Determination of Optimal Prime-Boost Vaccination Regimens against Zaire Ebolavirus

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Abstract

Zaire ebolavirus is a long filamentous single-stranded RNA virus belonging to the family *Filoviridae*. Due to the virus' high mortality rate, lack of an approved vaccine, and potential use as a bioterrorism weapon, research on this topic has been of high demand. To address this issue, several vector platforms have been investigated as vaccine candidates. DNA and adenovirus vaccine platforms are known to elicit robust cell-mediated immune responses, while adeno-associated virus and vesicular stomatitis virus vaccines are recognized for strong humoral responses. The leading hypothesis of the present project was to determine whether these four vaccination platforms, in a heterologous prime-boost regimen, increase survival and the breadth of the immune response. To test this hypothesis, the main objectives were to evaluate the cell-mediated and humoral immune responses, as well as correlate the induced immunity to protection against MA-EBOV. The heterologous pairings were strategically designed to induce both arms of the immune response. An optimized Zaire ebolavirus glycoprotein was inserted into each of the vaccine platforms and evaluated against mouse-adapted Zaire ebolavirus. Serum obtained from vaccinated mice was analyzed by a neutralizing antibody assay and IgG ELISA to determine the humoral response. The cell-mediated immune response was monitored via ELISPOT. Collectively, the data indicates that regardless of whether homologous or heterologous, a more robust immune response was observed in prime-boost strategies compared to an individual vaccination alone. In addition, the cell-mediated and humoral data show that heterologous combinations induce higher IgG specific titers in comparison to homologous regimens. As expected and consequent with immune responses, survival studies demonstrate that prime-boost

vaccinations, heterologous or homologous, are superior to vaccination regimens involving only one strategy. This data supports further evaluation of the prime-boost strategies to develop an optimal immunization strategy that can be applied to other disease models.

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List of Abbreviations

AAV = Adeno-associated virus

Ab = Antibody

ACE = Angiotensin Converting Enzyme

ACK = Ammonium-Chloride-Potassium

Ad = Adenovirus

Ad5 = Human Adenovirus Type 5

APC = Antigen Presenting Cell

BCG = Bacillus Calmette–Guérin

BEBOV = Bundibugyo ebolavirus

BSA = Bovine Serum Albumin

BSL2 = Biosafety Level 2

BSL4 = Biosafety Level 4

$CD3^+$ = Cluster of Differentiation 3

$CD4^+$ = Cluster of Differentiation 4

$CD8^+$ = Cluster of Differentiation 8

CO_2 = Carbon Dioxide

CPE = Cytopathic Effect

CT = cycle threshold

CTL = Cytotoxic T Lymphocytes

ddDNA = Double Stranded Deoxyribonucleic acid

ddH_2O = Double Deionized Water

DMEM = Dulbecco's Modified Eagle Medium

DNA = Deoxyribonucleic Acid

dNTP = Deoxyribonucleotide triphosphate

E. coli = Escherichia coli

EBOV = *Zaire ebolavirus*

EBOV-GFP = Zaire ebolavirus with Green Fluorescent Protein insertion

EBOV-GP = Optimized *Zaire ebolavirus* Glycoproptein Sequence

EBOV-GPc = *Zaire ebolavirus* Glycoproptein Consensus sequence

ELISA = Enzyme-Linked Immunosorbent Assay

ELISPOT = Enzyme-Linked Immunosorbent Spot Assay

EP = Electroporation

FBS = Fetal Bovine Serum

GC = Genome Copies

HA = Hemagglutinin

HEK = Human Embryonic Kidney cells

HEPES = 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HIV = Human Immunodeficiency Virus

