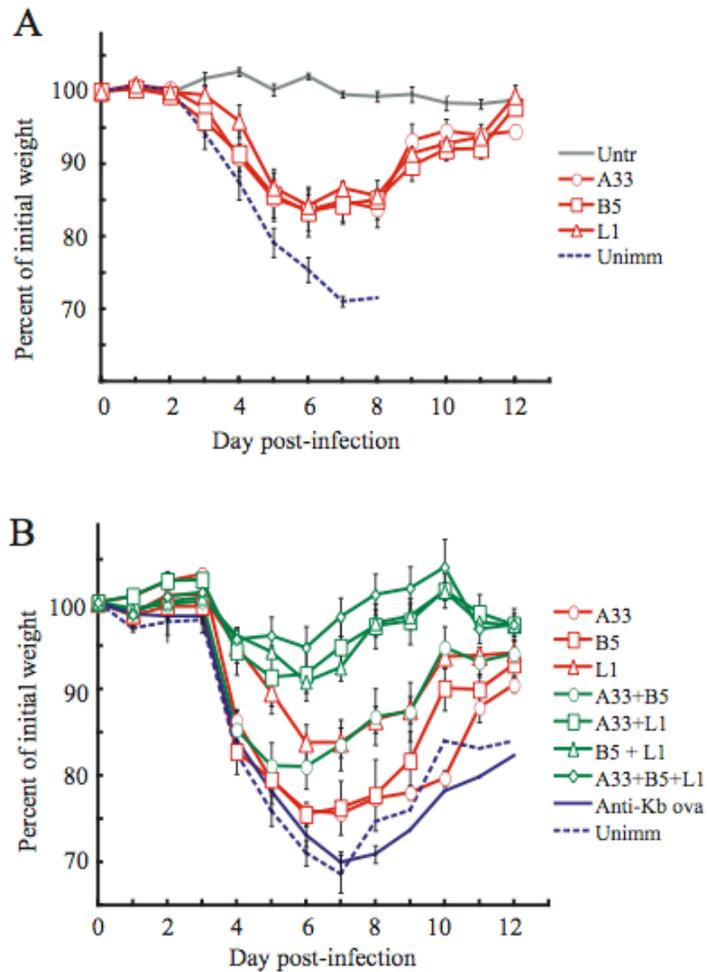
<sup>a</sup> Serum titers 24 hours after IP injection with a total of 5 mg PAb.

<sup>b</sup> Serum titers after three protein immunizations (geometric mean titers; chapter 3 and ref 222)

<sup>c</sup> --; not determined.

### 4.3.3 Protection of Mice with Individual or Combinations of MAbs

We used the same mouse challenge model as described above to examine protection of mice by passive immunization with A33, B5 and L1 MAbs. Mice were immunized IP with 200 µg of MAbs specific to A33, B5 or L1 and were challenged one day later. All unimmunized mice died, but all mice given MAbs survived challenge and lost approximately 15% of their initial weight prior to recovery (figure 4-3A). Likewise, practically all the mice shown in figure 4-3B survived, and were given only 100 µg of each MAb. Differences in weight loss were statistically significant in mice given 100 µg of L1 MAb compared to unimmunized mice on 4<sup>th</sup> and 6<sup>th</sup> day post-challenge ( $p=0.0356$  and  $p=0.0290$ , respectively), and similar significance was observed 6 days post-challenge with mice given anti-A33 or anti-B5 MAb ( $p=0.0400$  and  $p=0.0444$ , respectively; compared to unimmunized). Combinations of MAbs provided the best protection against challenge, similar to observations with mice immunized with PAbs. 25% survival was observed in unimmunized mice or those given an irrelevant MAb (K<sup>b</sup>-ova; specific to an ovalbumin determinant complexed with an MHC class I comprised of an H-2K<sup>b</sup> heavy chain) (figure 4-3B). On the other hand, complete survival was seen in mice immunized with 100 µg each of A33 and L1 MAbs, B5 and L1 MAbs, or all three MAbs (figure 4-3B).

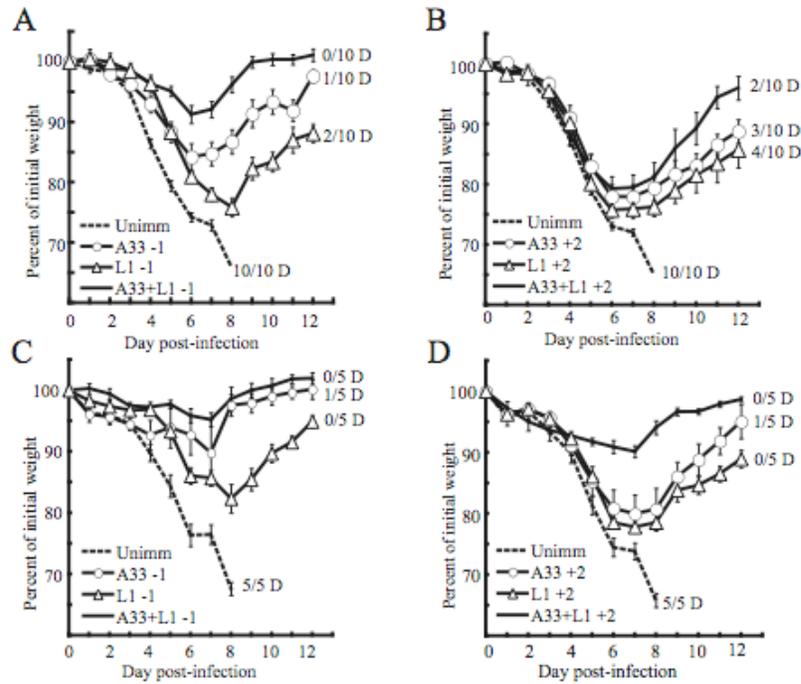


**Figure 4-3: Protection of mice by passive immunization with MAbs.** (A) 14-week old female BALB/c (n=4 mice/group) were immunized IP with 200  $\mu$ g of A33, B5 or L1 MAbs and challenged one day later as described in the legend for figure 4-3. All immunized mice survived and the average percentage of the initial weight  $\pm$  SEM was determined daily for each group. (B) 14-week old female BALB/c (n=4 mice/group) were immunized IP with 100  $\mu$ g of each antibody alone or in all possible combinations. Mice were challenged one day later in the same manner as described above, and all immunized mice but one given anti-B5 survived challenge. The average percentage of initial weight for each group  $\pm$  SEM is shown for each day after challenge.

The difference in weight in groups given any of these MAb combinations was statistically significant compared to unimmunized mice ( $p < 0.001$  on days 4, 5 and 6 for each combination). Mice given 100 mg each of the two EV antibodies against A33 and B5 showed greater weight loss than groups given combinations of IMV and EV MAbs, but weight loss was still significantly better compared to unimmunized mice ( $p = .0012$  on 6<sup>th</sup> day post-infection). Once again, we conclude that superior protection resulted from passive immunization with a combination of MAbs against IMV (e.g., L1) and EV (e.g., A33 and B5) targets.

#### **4.3.4 Effect of Time of Passive Immunization of MAbs Relative to Challenge**

The protective effect of passive immunization after challenge was tested next in light of the successful protection of mice immunized before challenge. In this experiment, 7-week old mice were used instead of 14-week old mice, and the younger age of mice was reflected in greater susceptibility to challenge since the challenge dose of  $10^6$  pfu was calculated to be equivalent to 6 LD<sub>50</sub> rather than 3 LD<sub>50</sub> (in 14-week old mice). The data shown in panels A and B of figure 4-4 is from two independent identical experiments, each with 5 mice per group, and the total number of dead mice is shown next to each trend line. The majority of mice immunized with anti-A33 or anti-L1 MAb, or a combination of both MAbs, survived challenge, although less weight loss was seen in mice given the combination of MAbs (figure 4-4A and B).



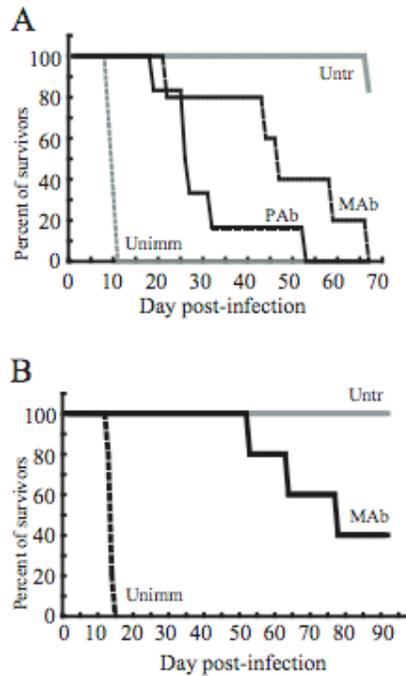
**Figure 4-4: Immunization before or after intranasal virus challenge.** (A,B) 7-week old female BALB/c mice (n=5 mice/group) were immunized with 100  $\mu$ g of anti-A33 or anti-L1 MAbs or a combination of both either one day before challenge (-1) or two days after challenge (+2) with 10<sup>6</sup> pfu of VACV WR. Mice were weighed daily and the number (#/10) indicates how many mice were sacrificed or found dead. These results represent the average change in weight and total number of dead mice for two independent experiments. (C,D) 7-week old female BALB/c mice (n=5 mice/group) were immunized in the same as described above but were challenged with 5 x 10<sup>5</sup> pfu of VACV WR instead. The number of mice that died or were euthanized is represented by the number (#/5). The average percentage of initial weight +/- SEM for each group is shown.

Mice given anti-A33 MAb prior to challenge were protected from significant weight loss compared to unimmunized control mice ( $p \leq 0.0003$  on days 4, 5 and 6 for anti-A33 compared to control). Protection from weight loss was also statistically significant compared to unimmunized mice for groups given anti-L1 or anti-L1 and anti-A33 MAbs before challenge ( $p \leq 0.0022$  on days 3, 4, 5 and 6 for anti-L1 or anti-L1 plus anti-A33 antibodies compared to control). Immunization of mice two days after challenge also protected the majority of mice from weight loss and death, although more immunized mice died and had greater weight loss (figure 4-4B).

The mice used in the experiments shown in figure 4-4 are younger than mice used in previous experiments, so we also challenged mice with the equivalent  $LD_{50}$  (equal to  $5 \times 10^5$  pfu) as used in earlier experiments in 14-week old mice (figure 4-4, panels C and D). None of the unimmunized mice survived challenge, but figure 4-4C shows that weight loss was significantly less severe and survival was improved in all groups of mice given antibodies before challenge ( $p \leq 0.0340$  on days 3 to 6 for anti-A33 and anti-L1 combination compared to control and  $p \leq 0.0064$  on days 4 to 6 for anti-A33 or anti-L1 compared to control). Figure 4-4D shows the lower challenge dose resulted in greater survival and less weight loss in mice given antibodies 2 days after challenge compared to control mice ( $p \leq 0.0042$  on days 5 to 7 for the combination of antibodies versus control and  $p=0.0266$  on day 7 for anti-A33 versus control). Most noticeably, relatively little weight loss was observed in mice given a combination of MAbs against A33 and L1 after challenge.

#### 4.3.5 Passive Immunization of SCID Mice

All of the animal challenge experiments described thus far that show protection by passive immunization use BALB/c mice. These mice have intact immune systems and can recruit an adaptive immune response in the face of challenge, thus passively administered antibodies are not the lone contributors to protection. We challenged SCID mice, which lack B- and T-cells and do not generate adaptive immune responses, as a way to better observe the contribution of passive immunization to protection. Mice were immunized with a equal amounts of all three MAbs or PABs against A33, B5, and L1 such that the total amount given was 300  $\mu$ g of MAb (100  $\mu$ g/MAb) or 5 mg of PAB (1.67 mg/PAB). All unimmunized mice challenged with  $10^6$  pfu of VACV WR died or were euthanized by the 11<sup>th</sup> day after challenge (figure 4-5A). Mice immunized with PAB or MAb had prolonged survival, with a 50% survival time 26 and 46.5 days, respectively (figure 4-5A). In spite of prolonged survival, all mice eventually exhibited typical symptoms of VACV infection prior to being sacrificed or dying, including labored breathing, ruffled fur, and a hunched posture. Passive immunization with a combination of MAbs, which yielded the longest survival time, was repeated with a lower challenge dose to see if survival could be further extended. Once again, all unimmunized control mice died or were euthanized within two weeks, but 2 of the 5 immunized mice were still alive after three months, at which time the experiment was terminated (figure 4-5B).



**Figure 4-5: Survival of passive immunized SCID mice following virus challenge.**

(A) 8-week old female BALB/c SCID mice (n=5 mice/group) were immunized IP with a mixture of 1.67 mg each of PABs against A33, B5 and L1 (5 mg total) or with 100 mg each of MAbs against A33, B5, and L1 (300 mg total). Mice were challenged one day later with  $10^6$  pfu of VACV WR and were weighed daily and euthanized if weight fell more than 30% of initial weight. (B) 8-week old female BALB/c SCID mice (n=5 mice/group) were immunized IP in the same manner as panel A and were challenged one day later with  $10^4$  pfu of VACV WR and weighed daily. Abbreviations: Unimm, unimmunized; Untr, untreated.

#### 4.4 Discussion

Antibodies have been shown to be essential for best possible protection against orthopoxvirus infection [92, 231, 232]. Optimal protection is also associated with immunity against both forms of infectious virus as immunization with inactivated virus, which is strictly IMV, is insufficient. Hence, we undertook a study to determine if polyclonal or monoclonal antibodies against IMV and EV targets would protect mice against a lethal respiratory virus challenge. Rabbits were immunized with recombinant forms of the EV proteins A33 and B5 and the IMV protein L1 to make specific PAbs since we had previously shown protection of mice immunized with the same proteins (chapter 3, reference 222). Characterization of these recombinant proteins is described in the preceding chapter, and biochemical analysis suggested that these proteins had similar characteristics as their viral counterparts and were recognized by monoclonal antibodies specific to viral epitopes of A33, B5 or L1. We also used these MAbs for passive immunization experiments, and initial characterization of these antibodies showed that anti-L1 PAb and MAb were able to neutralize IMV in vitro, and PABs and MAbs against A33 or B5 inhibited the spread of EV in cell culture.