HK = Hong Kong

HRP = Horse Radish Peroxidase

ICEBOV = Ivory Coastebolavirus

IFN = Interfereon

IgG = Immunoglobulin G

IgM = Immunoglobulin M

IL = Interleukin

IM = Intramuscular

ITR = Inverted Terminal Repeat

kb = Kilobase

LB = Lysogeny Broth

LD= Lethal Dose

LP = Lipofectamine

LP = Lipofectamine

MA-EBOV = Mouse Adapted*Zaire ebolavirus*

MARB = Marburg virus

MCS = Multiple Cloning Site

MEM = Minimum Essential Media

$MgSO_4$ = Magnesium Sulfate

MHC = Major Histocompatibility Complex

mRNA = Messenger Ribonucleic Acid

MVA = Modified vaccine Ankara

nAb = Neutralizing Antibody

NaCl = Sodium Chloride

NHP = Non-Human Primates

NML = National Microbiology Lab

NP = Nucleoprotein

PBMCs = Peripheral Blood Mononuclear Cells

PBS = Phosphate Buffered Saline

PCR = Polymerase Chain Reaction

pEBOV = DNA plasmid containing ZGPc

PHAC = Public Health Agency of Canada

rAAV = Recombinant AAV

rAd = Recombinant Adenovirus

RNA = Ribonucleic Acid

RPM = Revolutions Per Minute

RPMI = Roswell Park Memorial Institute Medium

RT-PCR = Real-Time Polymerase Chain Reaction

rVSV = Recombinant VSV

SARS = Severe Acute Respiratory Syndrome

SCID = Severe combined immunodeficiency

SD = Standard Deviation

SEB = Staphylococcal Enterotoxin B

SEBOV = Sudan ebolavirus

SIV =Simian Immunodeficiency Virus

ssRNA = Single Stranded Ribonucleic Acid

TAE = Tris-Acetate- Ethylenediaminetetraacetic acid

TB = Tuberculosis

$TCID_{50}$ = Median Tissue Culture Infective Dose

Th1 = T helper Cell Type 1

Th2 = T helper Cell Type 2

TNF = Tumor Necrosis Factor

Topo = PCR® 2.1-TOPO®

UV = Ultraviolet

VP = Viral Protein

VSV = Vesicular Stomatitis Virus

$VSVXN_2$ = VSV Wild Type Control

WT = Wild Type

1.0 – Zaire ebolavirus

1.1 – Introduction to Zaire ebolavirus

The family *Filoviridae* is composed of five separate *Ebolaviruses*species:*Bundibugyo ebolavirus* (BDBV), *Reston ebolavirus* (RESTV), *Sudan ebolavirus* (SEBOV), *Tai Forest ebolavirus* (TAFV), and *Zaire ebolavirus* (EBOV)(1), the latter causing the highest mortality of 90% (5,6). EBOV is a non-segmented, single-stranded RNA virus whose genomic RNA encodes the nucleoprotein (NP), viral proteins (VP35, VP40, VP30, and VP24), glycoprotein (GP), and RNA-dependent RNA polymerase (L)(2). All of the viral proteins produced by the virus have been evaluated in the vaccine field as potential antigens, the glycoprotein proving to induce the most robust immune responses and demonstrate the highest efficacy when associated animal models are challenged with a lethal dose (4, 7, 8).

Contraction of the virus leads to the development of a severe haemorrhagic fever, resulting in an abrupt onset of a temperature greater than 38.3^{o}C(3). Early symptoms are non-specific such as chills, vomiting, or muscle pain within a 2-21 day incubation period.

Since the prognosis mimic flu-like symptoms, late diagnosis of the hemorrhagic disease often leads to fatal outcomes due to severe clinical manifestations, resulting in coagulation abnormalities, and multiple organ failure.Ultimately this leads to severe shock and death (3,4). EBOV is known to cause the most severe form of haemorrhagic disease,where fatality occurs 6-16 days after initial symptom detection. This aggressive nature of EBOV apparent in the detection of viral particles at concentrations greater than $1x10^{8}$ copies/mL of blood in patients only 2 days following disease onset (5).

1.2 – Current Treatment against EBOV

Treatment of those infected primarily focuses on supportive measures. Outbreaks predominantly occur in developing countries with minimal healthcare services.Therefore the strategies in such areas revolve around fluid support. Modern healthcare facilities use equipment to maintain electrolyte balance and blood volume to help prevent organ failure during epidemics (6). Transmission of EBOV is by direct contact with contaminated bodily fluids, blood or even tissue.

Amplification of an outbreak ensues due to the reuse of medical equipment such as syringes, improper health care facilities, and poor application of prevention measures escalating transmission of the disease (7). Mucosal exposure and aerosol transmission (5, 13) occurs experimentally in NHPs, and although it has never been demonstrated in humans, it is believed that this mode of transmission can be applicable in anoutbreak setting. Unpredictable EBOV epidemics surfacing in Africa,further constitutes this virus as a major public health concern(4). Due to the high lethality rate (ranging from 20-90% in accordance to the WHO Safe Injection Global Network 2010 Meeting), the potential for bioterrorism, and no currently approved vaccine against EBOV, vaccine research in this field has been of highlypursued.