Animal challenge experiments used the BALB/c mouse respiratory infection model with VACV WR challenge as described in chapter 3. Intranasal infection of mice causes a severe respiratory illness from virus replication in the lungs, and this corresponds to weight loss and death typically within 7 to 10 days following inoculation [217]. This model is widely used for testing alternative smallpox vaccines [218, 223, 230, 233] and therapeutic treatments [216, 217, 234] because it

bears resemblance to the natural route of variola virus infection in humans.

Importantly, intranasal challenge allows us to passively immunize mice by the IP route and examine protection of systemically available antibodies.

Passive immunization with individual PABs or MABs partially protected mice against weight loss and death, but superior protection resulted from immunization with a combination of antibodies against IMV and EV targets, namely, L1, A33 and B5. Anti-L1 antibodies may inhibit infection caused by the virus inoculum comprised of purified IMV particles, since these antibodies neutralized IMV *in vitro*. Anti-L1 PAb or MAb may also neutralize EV particles with outer membranes disrupted during virus entry [235, 236] or by complement-mediated mechanisms, which both result in exposure of the IMV membrane. Recently, our lab has described a mechanism by which the presence of an anti-A33 Ab and complement disrupts the EV membrane and allows for neutralization by anti-L1 Ab. The combined effect of anti-A33 and anti-L1 Abs with complement *in vitro* is one possible reason for their effectiveness as a combination *in vivo*. Interestingly, mice passively immunized with anti-L1 antibody or actively immunized with L1 protein (chapter 3, reference 236) had an initial delay in weight loss, although this effect was not statistically significant. This observation suggests that anti-L1 antibodies may be neutralizing the virus inoculum and may be less effective when given after challenge. Our results refute this hypothesis since mice given anti-A33 and anti-L1 MAb after challenge were better protected compared to mice given anti-A33 antibody alone. Therefore, a combination of antibodies against EV and IMV effectively reduce virus replication and systemic spread *in vivo*.

SCID mice also showed protection against virus challenge by passive immunization with a combination of PAbs or MAbs. Immunized mice eventually succumbed to illness resembling the symptoms of VACV infection, indicating that passively administered antibody is not sufficient to clear virus in the absence of other adaptive immune responses. Circulating antibody levels were probably quite low when mice succumbed to disease 1 to 3 months following challenge, since the half-life of anti-A33 and anti-L1 MAbs was experimentally calculated to be 4.8 and 10.2 days, respectively. These calculations are similar to published reports of the half-lives of murine IgG2a and IgG3 [237], and likewise, the half-life of rabbit IgG in mice has been reported to be 5 to 6 days [238]. The half-life of these antibodies was not determined in SCID mice, but survival of immunized mice may have been further prolonged by additional passive immunizations after challenge.

The effective protection of mice with a combination of antibodies against A33, B5, and L1 is only evident in the face of animal challenge studies. To this end, evaluation of new antibody products or assessment of the potency of VIG can only be effectively tested in animal models in the absence of an *in vitro* assay that effectively corresponds to protection studies. *In vitro* assays can clearly delineate IMV neutralizing antibodies, but the anti-EV neutralization assay is less consistent since EV particles cannot be frozen or stored for long periods of time and the outer membrane of EV is easily broken. Anti-IMV antibodies must be used to neutralize damaged EV particles prior to addition of EV-neutralizing antibodies for an accurate measure of neutralization. Most importantly, EV neutralization assays do not effectively reveal antibodies that are protective *in vivo*, such as those against A33

[230]. For these reasons, ELISAs specific to EV proteins and comet reduction assays are preferred as a strategy to analyze the concentration and functionality of anti-EV antibodies. Nonetheless, animal protection studies are the most informative means to preliminarily evaluate the effectiveness of antibodies.

Mice were immunized with human VIG as a positive control for protection in the absence of other known and available protective antibodies. Serological analysis and challenge experiments showed that VIG was less effective than combinations of PAbs and MAbs against A33, B5, and L1. These results indicate that human MAbs against specific virus proteins may be a safer and a more effective alternative to VIG.

## **Chapter 5**

### **Protection of Mice Against a Lethal Challenge by Multiple Immunizations with Different Proteins of the Intracellular Mature Virus**

#### **5.1 Introduction**

The efficacy of recombinant VACV proteins as protective immunogens was initially evaluated by the experiments described in chapter 3. Mice immunized with recombinant forms of the EV proteins A33 and B5 and the IMV protein L1 were partially protected against a lethal challenge when given any one of the individual proteins, but superior protection was observed in mice given a combination of EV and IMV proteins. The success of this immunization strategy led us to explore the use of other VACV immunogens. This chapter will discuss experiments comparing the protection of mice immunized with A33, L1 and an alternative IMV protein, A27.

A27 is expressed late during infection as a 14-kDa protein and forms covalently-linked homotrimers [239, 240]. A27 does not contain a transmembrane domain and is targeted to the surface of IMV by interacting with another VACV protein, A17, through a C-terminal domain [241]. A27 has been considered to be important for virus-cell interactions [242] and may play a role in virus attachment, owing to the ability to bind heparan sulfate [26]. A27 has also been implicated as a fusion protein [17, 172, 239, 243], but Ward and co-workers recently constructed a

deletion mutant virus lacking A27 that was able to penetrate cells but was severely restricted in cell-to-cell spread [244]. A27 is also needed for EV formation and dissemination since repression of the gene or deletion of the 29 residues at the N-terminus causes a decrease in EV synthesis [245, 246].

A27 is the target of neutralizing antibodies [172, 247] and passive immunization with an anti-A27 MAb protects mice against an intraperitoneal (IP) virus challenge [226]. Mice immunized with an *E.coli*-expressed form of A27 formed IMV-neutralizing antibodies and were also protected against a lethal IP challenge [173]. Recent studies have shown that mice immunized with DNA encoding the A27 gene produce neutralizing antibodies [176] and show proliferation of T<sub>CD4+</sub> cells, as well as induction of antigen-specific T<sub>CD8+</sub> and humoral responses [248]. Surprisingly, mice immunized with A27 DNA alone were not protected against a lethal IP virus challenge, in spite of inducing A27-specific neutralizing antibodies [176]. Rhesus monkeys immunized multiple times with a DNA vaccine comprised of plasmids encoding A27, L1, A33 and B5 also produced neutralizing antibodies and were protected against a lethal intravenous monkeypox virus challenge [177].

The goal of the study described in this chapter is to determine if immunization of mice with A27 induces immune responses similar to or better than induced by L1. Our previous study analyzed two different EV proteins, and showed that A33 was consistently more immunogenic and protective than B5. Therefore, we wanted to ascertain if an alternative IMV protein would induce superior immune responses

compared to L1. A similar study design was used as described in chapter 3, and our results suggest that A27 is not a superior immunogen in contrast to L1.

## **5.2 Materials and Methods**

### **5.2.1 Cells and Viruses**

BS-C-1 monolayers and HeLas S3 suspension cells were maintained as detailed in chapter 3, section 3.2.1. Virus stocks of VACV WR, IHD-J, and VV-NP-SIINFEKL-EGFP were grown in HeLa cells and purified by sucrose density centrifugation as described in chapter 3, section 3.2.1. Viral titers were determined by plaque assay.

### **5.2.2 Recombinant Proteins**

Collaborators in the laboratory of Drs. Gary Cohen and Roselyn Eisenberg (University of Pennsylvania, Philadelphia, PA) prepared the recombinant proteins used in this study. Full length A27, and truncated forms of A33 and L1 lacking transmembrane domains were expressed in insect cells infected with recombinant baculoviruses as described in detail by Aldaz-Carroll and co-workers [214]. Each protein included a polyhistidine tag to facilitate purification by nickel affinity chromatography. The purity of each protein was confirmed by SDS-PAGE and staining of gels with a colloidal Coomassie blue dye (GelCode Blue, Pierce) as described in chapter 3.2, section 3.2.3. Biochemical analysis of the previously uncharacterized baculovirus-expressed A27 protein included SDS-PAGE of denatured protein that was non-reduced or reduced with  $\beta$ -ME. A27 was also characterized by Western blot analysis as explained in chapter 3.2, section 3.2.3.

Briefly, proteins were transferred to a nitrocellulose membrane (Invitrogen) following electrophoresis and blocked overnight in 5% BSA diluted in TBST. A polyclonal anti-A27 antibody raised by multiple immunizations of a rabbit was used as a primary antibody diluted 1:1000 in 5% BSA/TBST, and an HRP-conjugated anti-rabbit IgG (Amersham, diluted 1:10,000 in 5% BSA/TBST) and chemiluminescence (Pierce) was used for visualization. Purified IMV was also analyzed by Western blotting as a control to confirm that the anti-A27 PAb recognized the A27 protein. Purified WR IMV was thawed and sonicated and diluted in LDS sample buffer and deionized water with or without  $\beta$ -ME as a reducing agent. Samples were heated for 5 minutes at 95°C the equivalent of  $2.4 \times 10^7$  pfu of VACV WR was subjected to SDS-PAGE and western blotting in parallel with recombinant A27 protein.

### **5.2.3 Immunization Protocol**

5- to 6-week old female BALB/c mice were purchased from Taconic and housed in sterile microisolators at an animal facility at the National Institutes of Health. Protein immunizations were carried out in the same manner as described in chapter 3, section 3.2.5. Individual or combinations of recombinant proteins were diluted in an emulsion of MPL+TDM adjuvant (Sigma-Aldrich) and sterile PBS for a final dose of 10  $\mu$ g of each protein per 0.1 ml. Mice were immunized subcutaneously at the nape of the neck with 0.1 ml with a 25-gauge hypodermic needle. Three immunizations were given at three-week intervals and blood was collected from the tail vein prior to each immunization and prior to challenge. Serum was separated by centrifugation from clotted blood samples and sera pools were made from mice

immunized with the same proteins. Sera samples were heat-inactivated at 56°C for 30 minutes to destroy complement activity.

#### **5.2.4 ELISA**

Plates coated with recombinant protein antigens were used for ELISAs to detect specific binding antibodies in the sera of immunized mice. 96-well plates were coated with protein diluted in PBS and incubated at 4°C overnight. The optimal concentration for each protein used to coat plates was 90 ng/well for A33, 150 ng/well for A27, and 40 ng/well for L1. ELISAs were carried out as described in chapter 3, section 3.2.7 and an HRP-conjugated anti-mouse antibody was used to detect mouse IgG bound to antigen followed by visualization with a chromogenic substrate of a ready-to-use solution of 3,3',5,5'-tetramethylbenzidine (BM Blue, POD substrate, Roche). Spectrophotometric measurements were made at  $A_{370}$  and  $A_{492}$  and reciprocal endpoint titers were determined as the dilution with an absorbance of 0.1 following subtraction of background absorbance of serum samples incubated on plates not coated with protein.

#### **5.2.5 IMV Neutralization and Comet Reduction Assays**

The presence of IMV-neutralizing antibodies was measured using a flow cytometric-based assay with an EGFP-expressing recombinant VACV (VV-NP-SIINFEKL-EGFP). Twofold serial dilutions of serum was mixed with VV-NP-SIINFEKL-EGFP and used to infect HeLa S3 suspension cells as previously described in chapter 3, section 3.2.8 and reference 214. Virus replication was indirectly quantified by flow cytometric measurement of EGFP expression. The

neutralization titers described here are the sera concentrations that inhibit infection by 50% relative to values measured in HeLa cells infected with VV-NP-SIINFEKL-EGFP, but not treated with sera.

Anti-EV antibodies that inhibit secondary satellite plaques in cell culture were observed with the comet reduction assay. BS-C-1 cell monolayers in 6-well plates were infected with IHD-J and treated with a 1:50 dilution of serum as detailed in chapter 3, section 3.2.8. Plates were incubated for 36 hours at 37°C and comet-shaped plaques were visualized by staining of monolayers with crystal violet.

### **5.2.6 Virus Challenge**

Three weeks following the third protein immunization mice were challenged intranasally with VACV WR as explained in chapter 3, section 3.2.9. A fresh aliquot of purified virus was used for each challenge and was diluted in sterile PBS to a final concentration of  $10^6$  pfu or  $2 \times 10^7$  pfu per 20 ml, which is roughly equivalent to 5 and 100 times the  $LD_{50}$ , respectively. Sedated mice were inoculated intranasally with 20  $\mu$ l of virus (10  $\mu$ l/nostril) and weighed daily for two weeks. Mice were euthanized if their weight was below 70% of their initial weight. This mouse protocol was approved by the NIH Animal Care and Use Committee.

### **5.2.7 Statistical Analysis**

Mouse weight loss data was analyzed statistically to determine if any significant differences existed between mouse groups. The area under the curve (AUC) corrected for the follow-up period was calculated for days 2 through 9 as a summary statistic with a trapezoidal rule using all available measurements [249].

AUC values were compared between groups using a non-parametric Wilcoxon rank sum test adjusting  $p$ -values according to the method described by Holm [250] in order to control family wise error rate in multiple tests.

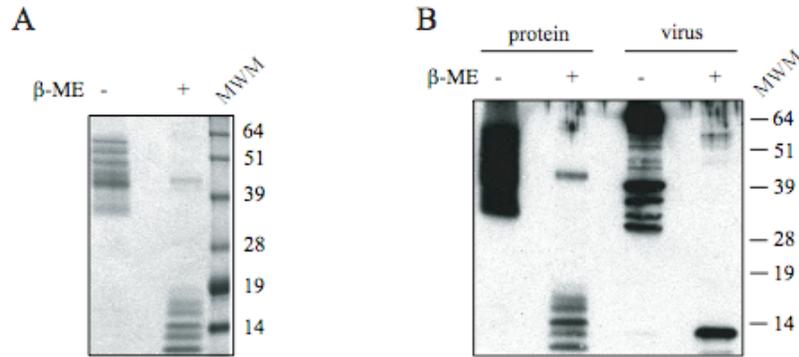
### **5.3 Results**

#### **5.3.1 Biochemical Characterization of Recombinant A27 Protein**

A recombinant form of the A27 protein of VACV was expressed in insect cells infected with a recombinant baculovirus and purified from the cell supernatant by nickel affinity chromatography. A27 is expressed late during virus infection as a 14-kilodalton protein and forms disulfide-bonded trimers that associate with the A17L protein on the surface of IMV particles. Figure 5-1, panel A shows SDS-PAGE and Coomassie staining of recombinant A27 and confirmed the molecular weight of the monomeric protein under reducing conditions (+  $\beta$ ME) as well as the formation of a trimer in non-reducing conditions (-  $\beta$ ME). Multiple bands are seen in both conditions, and the lower molecular weight bands may be truncated or cleaved forms of the protein. Previous investigators found that *E.coli*-based expression of A27 resulted in the synthesis of full length protein and a 12-kDa form truncated at the N-terminus [173]. They confirmed that the truncated protein lacked the first two amino acids but did not determine how or why they were missing. In addition, the recombinant protein may be glycosylated since one *N*-glycosylation site is predicted and may result the higher molecular weight forms observed by Coomassie staining (figure 5-1A).

Western blotting was used to confirm the identity of the recombinant A27 protein. A polyclonal antibody was purified from the serum of a rabbit immunized

multiple times with recombinant A27 protein, as described for the production of PABs in chapter 4, section 4.2.3. As expected, the anti-A27 antibody recognized both the reduced and non-reduced (+ or -  $\beta$ ME) forms of recombinant protein. This antibody also recognized protein of a similar electrophoretic mobility in purified virus but the viral protein did not show the same heterogeneity in molecular weight (figure 5-1B). This purified protein was used in further experiments as an immunogen and as an antigen to detect A27-specific binding antibodies.



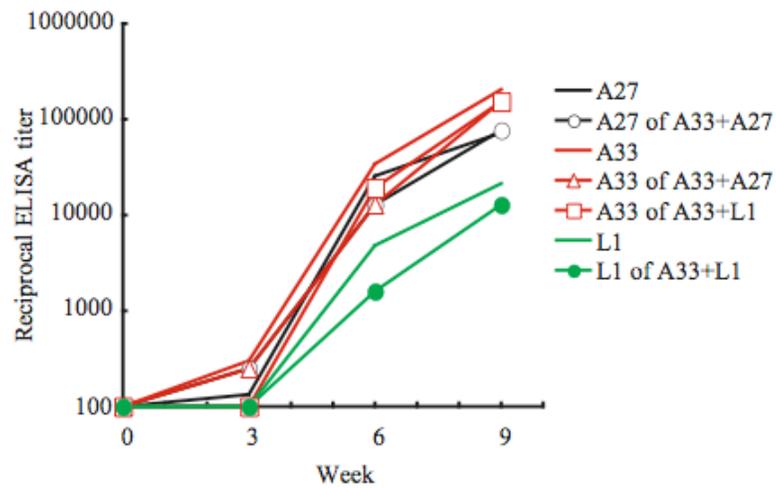
**Figure 5-1: Coomassie staining and western blot analysis of recombinant A27 protein.** (A) A27 expressed in insect cells with a recombinant baculovirus was purified by nickel affinity chromatography. Purified protein was denatured in LDS sample buffer and water with or without the reducing agent  $\beta$ -ME and was heated at 70°C for 10 minutes and 100 ng of protein was resolved in each lane by gel electrophoresis on a 12% NuPage Bis-Tris SDS-polyacrylamide gel with MOPS buffer. The gel was then stained with a colloidal Coomassie stain (GelCode Blue, Invitrogen). The symbols – and + denote the absence or presence of  $\beta$ -ME. A molecular weight marker (MWM) is shown in the rightmost lane and molecular masses are indicated in kilodaltons.

(B) Recombinant A27 protein (20 ng/lane, protein) was denatured and reduced as described for panel A. Reduced and nonreduced samples of purified IMV (virus) were also prepared and analyzed in parallel with protein samples. Resolved protein samples were transferred to nitrocellulose and analyzed by Western blotting with a polyclonal rabbit anti-A27 antibody and an HRP-conjugated anti-rabbit antibody followed by chemiluminescence visualization.

### 5.3.2 Antibody Responses Following Multiple Protein Immunizations

The results described in chapter 3 showed that mice immunized with L1 produced antibodies that were able to bind to L1 protein and virus lysate, and at least a subset of these antibodies neutralized IMV particles in vitro. Importantly, mice were partially protected against a lethal challenge when immunized with L1 alone, and protection was significantly improved by co-immunization with the EV proteins B5 or even more so with A33. This study expands on these results by comparing two different IMV proteins with respect to immunogenicity and protection of mice against a lethal intranasal challenge. Female BALB/c mice were immunized three times at three-week intervals with 10 µg of A33, A27, or L1 alone or with the EV and IMV protein pairings A33 and L1 or A33 and A27 (10 µg/protein). One day prior to each immunization or prior to challenge (at 9 weeks) mice were bled from the tail vein for serological analysis. ELISAs were carried out on pooled sera samples with A27, A33, or L1 used as binding antigens and the average reciprocal endpoints from two independent experiments are shown in figure 5-2. In each case, antibody titers were low after the first immunization but were boosted after the second and third immunizations. A33 induced the highest antibody responses after each immunization as an individual immunogen and this was similar to results observed in the study described in chapter 3 (figure 5-2). A33 responses were the same or slightly lower in mice immunized with A33 combined with L1 or A27 (figure 5-2). A27 protein alone induced higher antibody responses compared to L1, especially after two or three immunizations (figure 5-2). Mice immunized with pairs of proteins showed slightly lower responses to A27 or L1 after two immunizations, but there was very little or no

difference observed after three immunizations (figure 5-2). Overall, antibody responses were highest against A33 followed by A27 and L1. Mice immunized with combinations of proteins generated responses of a similar magnitude as mice given individual proteins, especially after three immunizations.

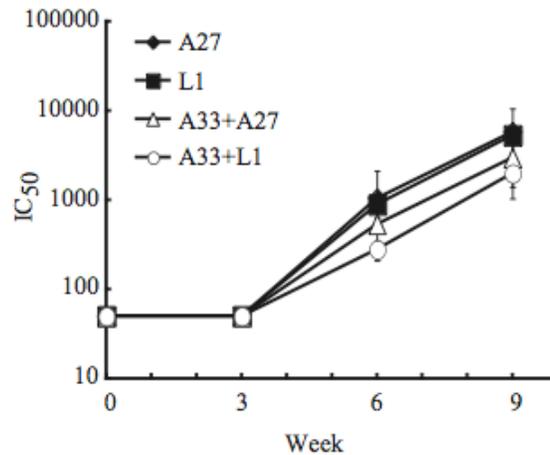


**Figure 5-2: Analysis of binding antibodies by ELISA.** Female BALB/c mice (n=5 mice/group) were subcutaneously immunized three times at 0, 3, and 6 weeks with 10  $\mu$ g of A27, A33, or L1 alone or with A33+L1 or A33+A27 (10  $\mu$ g of each protein) combined with MPL+TDM adjuvant and PBS. Mice were bled one day prior to each immunization or to virus challenge (at 9 weeks), and serum pools were analyzed by ELISA for binding antibodies specific to A33, A27 or L1. The reciprocal endpoint titers shown above are the average endpoints measured from two independent mouse experiments, and endpoint titers did not vary more than two dilutions between both experiments for all treatment groups.

### **5.3.3 Measurement of Neutralizing Antibodies Induced by Protein Immunization**

Neutralizing antibodies were measured using a flow cytometric-based assay with a recombinant VACV expressing an EGFP reporter gene as previously explained in chapter 3, section 3.2.4. Figure 5-3 shows that neutralizing antibodies were detected after two immunizations and further increased after a third immunization in each group as determined from two independent experiments. Standard deviations were calculated from the two independent experiments and are shown in figure 5-3. No significant differences between groups were detected after two or three immunizations, and neutralizing antibody titers were the same or slightly higher in mice given individual proteins compared to mice given protein combinations, similar to the results shown in figure 5-2. Surprisingly, neutralizing antibody titers were similar between mice given A27 or L1, in spite of A27 inducing higher binding antibody responses.

The comet reduction assay was used to observe the activity of anti-EV antibodies in vitro as described in chapter 3, section 3.2.4. Figure 5-4 shows the results of a comet reduction assay carried out with pooled serum samples from the second of two duplicate studies collected prior to the first immunization (0) or three weeks following the third and final immunization (3) and is representative of both studies. No comet reduction was observed in any groups prior to immunization, and also was not observed in mice immunized with either IMV protein (figure 5-4).



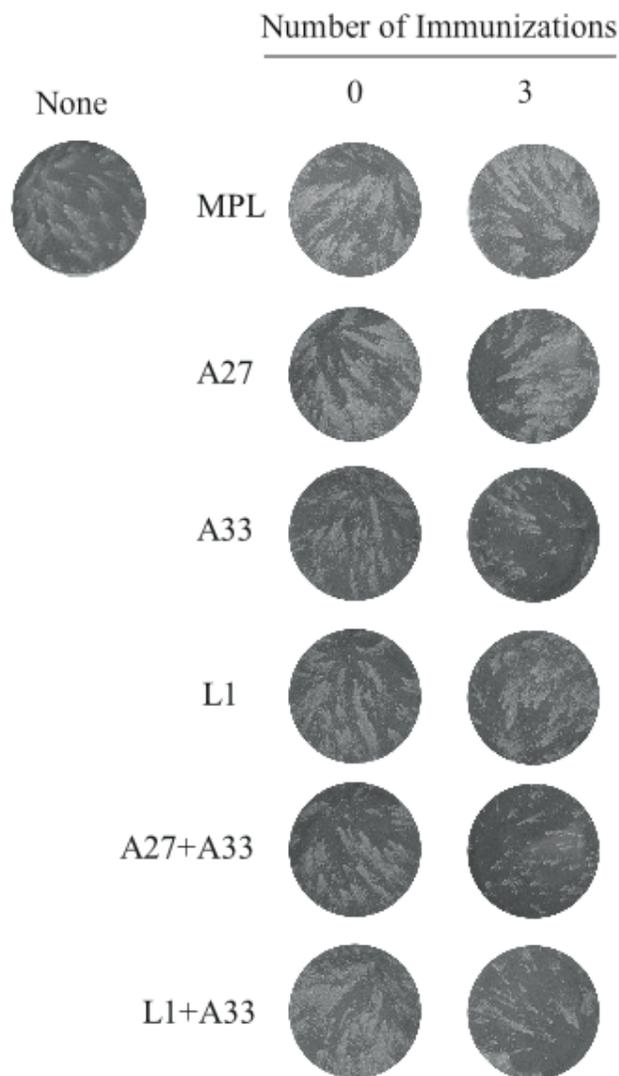
**Figure 5-3: Measurement of neutralizing antibodies following multiple protein immunizations.** Mice were immunized as described in figure 5-2. Neutralizing antibodies were measured in serum pools collected prior to each immunization at 0, 3 and 6 weeks or before challenge at 9 weeks. Neutralizing antibodies were measured in serum pools with an EGFP-based assay and titers were represented as the concentration of serum that inhibits infection by 50% relative to infected cells not treated with serum ( $IC_{50}$ ). The average titers + or – SD from two independent experiments are shown above.

Serum from mice immunized with A33 alone or combined with A27 or L1 noticeably reduced comets to a similar degree after three immunizations (figure 5-3), and was comparable to the results described in chapter 3 from mice immunized four times with A33.

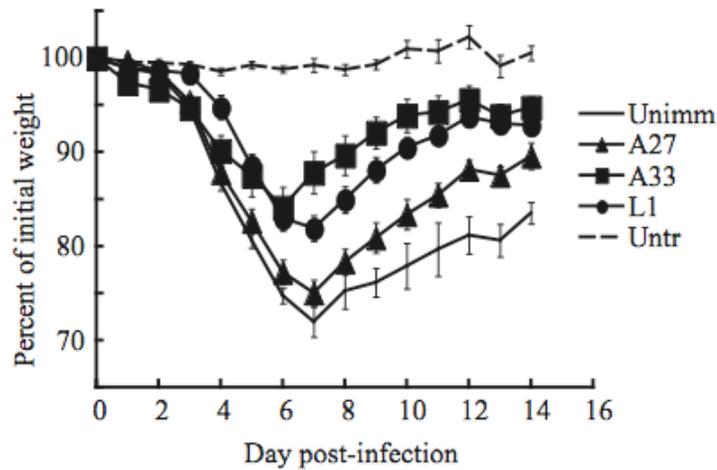
These results indicate that mice responded similarly to A33 and L1 protein immunizations in this study as previously observed in chapter 3. Mice immunized with A27 also showed a significant induction of antibodies and had a similar level of neutralizing antibodies as mice given L1 protein.

#### **5.3.4 Protection of Mice Following IN Challenge with $10^6$ pfu of VACV WR**

Female BALB/c mice were immunized three times with recombinant forms of A33, A27 or L1 and the results of serological analysis suggested that high levels of functional antibodies were produced. Only three immunizations were administered because the results of a dose response study described in chapter 3 showed little or no difference in protection following three or four immunizations with A33, B5, and L1 proteins. Mice were challenged intranasally with  $10^6$  pfu of VACV WR (equal to 5 LD<sub>50</sub>) as previously described in chapter 3, section 3.2.6. Mice were weighed daily for two weeks and were sacrificed if their weight fell below 70% of initial weight. The average percentage of initial weight for each treatment group from two independent experiments is shown in figure 5-5.