1.3 – Role of Innate Immunity in EBOV

Entry of the virus into the host is through skin lesions and the mucosa, the main targets of the virus being monocytes, macrophages and dendritic cells (3). Since innate immunity is heavily dependent on the activation and effector functions of these key players, impairment of the immune system to fight against this aggressive disease is inevitable. Dendritic cells play an integral role in antigen processing and presentation to prime the adaptive immune response. Presentation of the antigen to the adaptive immune system is required for proper T cell and B cell activation, and the prevention of this leads to removal of key cells responsible for mounting the proper immune response against the virus (8). The cytokines and chemokines produced play a role in activation of the adaptive immune response, where disruption of this process causes detrimental consequences.

Though the innate immune response is vital in the fight against infectious agents, over stimulation can cause harmful inflammatory responses. The main response of innate immunity is the inflammatory response alongside the production of cytokines such as interferon. In an immunocompetent mouse model, it has been shown that those treated with anti-IFN Abs succumbed to EBOV-WT challenge, while untreated immunocompetent mice survived (6). Two proteins that interfere with the production of IFN are VP35, which blocks an important IFN transcription factor, and VP24 which stops IFN signalling (9). Natural killer cells, whose effector functions include killing infected cells through apoptosis are decreased following EBOV infection. This was demonstrated in a NHP study where a dramatic drop in natural killer cell numbers occurred only 4 days

post-infection (10), with evidence suggesting that this decline is due to apoptosis of these cells.

In infected humans there is a comparable difference between survivors and non-survivors based on a qualitative comparison of their innate immune responses. High levels of IL-1β and IL-6 in the plasma have been associated with survival, while those with elevated levels of IL-10, and the IL-1A receptor detected in the early stages of infection reflect those that had contracted a fatal infection (17, 18, 19). In a large study, non-survivors were shown to develop alarmingly high pro-inflammatory cytokines and chemokines that escalated shortly after disease onset. Cytokine and chemokines also increase in surmountable amounts before the fatal outcome (11). This phenomenon is believed to be associated with the infection of monocytes and macrophages; leading to the increase in permeability of the endothelial later, which ultimately leads to septic shock and death.

1.4 – Role of Adaptive Immunity in EBOV

Majority of individuals who have been infected by the virus do not mount effective adaptive immune responses, primarily due to the severely compromised innate immune system early in the infection. Survivors demonstrate an increase in CTL levels following a rapid inflammatory response post-infection. This is in accordance with a detectable increase in IgM early on, followed by specific IgG antibodies against EBOV. Humoral immunity appears to have a key role in distinguishing between survivors and non-survivors. Survivors show IgM Abs as early as two days and detectable IgG Abs 5-8 days after first onset of symptoms. In fatal NHP cases, only 30% of the monkeys showed IgM

titers and no detectable IgG titers (17,21). Differences are also found in cell-mediated immunity. Though there is some discrepancy between studies, the common factor is a decrease of $CD8^+$ and $CD4^+$T cells from the immune system(11). Though the reason for this dramatic decrease is unknown, it is hypothesized that the Fas/FasL pathway leading to apoptosis may play a role (12).

1.5 - Cell-Mediated Correlates of Protection

The role of CTLs has been suggested as a correlate of protection marker for EBOV infections. In an adoptive transfer study, mice were vaccinated with alphavirus replicon particles expressing each of the EBOV proteins: GP, NP, VP24, VP30, VP35, and VP40 (13). $CD8^+$ specific T cell responses were mounted against all the viral protein except the polymerase. These $CD8^+$ T cells were then isolated, expanded by peptide stimulation, and adoptively transferred into naïve mice. The mice receiving the $CD8^+$ T cells were then challenged with MA-EBOV and showed 100% protection, demonstrating the ability to protect naïve mice from a lethal injection of EBOV. The protective capabilities of CTL cells were further explored in an NHP study through depletion of T cells through a monoclonal Ab against the CD3 surface antigen of T cells (14). This Ab was able to clear >85% of the T cells relative to starting levels. Those whose $CD8^+$ T cells have been depleted and vaccinated with rAd did not survive compared to those that were vaccinated with rAd-EBOV GP. This study suggests the integral role played by $CD8^+$ T cells in protection using the rAd EBOV GP. Further verification of the importance of $CD8^+$ T cells was explored by deletion of the $CD8^+$ T cells in the vaccinated NHPs that survived

by a $CD8^+$ α- chain specific Ab. In the study, four out of five monkeys succumbed to the diseasecompared to the survivors that received the rAd EBOV-GP and were not $CD8^+$ depleted. This NHP study suggest a key role played by $CD8^+$ T cells in the context of rAd EBOV GP and may provide some insight into which part of the immune response play an important role in viral clearance (14).

1.6 – Humoral Correlates of Protection

Humoral response has been identified to play a critical role in providing protection against EBOV. In the mouse model, IgG titers specific against EBOV-GP seem to be a quantitative measurement of B and T cell activity against the virus. Using an ELISA based IgG assay provides the tool to extrapolate results from the mouse model to the gold standard NHP model (15). The most promising candidates can therefore be screened using the rodent model and those yielding the highest IgG titers can be evaluated further on. The first NHP study demonstrating successful immune protection against EBOV was the DNA/rAd vaccination, which yielded the strongest antibody titers against the virus (16). Production of EBOV specific IgG Abs has shown protective immunity to many vaccine platforms such as DNA/rAd, rAd, and rVSV, resulting in 100% survival(15). Near complete survival can be predicted in the rAd model based on IgG titer alone, therefore it can be hypothesized that a certain threshold of IgG can be used as a correlate of protection. Another therapy that has proven the validity of this correlation is the use of monoclonal Abs as a post-exposure treatment. This has shown efficacy in rodent, guinea pigs and NHPs. In NHPs, a combination of three mAbs provided 100% survival 24 hours

post- exposure and 50% survival 48 hours (17). Different immune responses may be stimulated based on vaccine platform, though one common factor is that survivors in the NHP model all induced an antibody response (18).

1.7 – Vaccine Platforms used in EBOV Field

There are a variety of platforms that are utilized for vaccine development in the EBOV field. Outlined in the table below are the platforms which utilize GP as an immunogen, their efficacy in animal models, and issues surrounding the use of the strategy.

Table 1.7 – Summary of Platforms Utilized as Vaccines in EBOV Field

Platform	Efficacy in Animal Models	Concerns
Vesicular stomatitis virus	Rodents, NHPs	Safety issue: replication-competent
Adenovirus type 5	Rodents, NHPs	Pre-existing immunity, require a high dose
Virus-like particles	Rodents, NHPs	Boost required; production
Human parainfluenza virus type 3	Rodents, NHPs	Pre-existing immunity; Safety issue: replication-competent
Recombinant Ebola virus without VP35	Rodents	Safety
DNA	Rodents	Low immune response,

		several doses required
Vaccinia-virus based	Rodents (partial)	Low immunogenicity
Venezuelan equine encephalitisvirus	Rodents	Poor antigenicity
Baculovirus	Rodents (partial)	Low immunogenicity, require more than one vaccination
Cytomegalovirus	Rodents	Disseminating vaccine

(8,18-20)

The advantages of each platform can be assessed based on the category each strategy falls under. Replication-competent vaccines, such as VSV for instance, are known to illicit a sustainable immune response over a long period of time and therefore require few immunizations(20). Though non-replicating vaccines are safer due to their inability to replicate under any circumstances, their capability to produce a robust immune response is dampened(21). In general, viral-based platforms are known to stimulate immune responses through high antibody production, as well as the stimulation of the CTL cells. VSV is known to be advantageous in entering and targeting cells of interest for immune therapy, such as entry into mucosal cells (22). However, natural pre-existing immunity can impairviral vaccine efficacy and needs to be overcome in order to initiate a strong immune response.

DNA based vaccines fall under the category of sub-unit vaccine, which are acknowledged for their ability to favour Th1 phenotype, leading to the expansion of a cell-mediated immune response(23). Since there is no involvement or handling of

infectious materials, there is no concern for pre-existing immunity to the platform and these vaccines can be easily manufactured as a consequence of fewer safety obstacles. The low immunogenicity from these DNA platforms causes some concern, though current research in the field is concentrating on delivery systems which aid in creating a greater immune response(21). In this study four vaccine platforms: DNA, AAV, VSV and Ad5 will be evaluated for their ability individually and in combination to elicit a robust immune response and their efficacy against EBOV.

2.0 – Vesicular Stomatitis Virus Vaccine Platform

2.1 – Introduction to VSV Vector

Vesicular stomatitis virus belongs to the family *Rhabdiviridae* and is a negative sense ssRNA virus which contains an approximately 11kB genome encoding five major structural proteins: large protein of the RNA-dependent RNA polymerase, phosphoprotein, matrix protein, two nucleoprotein, and the glycoprotein(24,25). The glycoprotein plays a role in attachment and entry of the virus into its target cell. Transcription of the genome involves conserved stop and start sequences at the end of each gene to produce these five mRNA fragments. Production of the recombinant replication-competent VSV involves transfection of the cells lines with plasmids encoding the four structural genes, excluding the glycoprotein. In the rVSV platform, the glycoprotein is replaced by the gene of interest. To accommodate the foreign gene, a linker sequence is incorporated into the glycoprotein region between the start and stop sequences to accommodate an insert of approximately 4.5 kB. Due to the insertion in this

region, rVSV has the capability of expressing foreign transmembrane genes on its surface (26).