**Figure 5-4: Comet reduction assay.** The comet reduction assay was used as a way to detect anti-EV antibodies that inhibit the formation of secondary satellite plaques *in vitro*. Confluent BS-C-1 monolayers were infected with IHD-J virus and treated with heat-inactivated pooled sera (diluted 1:50) from mice prior to immunization (0) or three weeks following the final immunization (3). A well of cells that was infected but not treated with serum is shown above under the label “None.” Cells were stained with crystal violet 36 hours after infection to visualize plaques.



**Figure 5-5: Intranasal challenge with  $10^6$  pfu (5 LD<sub>50</sub>) of VACV WR.** Female BALB/c mice (n=10 mice/group) were immunized three times at three-week intervals with 10 µg of A33, A27, or L1 and MPL+TDM adjuvant. An additional group was unimmunized. Three weeks following the third immunization mice were challenged intranasally with  $10^6$  pfu of VACV WR (5 LD<sub>50</sub>) and were weighed daily for two weeks. Mice were sacrificed if their weight fell below 70% of their initial weight. Only 3 out of 10 unimmunized mice survived after the 7<sup>th</sup> day post-infection, while all immunized mice survived. The data shown above is the average percentage of initial weight for each group from two independent experiments +/- SEM. Abbreviations: Unimm, unimmunized; Untr, untreated.

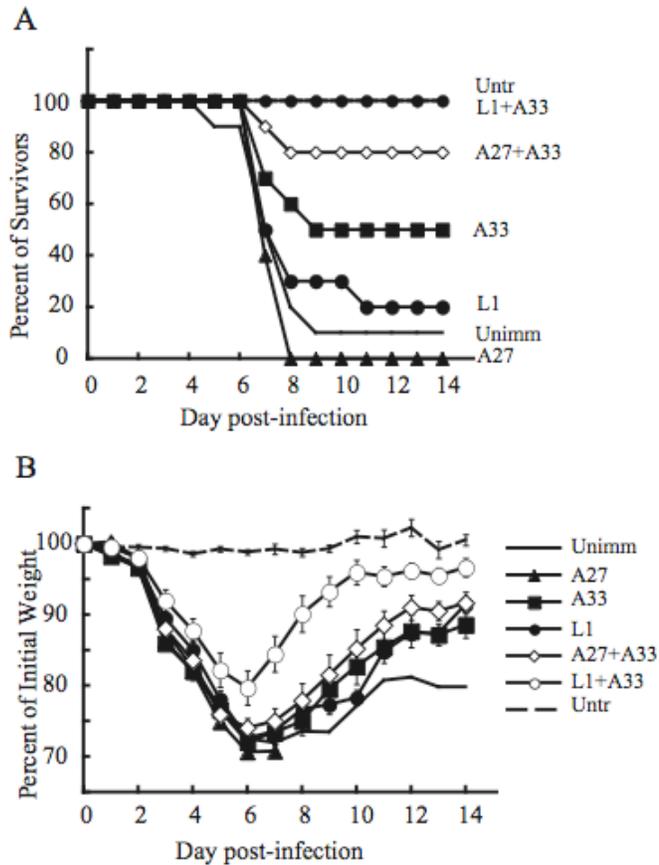
Additional control groups include unimmunized mice that were challenged with virus (Unimm) and untreated (Untr) mice that served as a control for daily weight fluctuations. Only 3 out of 10 unimmunized mice survived beyond the 7<sup>th</sup> day after challenge, but all protein-immunized mice survived challenge. Mice immunized with A33 and L1 had a similar degree of weight loss over time, while A27 immunization resulted in weight loss similar to what was observed in unimmunized mice (figure 5-5). Statistical analysis confirmed that L1 was significantly more protective against weight loss than A27 ( $p=0.01494$ ) and this comparison suggests that A27 is inferior to L1 as a protective immunogen.

### **5.3.5 Protection of Mice Following IN Challenge with $2 \times 10^7$ pfu of VACV WR**

Female BALB/c mice were immunized with recombinant forms of A27, A33, or L1 in the same manner as described in the preceding section. In addition, mice were immunized with two different combinations of IMV and EV proteins, A33 and A27 or A33 and L1. These combinations were included to determine if there were any differences in protection of mice immunized with A33 and different IMV proteins.

A challenge protocol described in chapter 3, section 3.2.6 showed protection of mice given IMV and EV protein combinations at a higher challenge dose of  $2 \times 10^7$  pfu (equal to 100 LD<sub>50</sub>) of VACV WR. Therefore, mice were challenged three weeks following the third protein immunization with this virus dose and were weighed for two weeks. Figure 5-6, panel A shows the percentage of survivors each day after challenge. The majority of unimmunized mice or those given A27 or L1 died, and

half of A33-immunized mice survived. A dramatic improvement in survival was seen in mice given either protein combination (figure 5-6A). Weight loss following challenge reflected the survival data to a degree. Overall, greater weight loss was seen in mice immunized with individual proteins compared to those challenged with  $10^6$  pfu of VACV WR (figure 5-6B). Weight loss in mice given A33+A27 did not differ from mice given single proteins, but mice immunized with A33+L1 showed the least weight loss (figure 5-6B). Statistical analysis confirmed these findings in that weight loss in A33+L1-immunized mice was significantly less than in mice immunized with A33 ( $p=0.02925$ ) or A27 ( $0.00329$ ) alone.



**Figure 5-6: Intranasal challenge with  $2 \times 10^7$  pfu (100 LD<sub>50</sub>) of VACV WR.**

Female BALB/c mice (n=10 mice/group) were immunized three times at three-week intervals with A33, A27, or L1, or the combinations A33+A27 or A33+L1 (10 µg/protein/immunization) and MPL+TDM adjuvant. An additional group was unimmunized. Three weeks following the third immunization mice were challenged intranasally with  $2 \times 10^7$  pfu of VACV WR (100 LD<sub>50</sub>) and were weighed daily for two weeks. Mice were sacrificed if their weight fell below 70% of their initial weight.

(A) Average percentage of survivors each day after challenge. (B) Average percentage of initial weight following challenge. The data shown above was calculated from the averages of two independent experiments +/- SEM.

Abbreviations: Unimm, unimmunized; Untr, untreated.

Despite the enhanced survival observed in mice immunized with A33+A27, the dramatic weight loss following challenge indicates that A27 is inferior to L1 as an immunogen given alone or in combination with A33.

#### **5.4 Discussion**

We investigated the immunogenicity and protection provided by immunization of mice with a recombinant form of the IMV protein A27. A previous study described in chapter 3 demonstrated that recombinant forms of A33, B5 and L1 induced specific antibody responses that were protective against infection in an intranasal virus challenge mouse model. L1 immunization also induced formation of IMV-specific neutralizing antibodies. In this study, a recombinant form of A27 was tested as an alternative IMV immunogen. This recombinant protein behaved similarly to its viral counterpart with respect to its electrophoretic mobility and its assembly into homotrimeric complexes. Specific binding antibodies were induced in mice following multiple immunizations with recombinant A33, L1 or A27. IMV neutralizing antibodies were also detected in mice immunized with L1 or A27. Protection against weight loss and death was not significant in A27-immunized mice, in spite of high binding and neutralizing antibody titers. Overall, A27 did not match or surpass L1 as an individual IMV-specific immunogen in the lethal intranasal mouse challenge model.

Our previous success of using a combination of IMV and EV proteins as immunogens was tested again by immunizing mice with A33 combined with L1 or A27. These results showed superior protection against both low and high virus challenge doses in mice immunized with A33 and L1, and this was confirmed in the

present study following challenge with  $2 \times 10^7$  pfu. The majority of mice immunized with A27 and A33 (80% survival) were protected against death, similar to mice given A33 and L1 (100% survival), but the former group was not significantly protected against weight loss. The relatively similar levels of neutralizing Abs measured in mice immunized with A27 or L1 was not predictive of subsequent protection, and this may be due to the kinds of Abs produced, including isotypes, and they function to inhibit infection *in vivo*. This data suggests that combining A27 with A33 does enhance protection compared to using either of these proteins alone, but A33 and L1 appear to provide even greater protection as a combination.

An earlier study by Lai and co-workers showed protection of mice following immunization with *E.coli*-expressed A27. Mice were immunized twice with either 50 or 10  $\mu$ g of protein with Freund's adjuvant and formed binding and neutralizing antibodies [173]. Both immunization doses protected mice against a lethal intraperitoneal virus challenge, but it is difficult to compare this data to our mouse model since a different challenge route was employed. A more recent study by Hooper and co-workers showed that multiple immunizations with a DNA vaccine encoding the A27 gene induced a specific and neutralizing antibody response [176]. Unlike the studies presented here and by Lai and co-workers, protection was not evaluated in mice immunized with A27 DNA alone and challenge experiments were only done in mice given the gene in combination with other VACV genes. On the other hand, mice were better protected against challenge when given B5 and L1 compared to B5 and A27, much like our observations.

The recombinant form of A27 is an effective protein for induction of both binding and neutralizing antibodies, but does not function significantly better than L1 as a protective immunogen. Given the difficulty and expense of developing a subunit vaccine with multiple immunogens, L1 appears to be the better choice over A27 for an MV protein in future vaccines.

## Chapter 6

### **Improved Antibody Responses to Recombinant Protein Immunization with Different Adjuvants Corresponds to Enhanced Protection of Mice and Monkeys against Orthopoxvirus Challenge**

#### **6.1 Introduction**

The vaccine studies described in chapter 3 and 5 provide compelling evidence that multiple recombinant VACV proteins induce protective immune responses in a mouse pneumonia model of infection. Individual EV proteins A33 or B5 or MV proteins L1 or A27 induced specific and neutralizing antibodies and partially protected mice after three or four protein immunizations combined with the MPL+TDM adjuvant system. Superior protection was observed in mice immunized with a combination of MV and EV proteins, especially with A33 and L1. These results were promising because high antibody levels are often induced by protein immunization, and the T<sub>CD4+</sub> cell-induced antibody response is presently considered the most important player for protection against orthopoxvirus infection in both mouse [127, 128, 132, 218] and monkey models [218, 251]. [218]

Immunostimulatory adjuvants can enhance the quality and scale of antibody responses to protein immunogens. Studies described by ourselves and others have been dependant on three or four protein immunizations for protection (Chapter 2, [174, 223]). A goal of the present study is to ascertain if different adjuvants can induce a more rapid and potent immune response to recombinant forms of A33 and

L1 in our mouse pneumonia model. Aluminum hydroxide gel (alum) is presently the only vaccine adjuvant licensed for human use and used in both pediatric and adult vaccines. Protein and DNA can be adsorbed to alum and these complexes are highly stable once formed [252]. Immunization with antigen-alum complexes forms an antigen depot at the site of injection, which enhances antigen uptake by antigen presenting cells. More recent studies have better characterized the immunostimulatory effects of alum, including the activation of cytokines and specific T-cell subpopulations [253, 254]. We are interested in exploring the efficacy of alum because of its long history as a safe adjuvant in a wide array of human and animal vaccines and its ability to be combined with other adjuvants to further enhance immunostimulation.

Several experimental adjuvants have been developed to induce higher immune responses to weak antigens or to optimize the type of immune response against a given pathogen. Lipopolysaccharide (LPS) from the cell membrane of Gram-negative bacteria is a potent, but toxic immunogen that was identified as an effective stimulator of the innate immune response via the toll-like receptor TLR4 [255]. Ribi and colleagues systematically modified LPS and developed a nontoxic derivative called monophosphoryl lipid A (MPL) that retained the same immunostimulatory qualities as LPS [256]. MPL has been safely used as a vaccine adjuvant in animal models and in human clinical trials against several infectious diseases and has been effective in shifting immune responses to some antigens from a Th2-dominant to a Th1-dominant response [257]. Trehalose dicorynomycolate (TDM) from the cord factor of the tubercle bacillus has also been used in combination with MPL to

enhance the adjuvant effect [258] and this emulsion adjuvant is often used as a safer alternative to Freund's complete adjuvant. Earlier work described in chapter 3 and 5 showed protection of mice following three or four immunizations with recombinant forms of A27, A33, B5 and L1 proteins combined with MPL+TDM [223].

QS-21 is a water-soluble saponin extracted from the bark of the *Quillaja saponaria* Molina tree that is also under development as an experimental adjuvant. QS-21 has been shown to enhance both humoral and cell-mediated immune responses and has been widely used in human clinical trials [259].

An emerging adjuvant strategy is the use of synthetic oligodeoxynucleotides with unmethylated CpG motifs (CpG ODNs). Bacterial DNA contains a high frequency of unmethylated CpG motifs, and these motifs alone have been shown to stimulate the innate immune response like MPL adjuvant but through recognition by the TLR 9 receptor [260-262]. CpG ODNs have been used in experimental vaccines and can induce a shift towards Th1-polarized responses in both animal models and humans [263] and can be combined with both mineral-based adjuvants like alum and emulsion adjuvants like MPL+TDM.

The study described in this chapter will compare the immunogenicity and protection of intranasally-infected mice immunized with recombinant forms of the VACV proteins A33 and L1 combined with alum, alum and CpG ODNs, MPL + TDM or QS-21 adjuvants. A small primate study will also be described that examines protection against monkeypox following multiple immunizations with recombinant VACV proteins A33, B5 and L1 and the QS-21 adjuvant.