2.2 – Application of VSV as Vaccine

This platform has been widely used in the past years due to its simple genome which allows easy insertion of foreign genes, the ability to achieve rapid growth titers in a variety of cell lines, the lack of recombination into the host genome, and its minimal pathogenicity in humans (31, 33). In the general population, there is a low rate of exposure to VSV, and those that have been tested seropositive have been predominantly against the VSV glycoprotein, a component that is not present in rVSV vaccines (27). This property allows the vector to circumvent pre-existing immunity, an obstacle present for other viral vectors such as Adenovirus. Concerns focus around rVSV being a replication competent vaccine platform, which brings to question whether they are appropriate for human use. Their safety was addressed in several studies that demonstrated no adverse effects whether the VSV platform was introduced by intranasal or intramuscular route (28), or in mouse(27), guinea pig (37), and NHP models (29).

2.3 – Immune Response Induced by VSV

VSV has been found to be a potent inducer of the humoral response in various disease platforms where immune sterility is strongly suggested to be associated with antibody production. This is prevalent in several disease models where humoral protection plays

an integral role. In influenza, there is the production of the annual seasonal flu vaccine which is based on the strains that were prevalent the previous year. Protection is evaluated by a haemagglutination inhibition test which determines the relative neutralizing antibody titers. Anything higher than a score of 40 is defined as protective, demonstrating that humoral protection is important in determining protection (30). One study incorporated the avian HK/136 hemagglutinin (HA) into rVSV vectors to determine whether these constructs could provide cross-protection (31). An inhibition assay revealed that mice that have been vaccinated twice with this rVSV construct induced nAbs against homologous and heterologous clades sufficient enough to provide full efficacy against a heterologous avian influenza challenge.

The rVSV platformwas also evaluated in severe acute respiratory syndrome (SARS) using the spiked glycoprotein cloned into the vaccine vector (32). Mice that had received a single immunization produced high nAbs titers that were sufficient enough to protect mice four months post-vaccination. Importance of the humoral response was highlighted in the protection of naïve mice through passive transfer of antibodies from vaccinated animals, inferring that antibodies play a key role in survival against SARS. As demonstrated in influenza and SARS, VSV is a potent stimulator of the humoral immune response in diseases whose antibody production is a strong correlate of survival.

Expression of the foreign gene is on the surface of rVSV and therefore initially presented to the immune system as an exogenous form of antigen. Antigen presenting cells, such as dendritic cells, B cells and macrophages, endocytose the rVSV, which results in the presentation of the foreign protein on an MHCII molecule(33). Recognition of the foreign protein by the T cell receptor of $CD4^+$ T helper results in their maturation. B cells

identify exogenous foreign proteins through their specific B cell receptor. Upon binding, the protein undergoes degradation and presentation on MHCII molecules to T helper cells. Activated T helper cells identify the antigen through their T cell receptors, resulting in the secretion of essential cytokines to allow the naïve B cell to undergo maturation(33).

2.4 – VSV as Vaccine against EBOV

In the EBOV field, rVSV has shown promising results both as a pre-exposure and post-exposure treatment. Full efficacy was observed in NHPs preimmunized once with VSV EBOV GP and then intramuscularly challenged with a lethal dose of EBOV (29). Protection was observed following challenge using different route including intramuscular, intranasal or oral in NHPs, proving the versatility of this platform against EBOV. However, a difference in immune responses was detected between the route of challenge, where an oral challenge yielded higher IgG titres against EBOV in comparison to intramuscular or intranasal administration (34). Following vaccination with rVSV, there is a general increase in IgG titers before infection with EBOV(35), indicating the importance of a strong humoral response.

As a post-exposure vaccination regime, rVSV demonstrated protection in guinea pigs 1h and 24h after challenge at an efficacy of 83% and 50%, while 100% protection was observed in mice (36). Though not all guinea pigs were protected, time of death was delayed in comparison to the control. When tested in NHPs, 50% protection was

observed 30 minutes after exposure, further strengthening rVSV as a potential candidate for post-exposure treatment against EBOV (36).