## **6.2 Materials and Methods**

### **6.2.1 Cells and Viruses**

BS-C-1 monolayers and HeLa S3 suspension cells were maintained as detailed in chapter 3, section 3.2.1. Vero E6 cells were maintained at 37°C and 5% CO<sub>2</sub> in Dulbecco's modified essential medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, Logan, UT), 2 mM L-glutamine (Invitrogen, Carlsbad, CA), 10 U/ml penicillin and 10 µg/ml streptomycin (Invitrogen). Virus stocks of VACV WR, IHD-J, and VV-NP-SIINFEKL-EGFP were grown in HeLa cells and purified by sucrose density centrifugation as described in chapter 3, section 3.2.1. Plaque assays were used to determine titers of viral stocks.

MPXV strain Zaire 79 (V-79-I-005) originally isolated from the scab of an infected human by incubation in LLC-MK2 cells and passaged twice in BS-C-40 cells was obtained from J. Esposito and propagated in MA-104 cells. A titered clarified lysate was used for the virus challenge.

### **6.2.2 Recombinant Proteins and ODNs**

Recombinant baculovirus-expressed forms of A33 and L1 were provided by our collaborators at the University of Pennsylvania in the laboratory of Drs. Gary Cohen and Roselyn Eisenberg, and were expressed, purified and characterized as described in chapter 3.2, section 3.2.3. A mixture of two CpG ODNs (GCTAGACCGTTAGCGT and TCAACCGTTGA) were used as vaccine adjuvants. No endotoxin or protein was detected in ODN preparations as determined by the chromogenic Limulus amoebocyte lysate assay and bicinchoninic acid protein assay (Pierce), respectively.

### **6.2.3 Immunization Protocol**

Female BALB/c mice (5-6 weeks old) were purchased from Taconic and housed in sterile microisolators at an NIH animal facility. Mice were immunized subcutaneously and boosted three weeks later with 10 µg each of A33 and L1 proteins in phosphate buffered saline (PBS) alone or with alum, alum and 50 µg of phosphorothioate oligodeoxynucleotides (ODNs) containing CpG motifs, a Ribi-adjuvant system (MPL+TDM; Sigma-Aldrich, St. Louis, MO), or a saponin adjuvant QS-21 (Antigenics Inc., New York, NY). Proteins or proteins and CpG ODNs were adsorbed to alum by vortexing tubes containing immunogens while adding alum in a dropwise manner and then adding PBS to dilute mixtures to the appropriate concentration. MPL+TDM was solubilized in PBS to 2x concentration and combined with immunogens and PBS and vortexed to create a stable oil-in-water emulsion. QS-21 adjuvant (2 mg/ml stock in sterile water) was diluted with proteins and PBS to a final concentration of 15 µg/ml. All immunization mixtures were administered subcutaneously at a final volume of 100 µl. Mice were bled one day prior to each immunization or prior to challenge by tail bleed for serological analysis.

### **6.2.4 Intranasal Virus Challenge of Mice**

Mice were challenged intranasally three to four weeks after the second protein immunization as detailed in chapter 3, section 3.2.9. An aliquot of purified VACV WR was thawed, sonicated and diluted in sterile PBS immediately prior to challenge to a final dose of  $10^6$  pfu/20 ml (approximately 5 LD<sub>50</sub>). Mice were lightly sedated by inhalation of isoflurane and intranasally infected with 20 µl of virus inoculum (10 µl/nostril), and were monitored and weighed daily for two weeks. Mice were

ethanized if they lost greater than 30% of their initial weight. The NIH Animal Care and Use Committee approved this protocol.

### **6.2.5 Monkey Immunization and Challenge Protocol**

Three female cynomolgous monkeys were subcutaneously immunized with 100 µg each of A33, B5 and L1 proteins mixed with 50 µg of QS-21 adjuvant on days 0, 28, 57, and 251 of the study. A control monkey was immunized in parallel with only 50 µg of QS-21 and two additional monkeys remained unimmunized. Mice were bled one day before each immunization or before challenge for isolation of serum for serological analysis. Mice were intravenously challenged four weeks following the fourth immunization with  $5 \times 10^7$  pfu of MPXV and monitored daily for signs of illness. Supportive care and intravenous fluids were provided as needed during the course of challenge and blood was collected every three to four days for further analysis. Monkeys were housed at Bioqual, Inc. (Rockville, MD) during the immunization period and transferred to US Army Research Institute of Infectious Diseases (USAMRIID, Ft. Detrick, Frederick, MD) at the time of challenge. The USAMRIID and NIH Animal Care and Use Committees approved the protocols.

### **6.2.6 ELISA**

96-well round bottom plates (Corning) were coated with recombinant A33, B5 or L1 proteins or a vaccinia virus-infected cell lysate to detect specific binding antibodies as previously described chapter 3, section 3.2.7. Serum was heat-inactivated at 56°C for 30 minutes prior to analysis and reciprocal endpoint titers were determined by serial two-fold dilution of pooled mouse serum or individual

monkey serum samples. Total mouse IgG was detected by addition of anti-mouse ( $\gamma$ -chain) horseradish peroxidase (HRP)-conjugated antibody (Roche Diagnostics, GmbH, Mannheim, Germany) and isotype-specific antibodies were distinguished by using horseradish peroxidase-conjugated antibodies against murine IgG1 or IgG2a (BD Pharmingen, San Diego, CA). Monkey antibodies were detected with an anti-monkey immunoglobulin, Fc-specific peroxidase-conjugated antibody used at a 1:4000 dilution (Nordic Immunology, Tilburg, The Netherlands). A ready-to-use solution of soluble 3,3',5,5'-tetramethylbenzidine (BM Blue, POD substrate; Roche Diagnostics) was added to plates after removal of HRP-conjugated antibody and the  $A_{370}$  and  $A_{492}$  was measured with a spectrophotometer after incubation for 30 minutes at room temperature. Reciprocal endpoint titers were determined for mouse samples as the dilution with an absorbance of 0.1 after subtraction of background absorbance of serum samples incubated on plates not coated with protein. Similarly, titers of monkey samples were determined as the dilution with an absorbance two standard deviations above that measured in wells not treated with serum.

### **6.2.7 MV Neutralization and Comet Reduction Assays**

MV neutralizing antibodies were measured with a flow cytometric-based assay using a recombinant VACV that expresses EGFP (VV-NP-SIINFEKL-EGFP) as an indirect indicator of virus replication as previously described in reference [215] and chapter 3, section 3.2.8. The 50% neutralization titers were determined for both mouse and monkey sera samples as the concentration correlating to inhibition of infection by 50% relative to infection in the absence of serum.

The comet inhibition assay was used to observe inhibition of the spread of secondary satellite plaques by anti-EV antibodies. Mouse or monkey sera diluted 1:50 was added to BS-C-1 cell monolayers infected with IHD-J as detailed in chapter 3, section 3.2.8. Plates were incubated for 36-40 hours at 37°C and were stained with crystal violet to visualize the comet-shaped plaques.

### **6.2.8 Determination of MPXV Genomes in Blood**

Viral DNA was extracted from whole blood using the QIAGEN QIAamp DNA Mini Kit. A quantitative TaqMan-Minor Groove Binder polymerase chain reaction was set up with a pan-orthopoxvirus probe as previously described . Each sample was run in duplicate and the limit of detection for this assay was 200 genomes/ml of blood.

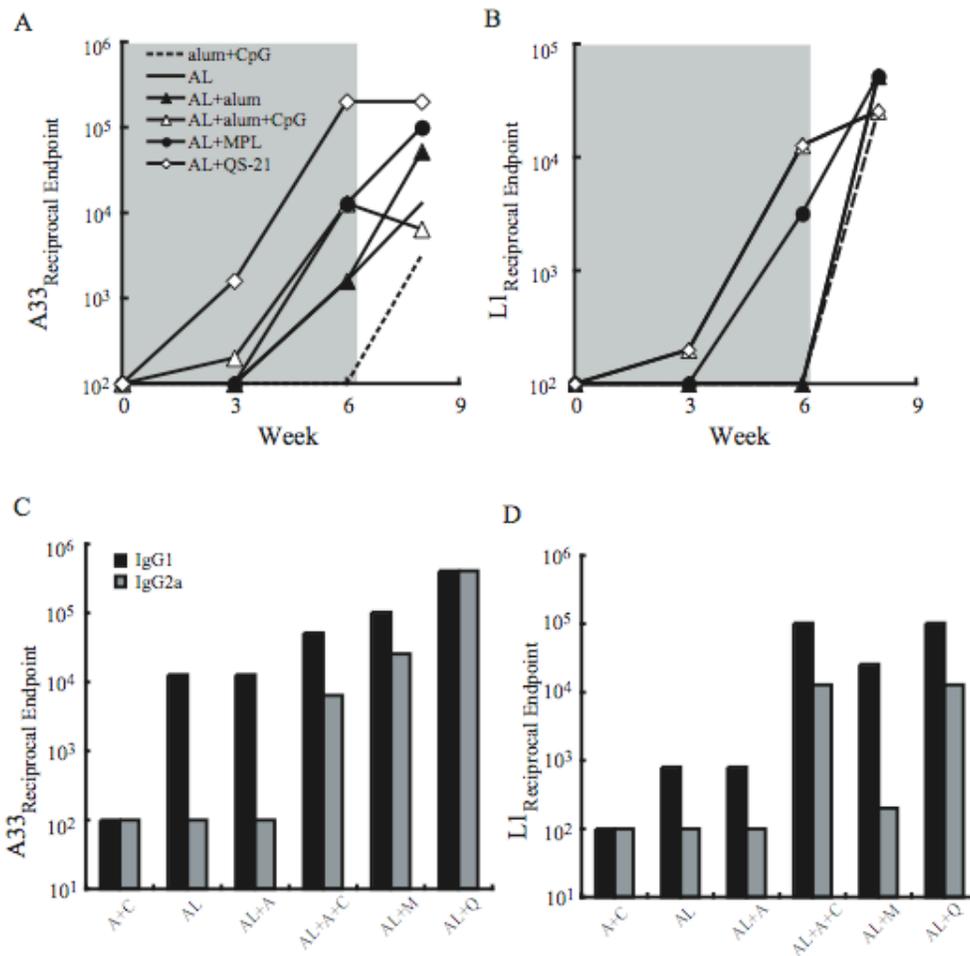
### **6.2.9 Statistical Analysis**

Statistical analysis was carried out with mouse weight loss data collected following intranasal challenge. To compare treatment groups, the area under the curve (AUC) corrected for the follow-up period was calculated for each mouse for days 2 through 14 post-infection as a summary statistic with a trapezoidal rule using all available measurements [249]. AUC values were compared between all treatment groups with the non-parametric Wilcoxon rank sum test adjusting *p*-values according to Holm [250] was used to control family wise error rate in the multiple tests. AUC analysis was also used to compare ELISA titers in individual mice, as well as monkey viral load and lesion count data, and a t-test was employed to compare groups.

## **6.3 Results**

### **6.3.1 Antibody Responses to A33 and L1 Proteins Following Immunization with Different Adjuvants**

In chapter 3, I described a multiprotein subunit vaccine that included the MV protein L1 and the EV proteins A33 and B5 that effectively protected mice against a lethal intranasal virus challenge following three or four immunizations [223]. A goal of the study described in this chapter is to determine if mice can be better protected from death and illness with fewer protein immunizations by using alternative adjuvants. The earlier study showed that mice were protected nearly as well with a combination of A33 and L1 compared to A33, B5 and L1. We decided to immunize mice with only A33 and L1 for this reason and because we thought we would better discern the effects of different adjuvants. Female BALB/c mice were immunized subcutaneously and boosted three weeks later with recombinant forms of the VACV proteins L1 and A33 with or without an adjuvant. The following adjuvants were used: alum, alum+CpG ODNs, MPL+TDM or QS-21. A group of mice was immunized with alum+CpG ODNs as a negative control for protein immunization. Serum was collected from mice prior to each immunization or prior to virus challenge to measure antibody responses. Mice immunized with protein and QS-21 were the only group to show a potent antibody response after one immunization and were further boosted after the second immunization to levels that were comparable to titers reached after three immunizations in our previous study with the MPL+TDM adjuvant (figure 6-1A, shaded box).



**Figure 6-1: ELISA values of pooled mouse sera following immunizations with A33 and L1 proteins combined with different adjuvants.** Mice (n = 5) were immunized twice with a mixture containing 10 mg each of A33 and L1 proteins (AL) alone or combined with the alum, alum+CpG, MPL+TDM (MPL) or QS-21 and challenged with 10<sup>6</sup> pfu of VACV WR at 3 weeks after the last immunization. Serum was collected prior to immunization (week 0), three weeks following immunizations 1 and 2 (shaded area), and two weeks after challenge (unshaded area). Antibodies to A33 (A) and L1 (B) were determined on pooled sera by ELISA and reciprocal endpoint values are plotted. Serum collected three weeks after the second immunization was re-analyzed for IgG1 or IgG2a isotype antibodies to A33 (C) and L1 (D).

In this study, protein-immunized mice given alum+CpG ODNs or MPL+TDM adjuvants showed similar anti-A33 titers, albeit lower than in mice given QS-21, but were higher than in mice immunized with alum or no adjuvant (figure 6-1A). L1 was less immunogenic than A33, as seen in our earlier studies, but a strong antibody response was observed after two protein immunizations with QS-21 or alum+CpG ODNs. Anti-L1 responses were lower with MPL+TDM adjuvants and undetectable with alum or no adjuvant (figure 1B). The effectiveness of adjuvants on antibody titers can be ordered as was QS-21 > alum+CpG ODNs > MPL+TDM > alum = no adjuvant. Antibody titers following challenge, shown in the non-shaded areas of figure 6-1, panels A and B, will be discussed in section 6.3.2.

The analysis of binding antibodies was done with pooled serum from mice in each immunization group. The serum of individual mice was analyzed after the second immunization to determine if there were statistically significant differences between groups and the titers are shown in table 6-1. Table 6-2 shows the *p*-values determined by statistical analysis for both A33 and L1 ELISAs. Antibody titers against A33 were significantly higher in mice immunized with protein and QS-21 compared to any other group ( $p < 0.00002$ ), and A33 titers were significantly higher in mice immunized with proteins and alum and CpG ODNs or with MPL+TDM compared to protein alone or with alum, but the former adjuvants did not differ significantly from each other. Similar trends were observed with L1 titers, but protein-immunized mice given QS-21 or alum and CpG ODNs did not differ significantly.