3.0 – Adeno-Associated Virus Vaccine Platform

3.1 – Introduction to AAV Vector

The Adeno-associated virus belongs to the *Dependoviridae* genus, which is part of the family Parvoviridae. This virus contains a ssDNA genome and is dependent on co-infection with helper viruses, such as adenovirus or herpesvirus, for proper transfection (37). The tropism of AAV is based on different serotypes, allowing transduction of specific cell types based on the cap gene of the virus. Unlike other viral platforms, the foreign gene does not integrate into the host genome and is engineered so that no part of the virus is included in the recombinant vector. A triple transfection is required to produce the rAAV virus *in vitro*. The first AAV vector is the *cis* plasmid: a transgene expression cassette which can accommodate approximately 5kb and is flanked by two packaging signals called inverted terminal repeats (ITR)s. The second is the *trans* plasmid containing the rep and cap genes. The rep genes are responsible for replicating the recombinant genome, and the cap gene for forming the viral shell which determines the serotype of the rAAV virus. Third, is the *helper* plasmid pAd.DELTA F6, that contain the adenovirus accessory genes necessary to drive AAV replication and play an integral role in particle formation (37).

3.2 – Application of AAV as Vaccine

Recombinant AAV has been shown to be a promising vector for human gene therapy due to its ability to infect replicating and non-replicating cells, being non-pathogenic in humans, and sustaining expression of foreign genes (38). Currently, the best characterized AAV serotype 2 (AAV-2) which has been continuously improved as a vector, leading to a wealth of information from pre-clinical trials. Though there were promising results for AAV-2 as a vector, transduction of particular cells of clinical interest, such as liver or muscle cells, required a high dose to attain therapeutic gene transfer in phase I clinical patients (39). Another limiting use of AAV-2 is the prevalence of natural antibodies against the virus, an estimation of up to 20% of humans being seropositive (40). This would hamper the effect of the vaccine and would cause severe decrease of effect in any subsequent injections. Due to these drawbacks, research has been focussed on isolating AAV of different serotypes to evade pre-exisiting immunity. Other AAV serotypes, such as AAV-5, AAV-7, and AAV-8 have shown lower pre-existing immunity by detection of minimal to non-existent nAbs in comparison to AAV-2 (45, 46). This shows promising results for the use of various AAV serotypes in the clinical field.

3.3 – Immune Response Induced by AAV

Similar to the VSV platform, the rAAV platform is known to induce strong humoral immune responses in a variety of diseases. In SARS a rAAV containing the SARS spiked glycoprotein resulted in strong IgG and IgA levels, where a PBS wash of the lungs

indicated long-lasting nAbs against SARS 6 months post vaccination (41). This data strongly correlates to virus protection, where a strong IgG titer lead to stronger nAbs and a higher protection against a SARS challenge. Vaccine development against Nipah virus infection is another instance where high antibody titer against an important antigen, in this case the glycoprotein, was correlated to survival. In one study, the Nipah glycoprotein is incorporated into the rAAV vector and demonstrates that one vaccination is sufficient to induce a strong antibody response to confer 100% protection against a lethal challenge in the hamster model (42). Classification of the humoral response appeared to correspond to the route of administration, where those injected intramuscularly primarily induced an IgG1 response which correlates to a Th2, or strong humoral, response. Both in the context of SARS and Nipah, rAAV induce a potent humoral immune response, an indication of its preferential ability for antibody production.

Tropism of rAAV is attributed to the cap protein of the viral shell and plays an integral role in targeting specific tissues for gene therapy. The PO6 cap protein utilized in this study has been previously characterized in the lab, demonstrating substantial transduction of muscle tissue when administered intramuscularly. Expression of the foreign gene by the myocytes causes uptake of the exogenous protein by antigen presenting cells, and expression of the immunogen on MHCII molecules(33). Similarly to rVSV, MHCII antigen presentation results in the stimulation of the humoral response by the activation of B cells.Recombinant AAV are known to be poor inducers of cellular immunity, where particular isotypes were found to stimulate non-functional T-cells leading to cell-

mediated immune tolerance, while a robust antibody response is generated (43).The underlying mechanisms explaining poor cellular response by rAAVs is currently an enigma and the subject of intense research.