**Table 6-1: Individual ELISA titers against A33 and L1 after two immunizations.**

Immunization	Mouse ID	ELISA antigen	
		A33	L1
AL	2840	100	200
AL	2841	200	200
AL	2842	100	400
AL	2843	200	200
AL	2844	400	100
<b>AL</b>	<b>average</b>	<b>200</b>	<b>220</b>
AL+A	2850	400	100
AL+A	2852	400	100
AL+A	2853	400	100
AL+A	2854	400	100
<b>AL+A</b>	<b>average</b>	<b>400</b>	<b>100</b>
AL+A+C	2880	800	1600
AL+A+C	2881	25600	800
AL+A+C	2882	1600	3200
AL+A+C	2883	12800	12800
AL+A+C	2884	3200	12800
<b>AL+A+C</b>	<b>average</b>	<b>8800</b>	<b>6240</b>
AL+M	2860	12800	800
AL+M	2861	6400	6400
AL+M	2862	3200	800
AL+M	2863	3200	1600
AL+M	2864	51200	800
<b>AL+M</b>	<b>average</b>	<b>15360</b>	<b>2080</b>
AL+Q	2870	204800	3200
AL+Q	2871	409600	51200
AL+Q	2872	409600	25600
AL+Q	2873	204800	6400
AL+Q	2874	409600	3200
<b>AL+Q</b>	<b>average</b>	<b>327680</b>	<b>17920</b>

Abbreviations: AL, A33+L1; AL+A, A33+L1+alum; AL+A+C, A33+L1+alum+CpG

ODNs, AL+M, A33+L1+MPL+TDM; AL+Q, A33+L1+QS-21.

**Table 6-2: *P*-values determined by pairwise comparisons of individual ELISA data using t-tests with pooled standard deviations.**

**A33 ELISA**

	AL	AL+A	AL+A+C	AL+M
AL+A	0.3033	-	-	-
AL+A+C	<b>6.8e-05</b>	<b>0.0013</b>	-	-
AL+M	<b>6.6e-06</b>	<b>9.5e-05</b>	0.3033	-
AL+Q	<b>1.8e-10</b>	<b>1.4e-09</b>	<b>1.7e-06</b>	<b>1.6e-05</b>

**L1 ELISA**

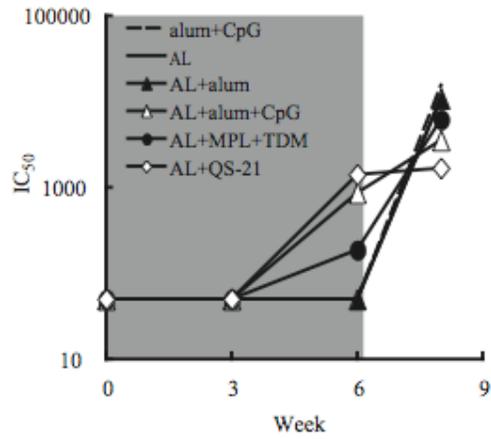
	AL	AL+A	AL+A+C	AL+M
AL+A	0.49920	-	-	-
AL+A+C	<b>0.00073</b>	<b>0.00017</b>	-	-
AL+M	<b>0.02188</b>	<b>0.00533</b>	0.37190	-
AL+Q	<b>2.6e-05</b>	<b>6.8e-06</b>	0.37190	<b>0.02188</b>

Significant *p*-values ( $p < 0.05$ ) indicated by numbers in **bold**.

Abbreviations: AL, A33+L1; AL+A, A33+L1+alum; AL+A+C, A33+L1+alum+CpG

ODNs, AL+M, A33+L1+MPL+TDM; AL+Q, A33+L1+QS-21.

Typically, protein immunization induces a predominantly Th2 response, which is characterized by IgG1 as the dominant immunoglobulin isotype in BALB/c mice. Th1-dominant responses are associated with virus infection and the IgG2a antibody isotype, but different adjuvants can also favor Th2 responses to protein immunogens. Figures 6-1A and 6-1B show the IgG1 and IgG2a levels generated by protein immunization with different adjuvants in pooled serum collected three weeks after the second immunization. IgG1 antibodies against A33 were seen exclusively in mice given protein alone or with alum, but IgG2a antibodies increased in mice given alum and CpG ODNs or MPL+TDM (figure 6-1C). Remarkably, the highest level of IgG2a was observed with QS-21. L1 antibodies were mostly of the IgG1 isotype in all groups but IgG2a titers increased in groups given protein with alum and CpG ODNs or QS-21 (figure 6-1D). These results indicate that both the scale and the isotype specificity of the antibody response are affected by the protein immunogen and adjuvant. QS-21 gave the most balanced response, indicated by the relatively similar levels of IgG1 and IgG2a antibodies. Alum+CpG ODNs or MPL+TDM were the next most effective adjuvants for increasing both the magnitude of the overall response and specific IgG2a titers. The isotype may be dictated by the type of antigen presenting cells that are most effective in stimulating the T-helper response, such as B-cells.



**Figure 6-2: Induction of neutralizing antibody.** The sera described in Figure 6-1 obtained from mice immunized with A33 and L1 (AL) plus the indicated adjuvants were used. MV neutralizing antibodies were measured with a flow cytometry-based GFP assay and the 50% inhibitory concentration ( $IC_{50}$ ) was determined for each pool of mouse sera.

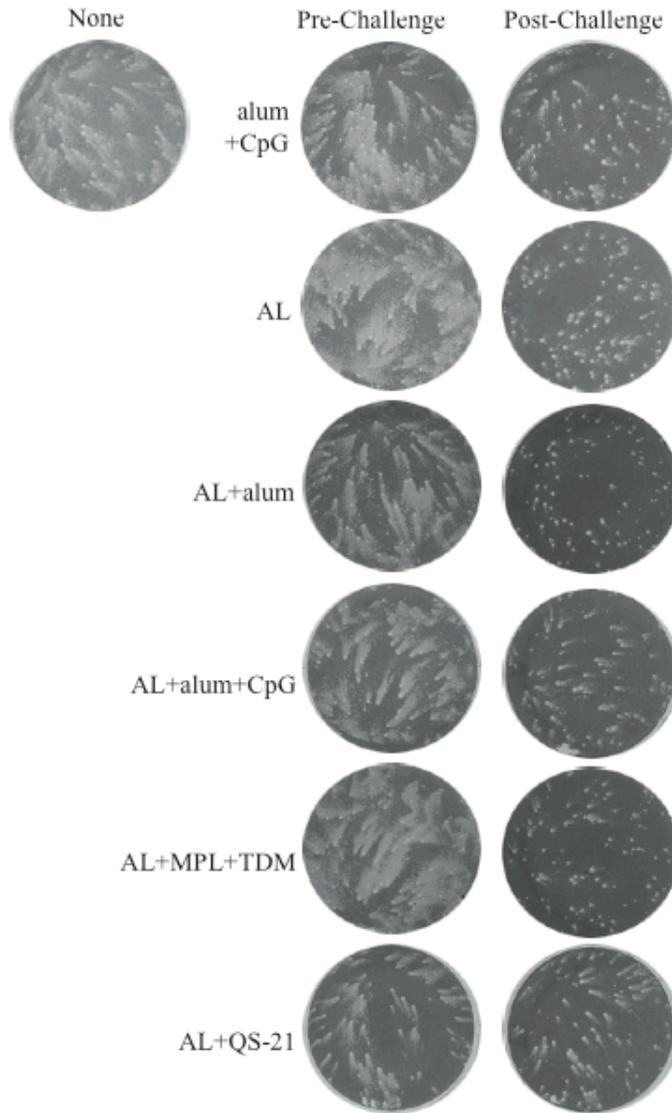
### **6.3.2 Induction of Neutralizing and Comet-Reducing Antibodies**

Neutralizing antibodies were measured with a GFP-based flow cytometry assay previously described in chapter 3, section 3.2.4. Figure 6-2 shows that neutralizing antibodies were undetectable after one immunization but were observed three weeks after the boost. Protein immunization with QS-21 or alum+CpG ODNs induced the highest neutralizing antibody titers in mice, while titers were somewhat lower with MPL+TDM and undetectable with alum or no adjuvant (figure 6-2). Post-challenge neutralizing antibody titers are discussed in a following section.

Once again, EV-specific antibodies that inhibit the formation of satellite plaques in vitro were detected by using the comet reduction assay. Pooled sera samples collected three weeks after the booster immunization were added to IHD-J infected cells as shown in figure 6-3 in the column labeled “pre-challenge.” As expected, sera from mice immunized with only alum and CpG ODNs or protein without adjuvant showed no noticeable plaque reduction. Protein immunization with the other adjuvants induced different degrees of comet reduction, and mice given QS-21 showed the most dramatic reduction, which also correlates to the highest titers against the EV protein A33 (figure 6-3). Post-challenge comet reduction will be discussed in the following section.

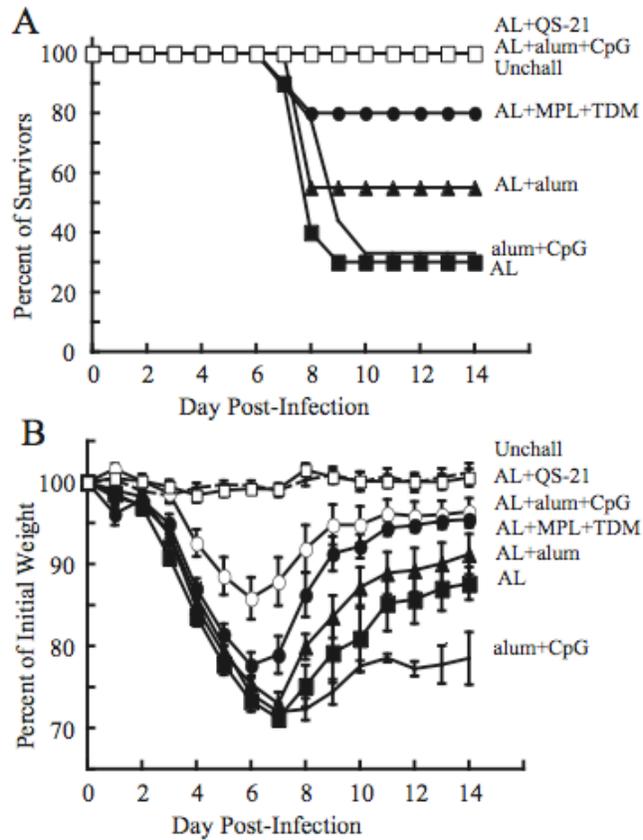
### **6.3.3 The Effect of Different Adjuvants on Protection of Mice Against Lethal Intranasal Virus Challenge**

Our previous studies discussed in chapters 3 and 5 that characterized protection of mice following multiple protein immunizations used an intranasal challenge model as a tool to judge protection [223]. The reasons for using this



**Figure 6-3: Induction of comet-reducing antibody.** The sera described in Figure 6-1 obtained from mice immunized with A33 and L1 (AL) plus the indicated adjuvants were used to detect antibodies that inhibit the formation satellite plaques due to spread of EV in liquid medium. BS-C-1 cells were infected with VACV strain IHD-J (80 pfu/well), overlaid with medium containing a 1:50 dilution of pooled mouse serum, and 40 h later stained with crystal violet. Comet-shaped plaques were counted. The column labeled pre-challenge represents samples collected three weeks after the second immunization and one day prior to intranasal virus challenge. The post-challenge column shows samples collected from surviving mice two weeks following challenge. The well shown in the upper left corner shows the typical formation of comet-shaped plaques in the absence of serum.

challenge model were previously described in chapter 3, section 3.2.6, including the measurement of weight loss as an indirect measurement of virus replication and disease severity. In this study, mice were challenged with  $10^6$  pfu (5 LD<sub>50</sub>) of VACV WR three to four weeks after their second immunization and were euthanized if they lost greater than 30% of their initial weight following challenge. Unlike previous studies, we did not challenge a subset of mice with a higher challenge dose because we expected to discern differences between groups using 5 LD<sub>50</sub> after only two immunizations. The majority of mice given only alum and CpG ODNs or the A33 and L1 proteins without adjuvant did not survive challenge (figure 6-4A). Half of the mice given proteins with alum and 80% of those given protein and MPL+TDM survived challenge. Most notably, no deaths were observed in mice immunized with proteins and QS-21 or alum+CpG ODNs adjuvants (figure 6-4A). Nearly all of the surviving mice showed steep weight loss in the first week following challenge, except for those immunized with proteins and QS-21 (figure 6-4B). In fact, this group showed little weight loss and no outward signs of illness during the two-week period following challenge. Immunization with proteins with alum and CpG ODNs provided the next best degree of protection against weight loss compared to the remaining treatment groups. A study by Rees and co-workers showed protection of mice infected with VACV in the upper respiratory tract by prophylactic treatment of mice with CpG ODNs alone [217]. Protection required administration of ODNs near the time of challenge, and no protective effect was observed in mice treated three weeks before challenge, which was the shortest time interval between immunization and challenge in this study. CpG ODNs presumably trigger an innate immune



**Figure 6-4: Survival and weight loss in mice immunized with A33 and L1 proteins and different adjuvants followed by intranasal VACV challenge.** Mice were immunized as described in Fig. 6-1, and were intranasally challenged three weeks following the second immunization with  $10^6$  pfu of VACV WR. The untreated (Untr) mice were not immunized or challenged. The alum+CpG ODN group received no recombinant protein. Mice were weighed daily for two weeks and sacrificed if their weight fell below 70% of the initial value. The percent of survivors (A) and the percent of initial weight of surviving mice (B) are shown for each group. The data shown here represents two independent experiments and each group had 4-5 mice/group. Each data point is the average weight  $\pm$  SEM of mice in each group from the two challenge experiments.

response, which may protect against virus challenge. This effect does not appear to influence our study since no protection was seen in a group of negative control mice given only alum with CpG ODNs.