3.4 – AAV as Vaccine against Hemophilia B disease

One study evaluated the rAAV platform in Hemophilia B disease, an X-linked bleeding disorder. The cause of the disease is attributed to the lack of clotting factor IX, and it has been shown that a physiological increase of 1% would improve the severe phenotype (44). A single administration of AAV-2 has consistently demonstrated no toxicity in either the mouse or canine models after following administration either intramuscularly or in the liver (48, 49). Due to the success in these studies, evaluation of this rAAV-2 expression clotting factor IX has been clinically assessed in more than 100 patients with Hemophilia B disease using the liver as the target tissue (45). Safety of the AAV-2 platform was validated though the aforementioned studies, and three Phase I clinical trials have been performed as a result. Avigen, Inc conducted the first clinical trial using the rAAV-2 clotting factor IX construct (46). Even though the toxicity was low as predicted by the earlier animal model studies, effective therapeutic results were only obtained at high doses of the vector. The therapeutic effects wore off and the clotting factor XI concentration returned to baseline only after 8 weeks due to cytotoxic clearance (45).

To overcome this immune response, another group attempted a different route of administration using the skeletal muscle (39). It was discovered that detection of the clotting factor lasted for at least 10 months, one patient even maintain expression after

almost 4 years. This demonstrated that transduction with rAAV-2 can result in a long lasting effect in a clinical setting, but is dependent on the route of delivery, dose and immune response induced.

4.0 – Adenovirus Vaccine Platform

4.1 – Introduction to Ad

Adenovirus serotypes are responsible for most upper respiratory tract infection in humans. The most characterized serotype is human adenovirus serotype 5 (Ad5). Deletion of early and intermediate genes in the vector backbone prevents replication and allows insertion of up to 8 kB of foreign DNA. Due to the natural frequent exposure to this virus, approximately 30-50% of the North American population have pre-exisiting antibodies against Ad5(20).

4.2 – Application of Ad as Vaccine

As with other viral vectors, a major concern that affects vaccine efficacy is natural pre-existing immunity. Vaccination of NHPs with the Ad5-ZGP that had previous exposure to Ad5 showed no protection against an EBOV challenge (47). There have been several efforts to avoid the adverse effect of pre-exisiting immunity, such as utilizing different adenovirus serotypes. A chimpanzee-based adenovirus platform (AdC7) expression EBOV GP has was evaluated, resulting in strong immune responses and complete protection against a lethal challenge in the mouse model (48). Full efficacy was also

observed in the guinea pig model with low doses on the vaccine, $5x10^9$ virus particles/kg. Pre-existing immunity to the Ad5 vector resulted in no change in efficacy, suggesting a potential strategy to circumvent the drawbacks of the Ad5-based vaccines.

Another alternative to avoid pre-existing immunity is to alter the vaccination route. Ad-based vectors that have been introduced orally or nasally have shown an improvement in immune responses in comparison to intramuscular injection through stimulation of effector $CD8^+$ T cells and effector memory cells(49). Another method to improve the Ad system is the insertion of an improved expression cassette into the Ad system (Ad-CAGoptZGP). This modification significantly amplified the humoral and cell-mediated responses in doses that were 10-100x higher than Ad-CMVZGP, the vector which the Ad5 platform is based on. This in turn correlated to 100% efficacy in mice against a lethal challenge at a dose 100x lower than the previous Ad5-CMVZGP vector. When used in a post-exposure setting, it was protective when delivered 30minutes post-challenge, indicating a promising post-exposure treatment (50).

4.3 – Immune Response Induced by Ad

Recombinant adenovirus vectors are associated with strong cell-mediated responses, primarily through antigen specific $CD8^+$ T cell activation and effector functions. Human recombinant Ad5 elicits a prevailing effector memory phenotype, where low transgene expression maintains the $CD8^+$ T cell memory population (51). Studies that had examined rAd $CD8^+$ T cells in humans and NHPs have discovered that the effector T cells dominate over other memory populations. The cytokine profiling of NHPs further

demonstrated strong IFNγ secretion which is consistent with stimulation of a Th1 immune response. In humans, the memory $CD8^+$ T effector properties are highly persistent and are found to hold the same cytokine profile(52).

Since rAd5 can express the transgene at low and persistent levels, it contains the ideal characteristics to maintain effector memory cells. This ability of rAd vectors has proven to be very successful in controlling SIV challenge in NHP models. In one study, one third of NHPs that were exposed to repeated challenges of SIV were able to contain the virus and thus prevent systemic infection, indicating that effector memory T cell population are important in protection (53).