Statistical analysis was carried out with weight loss data in a similar manner as described in chapter 5, section 5.3.4. Area under the curve (AUC) was calculated as a summary statistic for the weight loss measured in each animal and the nonparametric Wilcoxon rank sum test with the Holm  $p$ -value adjustment method for multiple tests was used to compare groups. Table 6-3 summarizes the  $p$ -values calculated between each group. This analysis confirms that immunization with proteins and QS-21 was significantly more protective against weight loss than protein immunization with any other adjuvant. Mice given proteins with alum and CpG ODNs showed the next best degree of protection followed by those given proteins and MPL+TDM adjuvant. Immunization with alum and CpG ODNs and no protein or with proteins and no adjuvant did differ significant from each other. These results paralleled the number of survivors in each group and the levels of antibody responses induced by immunization since the most protective vaccines had the greatest number of survivors and induced the highest antibody titers.

Virus challenge induces antibody responses in both naïve and immunized mice, and the scale and nature of the antibody response is an indirect indicator of the level of virus replication. Serum was collected from surviving mice two weeks after virus challenge and was analyzed by ELISA for binding antibodies to A33 and L1 (figure 6-1A and B). The mice that showed the best protection, specifically those given proteins with QS-21 or alum and CpG ODNs, showed little or no increase

**Table 6-3. Table of p-values calculated with area under the curve analysis followed by the Wilcoxon rank sum test using the Holm *p*-value adjustment.**

	Alum+CpG	AL	AL+A	AL+A+C	AL+M
AL+A	0.4	0.4	-	-	-
AL+A+C	<b>0.002</b>	<b>0.002</b>	<b>0.02</b>	-	-
AL+M	<b>0.02</b>	<b>0.02</b>	0.4	0.4	-
AL+Q	<b>0.0005</b>	<b>0.0003</b>	<b>0.0005</b>	<b>0.0008</b>	<b>0.0003</b>

Significant *p*-values ( $p < 0.05$ ) indicated by numbers in **bold**.

Abbreviations: AL, A33+L1; AL+A, A33+L1+alum; AL+A+C, A33+L1+alum+CpG

ODNs, AL+M, A33+L1+MPL+TDM; AL+Q, A33+L1+QS-21.

antibody titers against A33 or L1 (figure 6-1A and B, respectively). Conversely, mice with lower antibody responses prior to challenge that not well protected against disease showed a greater boost in antibodies against these same proteins (figure 6-1A and B).

A similar trend was seen with respect to IMV neutralizing antibodies and EV-specific comet reducing antibodies. Virus challenge induces a polyclonal antibody response against many viral proteins, including several IMV proteins that induce neutralizing antibodies. Therefore post-challenge measurement of neutralizing antibodies is not specific to L1 as seen prior to challenge. Mice immunized with proteins and CpG ODNs showed little change in IMV-specific neutralizing antibodies after challenge (figure 6-2). All other groups showed a significant boost and the rise in post-challenge neutralizing antibodies is probably against multiple IMV proteins. Likewise, sera from all groups caused significant comet inhibition after challenge, especially in the groups with lower anti-A33 antibody titers prior to challenge (figure 6-3).

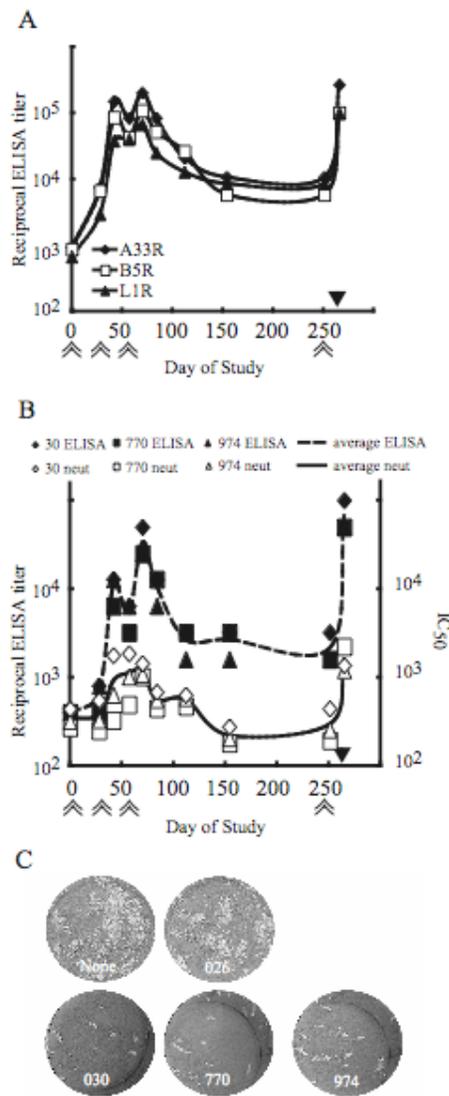
#### **6.3.4 Induction of Binding and Neutralizing Antibodies in Cynomolgous Monkeys Immunized with Recombinant A33, B5 and L1 Proteins with QS-21.**

A small experiment with cynomolgous monkeys was initiated prior to completion of the mouse experiments to test the efficacy of recombinant proteins as a vaccine in a non-human primate model. Three cynomolgous monkeys (monkeys 030, 770, 974) were immunized three times at one-month intervals with 100 µg each of recombinant A33, B5 and L1 proteins with QS-21 adjuvant. One monkey was

immunized with QS-21 adjuvant alone (monkey 026) and two remained unimmunized (monkeys 398, 419) as negative controls. Figure 6-5A shows the ELISA titers from the immunized monkeys at each time point. One month after the first immunization antibodies were detected against A33, B5 and L1 in each of the monkeys and were boosted after the second immunization. Titers were also boosted after the third immunization, in spite of falling off after the prior immunization. Titers fell again during the six-month period between the third and fourth immunizations more than one log lower than the highest values, but they were boosted again after the fourth immunization. Figure 6-5B shows titers measured with a VACV-infected cell lysate ELISA that detects binding antibodies against many viral antigens, and antibody responses closely parallel those observed with the protein ELISA in figure 6-5A.

Neutralizing antibodies against IMV, presumably specific to L1, were distinguished after one immunization and boosted with each succeeding immunization (figure 6-5B).  $IC_{50}$  titers fell to background levels between the third and final immunizations, similar to results obtained for binding antibodies, but were boosted to their highest levels after the last immunization.

Murine antibodies specific to the EV proteins A33 and B5 have been shown to reduce comet formation by inhibiting the spread of EV particles to nearby cells in tissue culture. Figure 6-5C shows that protein-immunized monkeys (030, 770, and 974) also produce specific antibodies that inhibit comet formation, while the monkey immunized with adjuvant only shows no detectable comet inhibition.

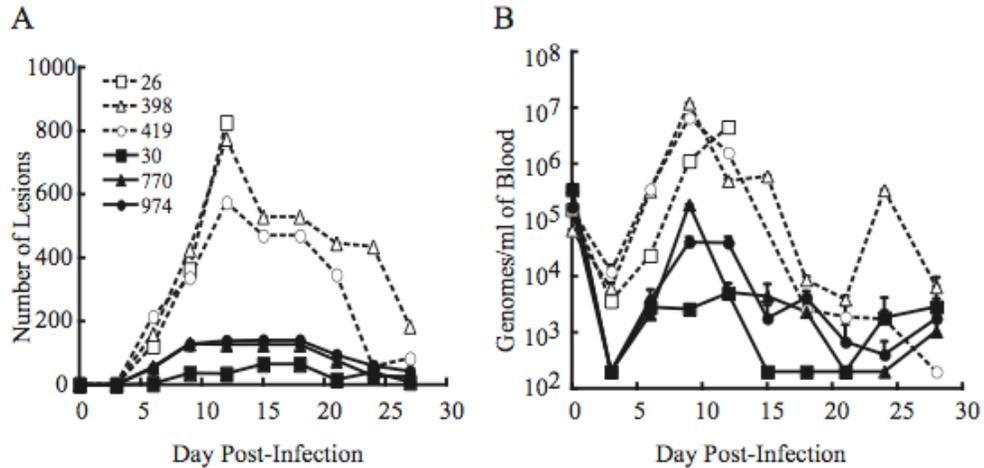


**Figure 6-5: Analysis of monkey sera following immunizations with A33, B5 and L1 combined with QS-21.** Cynomolgous monkeys were immunized with 100 mg each of recombinant A33, B5 and L1 proteins combined with the adjuvant QS-21 at days 0, 28, 57 and 251 as indicated by double arrows below the x-axis. Four weeks after the fourth immunization, monkeys were challenged intravenously with  $5 \times 10^7$  pfu of MPXV as indicated by the solid black triangle above the x-axis. ELISAs specific for A33, B5, and L1 (A) and against an infected cell lysate (B) were performed on sera collected prior to each immunization and challenge. IMV-neutralizing antibodies were also measured and the 50% inhibitory concentrations (IC<sub>50</sub>) from different serum samples are represented by the y-axis on the right (B). The presence of EV-neutralizing antibodies in sera collected 4 weeks after challenge was determined using the comet reduction assay (C). BSC-1 cells were infected with 80 pfu of VACV-strain IHD-J for 2 h. Following removal of the virus inoculum, the cells were overlaid with medium containing heat inactivated serum and a 1:50 dilution of monkey serum. Cells were incubated for 36 h at 37°C and stained with crystal violet. Key: None, no serum; 026, adjuvant only monkey serum; 030, 770, 974, sera from immunized monkeys.

### **6.3.5 Protection of Monkeys Against an Intravenous MPXV Challenge Following Multiple Protein Immunizations**

Initially, monkeys were to be challenged following the third protein immunization, but logistical difficulties did not allow for challenge at that time. Several months later, we were presented with an opportunity to challenge our monkeys with collaborators at USAMRIID in Fort Detrick, Maryland. We immunized monkeys with a fourth dose of proteins to boost antibody titers that had fallen off in the intervening months. The monkeys immunized with protein and QS-21 (030, 770, 974) and the negative control monkeys that received only QS-21 (026) or nothing (398, 419) were subsequently challenged intravenously four weeks later with  $5 \times 10^7$  pfu of MPXV. Monkeys were monitored daily for signs of illness and all three negative control monkeys became gravely ill with severe weight loss and fever as previously described [167], and the QS-21-immunized monkey (026) died 12 days after challenge.

In contrast, all monkeys immunized with protein and QS-21 adjuvant appeared healthy after challenge during the one-month observation period. Virus lesions were counted every three to four days as an indirect measure of virus replication and disease severity as shown in figure 6-6A. At their peak 12 days after infection, all unimmunized monkeys had greater than 500 lesions (figure 6-6A). The three immunized monkeys had significantly fewer lesions compared to unimmunized monkeys as determined by statistical analysis ( $p=0.02$ ). The lesions observed in immunized monkeys were smaller and atypical compared to naïve monkeys and developed less synchronously.



**Figure 6-6: Protein immunization reduces skin lesions and circulating virus in macaques.** (A) Skin lesions. Monkeys immunized as described in the legend to figure 6-5 were challenged intravenously with  $5 \times 10^7$  pfu MPXV and skin lesions were counted at 3 to 4 day intervals. (B) Blood samples were collected at 3 to 4 day intervals and the number of viral genomes was determined in duplicate by real-time quantitative PCR. The average  $\pm$  standard deviation is shown for each time point. The limit of sensitivity was 200 genomes/ml. Key to monkeys: 026 (QS21 adjuvant only); 398 and 419 (no immunization); 030, 770 and 974 (QS21 and A33, B5 and L1 proteins).

Virus replication was monitored with a real-time PCR assay to detect viral genomes in blood samples collected multiple times after challenge, including immediately after intravenous infection (day 0). Figure 6-6B shows a rise in the number of genomes detected by 6 days after infection, and peak levels were typically observed by day 9. In spite of the small number of experimental animals, statistical analysis showed that concentration of circulating genomes was significantly higher in unimmunized monkeys (026, 398, 419) compared to those immunized with proteins ( $p=0.04$ ) and the average difference between the two groups was approximately 2.5 logs.

#### **6.4 Discussion**

Recent studies in both murine and non-human primate models have indicated that the humoral response is essential to protection against orthopoxvirus infection. Studies with knockout mice [188] or B- and T-cell [130, 131] depletion have demonstrated that induction of the antibody response by  $T_{CD4+}$  cells is central to protection against VACV infection. The pivotal role of antibody has also been confirmed in the murine ectromelia virus model [126, 128, 219] and in MPXV-infected immunosuppressed macaque monkeys [251]. Protein immunization typically induces an antibody-dominated response, thus we sought to test the effectiveness of recombinant VACV proteins as protective immunogens.

The preliminary characterization of protein subunit vaccines in our mouse model has been discussed in chapters 3 and 5 and in reference [223]. Our results showed that a combination of IMV and EV proteins protected mice against intranasal virus challenge with VACV, but a protective response was dependent on at least three

immunizations with proteins and the MPL+TDM adjuvant. The study described in this chapter set out to test alternative adjuvants in mice immunized with the A33 and L1 proteins in search of a more potent and rapid vaccine formulation. To this end, we have shown that mice immunized twice at three week intervals with proteins and the QS-21 adjuvant were completely protected against death and significant weight loss following intranasal challenge with 5 LD<sub>50</sub> of VACV WR. Mice vaccinated in parallel with the same proteins and alum and CpG ODNs showed the next best protection, followed by those immunized with proteins and MPL+TDM. Alum was the least effective adjuvant and mice were not significantly better protected from challenge compared to those given protein without adjuvant. Serological analysis confirmed that protection correlated with the level of the antibody response, as measured by ELISA, IMV neutralization and comet reduction assays. Analysis of sera collected from surviving mice after challenge revealed a consistent trend in ELISA and neutralizing antibody titers. Mice with the highest titers prior to challenge, especially those immunized with proteins and QS-21 or alum+CpG ODNs had little or no boosting of titers after challenge. On the other hand, immunization groups with lower antibody responses before challenge and poor protection against infection showed the greatest boost in ELISA and neutralizing antibody titers. This trend is most likely due to virus replication since antibody titers against both IMV and EV proteins were affected in the same manner.

Protein immunization typically drives production of high levels of antibodies through activation of a Th2-polarized T-helper cell response. This response activates B cells to produce antibodies that are predominantly of the IgG1 isotype in mice.

Conversely, Th1 responses are characteristically activated by virus infection and stimulate T<sub>CD8+</sub> and production of IgG2a antibodies. Analysis of the isotype profile induced by immunization with different adjuvants showed that QS-21, and to a lesser degree, alum+CpG ODNs or MPL+TDM, induced greater levels of IgG2a. This shift towards an Th1 response has been observed with other immunogens delivered with immunostimulatory adjuvants, including QS-21 [264-268] and CpG ODNs [269, 270]. These adjuvants can also activate T<sub>CD8+</sub>, but we did not analyze this immune response because of the well-documented role of antibody in protection against orthopoxvirus disease.

This chapter included a small protection study with cynomolgous monkeys immunized with recombinant forms of A33, B5 and L1 with QS-21 adjuvant. Monkeys produced robust antibody responses to each immunogen following multiple immunizations, as seen in our previous study with mice (chapter 3 and ref. 223) and these responses were dramatically boosted after an intervening period of seven months. We decided to include B5 in the monkey study because it has been shown to induce EV-neutralizing antibodies [174, 223], and anti-B5 antibodies provide passive protection against a virus challenge [188, 271]. B5 has also recently been characterized as the major EV neutralizing antibody in human-derived VACV immune globulin. We expected that immunization with VACV proteins would generate cross-protective responses against MPXV since all three proteins show a high rate of amino acid identity to MPXV homologs, which is 93%, 96%, and 98% for A33, B5, and L1, respectively. QS-21 has been used successfully in monkeys with recombinant HIV proteins [272], but we did not know the result of the

corresponding protection study in mice when we began immunizations in monkeys. Binding antibodies were detected after the first immunization and were boosted after each subsequent boost. Neutralizing and comet-reducing antibodies were only detected after the second immunization and neutralizing antibodies were boosted to peak levels after the fourth immunization. Both binding and neutralizing antibody levels fell significantly during the seven-month period between the third and fourth immunization, but titers rose impressively after the fourth immunization when measured just prior to virus challenge. Virus challenge by intravenous delivery of MPXV is a well-characterized model that has been used to test protection of MVA as protective vaccine [167]. Following challenge, monkeys were observed daily for several indicators of disease, including the number of lesions and the virus load in blood. Unimmunized monkeys developed greater than 500 lesions and one succumbed to disease, while immunized monkeys were generally healthy, they developed between 65 and 140 smaller atypical lesions and had significantly lower virus loads. Previous studies suggest that protein immunization was not as protective as immunization with MVA or Dryvax [167], but comparable to immunization with a multi-gene DNA vaccine [177]. In the future, immunization of monkeys with MPXV proteins instead of VACV proteins would be one possible strategy to improve protection in this animal model.

## **Chapter 7**

### **Conclusion**

In this dissertation, I have sought to develop new active and passive immunization strategies against orthopoxvirus infection and test their efficacy. Significant interest in the development of safer smallpox vaccines and therapeutics was emerging at the time of the inception of my dissertation project in the spring of 2002. The presently licensed smallpox vaccine is associated with a wide array of side effects, including heart attacks, that has left the public wary of being vaccinated in the absence of a clear threat or outbreak. Passive antibody therapy with human-derived vaccinia immune globulin has been used in the past for treatment of smallpox or vaccine-related complications. The development of new antibody products to replace VIG is also urgently needed in light of its indefinite efficacy and derivation from human blood products, ideally with the use of hybridoma technology.

My dissertation project has focused on both active and passive immunization strategies based on previous studies that have demonstrated the importance of protecting against the two infectious forms of virus. Intracellular mature virions (IMVs) are formed in the cytoplasm of infected cells, are released by cell lysis, and are considered the primary form responsible for spread between hosts. Enveloped virions (EVs) are IMVs wrapped by an additional membrane and are released from cells on the tips of actin tails. The membranes encasing each particle contain a unique set of viral proteins, and superior protection in animal models is associated with immunity to proteins from the outer membrane of both particles. The first active immunization study, which is described in chapter 3, demonstrated that mice

immunized multiple times with recombinant forms of the EV proteins A33 and B5 and the IMV protein L1 formed specific binding antibodies that also recognized viral antigens. Antisera from mice immunized with A33 or B5 inhibited the spread of EV *in vitro*, as shown by the comet reduction assay, and IMV particles were neutralized *in vitro* with antisera from L1-immunized mice. A murine intranasal virus challenge model with vaccinia virus WR (VACV WR) was used to examine protection since it resembles the respiratory infection seen in humans infected with variola virus. Each of these proteins protected mice from challenge with a low dose of virus, and mice immunized with A33 showed the best protection, which was also correlated with the highest antibody responses. Individual proteins protected less well against a higher challenge dose, while mice immunized with a combination of IMV and EV proteins showed superior protection. Comparable protection was observed between groups immunized with all three proteins and those given A33 and L1, and three or four immunizations with A33, B5 and L1 also protected similarly. This study was the first to demonstrate that significant protection was provided by immunization with a combination of recombinant IMV and EV VACV proteins.

The results of this first study suggested that passive immunization with antibodies against A33, B5 and L1 may also be feasible, especially since protein immunization is associated with a mainly antibody-mediated response. Chapter 4 presents experiments in which polyclonal (PAb) or monoclonal (MAb) antibodies specific to A33, B5 or L1 were administered passively before intranasal challenge. Mice given individual antibodies were partially protected, while those given a combination of EV- and IMV-specific antibodies showed superior protection, much

like the results of the previous active immunization study. Another noteworthy finding was the ability to protect mice by passive administration of a combination of anti-A33 and anti-L1 MAbs one day before or two days after challenge. SCID mice are unable to recruit an adaptive immune response, but passive immunization with a combination of all three MAbs or PAbs prolonged survival after virus challenge. The mechanisms of antibody protection may be dependent on the target antigen. IMV-neutralizing antibodies can neutralize the virus inoculum as well as EV particles with damaged outer envelopes that expose the IMV membrane. Anti-EV antibodies may use different mechanisms since previous work has shown *in vitro* neutralization of EV by anti-B5 antibodies [174, 185] but not with anti-A33 antibodies, in spite of their ability to protect mice and monkeys against virus challenge [174-177, 223, 271]. A study from our laboratory suggests that anti-A33 antibodies may be working in concert with complement to disrupt the outer EV membrane, thus rendering it susceptible to neutralization by IMV antibodies [273]. The results of these passive immunization experiments attest to the efficacy of antibody-mediated immunity and support the use of a protein-based vaccine that primarily recruits an antibody response. These findings also give hope to the development of a next generation VIG comprised of specific antibodies and a recent publication has demonstrated the efficacy of a chimeric chimpanzee/human MAb against B5 to passively protect mice against disease [188]. The hope for the future is that more humanized MAbs will be developed and tested in non-human primates and subsequently be advanced to human clinical trials.

The efficacy of recombinant forms of A33, B5 and L1 as protective immunogens suggested that we examine the use of other viral proteins as potential vaccine candidates. A27 is an IMV protein that is the target of neutralizing antibodies that has been successfully used to immunize mice as a recombinant *E.coli*-expressed protein [173]. A multicomponent DNA vaccine including the A27 gene protected mice and monkeys against lethal orthopoxvirus challenges in combination with A33, B5, and L1 [176, 177]. The work described in chapter 3 showed that A33 was the more immunogenic and protective EV protein compared to B5, but L1 was the only IMV protein examined. The goal of the study described in chapter 5 was to determine if a baculovirus-expressed form of A27 would be more protective than L1, such that A33 could be paired with a highly immunogenic IMV protein. Mice immunized with A27 produced higher levels of binding antibodies and similar levels of IMV-neutralizing antibodies, but protection from weight loss was better with L1 alone compared to A27. The majority of mice immunized with A33 combined with A27 or L1 survived challenge, but weight loss was significantly less with A33 and L1 compared to A33 and A27. It is important to note that the inferior protection with A27 was not directly correlated with antibody responses, thus reaffirming the importance of carrying out animal challenge studies. This is the first study to compare A27 with another IMV protein, and L1 appears to be the better choice as an IMV antigen.

The two active immunization studies described in chapters 3 and 5 were dependent on three or four protein immunizations accompanied by the MPL+TDM adjuvant. A goal of the final study that is detailed in chapter 6 was to evaluate the use

of different adjuvants in order to stimulate a more rapid and potent immune response to the recombinant A33 and L1 proteins. Mice were immunized twice with proteins combined with the adjuvants alum, MPL+TDM, QS-21, or alum with CpG ODNs and challenged intranasally in the same manner as used in earlier mouse studies.

Antibody responses to each protein were noticeably higher after each immunization in mice immunized with proteins and QS-21 adjuvant, and to a lesser extent in mice given proteins with alum and CpG ODNs. The antibody response with QS-21 or alum and CpG ODNs also suggested a shift towards a Th1-polarized response, indicated by an increasing ratio of IgG2a to IgG1 titers. Mice immunized with proteins and QS-21 showed little weight loss or signs of illness following challenge, and mice given proteins with alum and CpG ODNs showed the next best protection. The worst protection was observed in mice given proteins alone or with alum and the overall trend in protection was correlated to the scale and of the antibody responses. Analysis of convalescent sera from surviving mice also brought to light an interesting trend. Mice given proteins with QS-21 or alum and CpG ODNs showed little or no change in antibody titers after challenge, while mice with the lowest pre-challenge antibody responses had the greatest boosts after infection. This result indicates that well-protected mice were able to quell the initial infection so completely that anamnestic responses were minimized.

A small monkey study was also initiated at the time of this mouse study in which three cynomolgous monkeys were immunized four times with A33, B5, and L1 and QS-21 adjuvant. Specific and neutralizing antibodies were induced against each protein, and A33 was the most immunogenic protein, much like the results of

serological analysis in earlier mouse studies. Monkeys were challenged intravenously with monkeypox and protein immunization provided significant protection from disease. Successful protection of both mice and monkeys lends further support to future study of these proteins as a subunit vaccine against smallpox.

Many questions remain surrounding the true efficacy of a multiprotein subunit smallpox vaccine, in spite of the many indications from my research that it may be a viable alternative. These proteins are trafficked through the secretory pathway as a result of expression using recombinant baculoviruses and are subject to post-translational modifications, including glycosylation. EV proteins are also trafficked through the secretory pathway, but IMV proteins are expressed in the cytoplasm of infected cells and are not modified in the same manner. Therefore, immune responses to recombinant IMV may be altered by the presence of these uncharacteristic modifications. Future recombinant proteins can be engineered to lack glycosylation motifs, although these changes may alter the protein structure in other ways. The effect of mutagenesis on the efficacy of a protein as an immunogen can only be fully examined by further immunization studies. Enzymatic deglycosylation may also be considered, but this is not a realistic option for large-scale protein production. Alternatively, bacterial expression may be considered as a way to avoid post-translational modifications, but the ease and convenience of purification of secreted protein from the medium would be lost.

These studies were carried out with vaccinia virus proteins, but the highly similar monkeypox and variola virus counterparts to these proteins can be expressed in the same manner. The protection offered by any new smallpox vaccines against

variola virus remains uncertain in the absence of endemic smallpox. Thus, recombinant forms of the variola virus proteins may offer greater assurance of protection, and monkeypox challenge studies may be more informative in monkeys immunized with monkeypox proteins. In this same manner, a recent study showed protection of mice against challenge with ectromelia virus by immunization with a recombinant form of the ectromelia virus counterpart of A33 [274]. The use of viruses in their natural hosts, such as ectromelia virus in mice, is one strategy for understanding protection against smallpox in the absence of a better animal model. The presently licensed smallpox vaccine (Dryvax) is acceptable for use in the general population, and next generation smallpox vaccine is primarily targeted towards individuals excluded from vaccination with live virus. Modified vaccinia Ankara (MVA) is an attenuated vaccinia virus that has been used in non-human primates to protect against monkeypox [167]. MVA is considered a leading candidate for an alternative smallpox vaccine that can be administered as a primary vaccination to be followed by Dryvax with the intention of providing the benefits of live virus vaccination with reduced side effects. Protein subunit vaccines can also be considered as primary vaccines, and future studies should address this scenario. Ideally, a safer alternative primary vaccine with few side effects would be used at any time while a Dryvax boost would be administered in the face of a true threat or outbreak. Perhaps this vaccination regime would be more widely accepted by members of the general population who are reluctant to be vaccinated but are otherwise not contraindicated for vaccination.

The recent elucidation of the VACV entry-fusion complex presents new candidate proteins for use as immunogens. Expression of recombinant forms of these proteins can be carried out in a similar manner as used for A33, B5, L1 and A27, and immunization studies in mice may not only identify better immunogens, but may also enrich our knowledge of the nature of virus entry and how it can be blocked.

In conclusion, the results described here have provided a firm foundation for future work in the development of a multiprotein subunit vaccine for protection against smallpox. Further animal studies, especially those using challenge models with viruses in their natural hosts, can better assess the potential efficacy of such a vaccine. Of course, a long term goal of my work is to test a subunit vaccine in human clinical trials, but I hope the need for this or any smallpox vaccine never arises.

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