Application of the rAd platform has also shown success in protection against tuberculosis in the mouse model. Intranasal administration using a rAd5-based tuberculosis vaccine managed to induce and maintain an antigen specific $CD8^+$ T cell population in the airway, an establishment important in mucosal immunity (54). The population characterized was found to be distinct of the peripheral T cell population and elicited a persistent $CD8^+$ T cell population expressing an activated effector memory phenotype. This population of T cellshas been identified as a correlate of protection against tuberculosis challenge in the mouse model. In conclusion, rAd induces vital cell-mediated responses necessary for protection in tuberculosis, SIV and various other infections through a sustained memory $CD8^+$ T cell population (51).

Thebroad tropism of Ad is associated with its ability to infect the majority of human cell types, including skin, muscle, bone, nerve, and liver cells and lymphocytes. Infection of professional antigen presenting cells, such as dendritic cells and macrophages, by rAd

causes intracellular expression of the foreign gene, resulting in the degradation and presentation of the foreign peptide on MHCI molecules(33). Identification of the foreign peptide by $CD8^+$ T cells is accomplished through their T cell receptor and results in the induction of the cell-mediated immune response. Cellular immune responses are also generated following stimulation by Ad5 via infection of other cell types excluding immune cells, resulting in presentation of the foreign gene though the MHC I pathway.

4.4 – Ad as Vaccine against EBOV

In the EBOV field, a two vaccination regimens using Ad-5 EBOV GP resulted in full protection in NHPs with no detectable EBOV virus replication post-challenge. The second vaccination however, did not result in an increase in antibody titer, seemingly due to the vector immunity from the first vaccination (55). A single vaccination of the Ad5-ZGP construct demonstrated 100% efficacy after challenging 1 month post-vaccination in the gold standard NHP model. A subsequent vaccination in this study did not produce any significant ZGP specific immune responses, indicating that a single immunization is only required to confer 100% protection in NHPs. The dose of vaccination has been shown to play a critical role in protection where a minimum of $1x10^{10}$ virus particles was required to achieve 100% efficacy in NHPs (56). Both the cellular and humoral immune responses appeared to play a role in eliciting protective immunity and may be tentatively correlated with protection. With promising results as a vaccine against EBOV, this platform was evaluated in a Phase I clinical trial which showed both safety and an immunogenic response in adults (57).

5.0 – DNA Vaccine Platform

5.1 – Introduction to DNA Platform

DNA vaccines are bacterial plasmids optimized in certain aspects to express a foreign gene of interest, such as a viral protein. One major advantage of DNA vaccines is there is no direct contact with any infections agents during the manufacturing process, allowing the production and scaling up process to be completed more efficiently. Since the expression of the viral antigen of interest does not involve a viral delivery system, the immune system will not develop any anti-viral vector antibodies(23). This also circumvents any pre-existing immunity, a common hindrance with many viral platforms. Early clinical trials showed safety and immunogenicity with a variety of antigens, such as in Hepatitis B, HIV, and influenza (23). However, their immunogenicity in humans fell short to the anticipated pre-clinical studies. This drawback led to research into delivery, plasmid expression, and other methods to improve immunity and efficacy against diseases.

In this study, electroporation technology developed by Invitrogen was utilized as a delivery tool. The electroporation device created an electric field to open small pours into cell membranes and facilitate entry of the DNA vaccine into these cells. In general, electroporation of either muscle of skin has shown improvement in vaccine delivery and creating an antigen specific immune response (21). Several parameters were tested to maximize plasmid delivery efficiency. Those that were considered were pulse pattern, number of pulses, electric field intensity, along with other variables using a constant current device (58). In the mouse model, the optimum settings that achieved the highest

expression level were utilized. This involved four pulses, the first two with a 0.2 second delay, the second and third having a 3 second delay, followed by the third and fourth having a 0.2 second delay in between. Each pulse was 52 milliseconds in length, with a constant current of 0.1 amps.

5.2 – Application of DNA as Vaccine

One of the prominent features of DNA vaccines is to induce strong CTL responses, which is primary immune response required against intracellular infections such as *Plasmodium falciparum*, a parasites responsible for malaria. In one study (59), a cohort of adults wassubjected to natural infection over the course of a malaria transmission season. These individuals were subjected to three immunizations of a DNA recombinant malaria vaccine RTS,S/AS02, which contains a part of the circumsporozoite protein fused to the hepatitis B surface antigen together with the AS02 adjuvant. ELISPOT assays were performed on samples collected throughout the vaccination regime and revealed a possible correlate of protection. Specifically the response to a peptide in the pools, peptide 22, which is conserved between the strains of *P.falciparum*, was found to significantly correlate with protection against infection as well as lower risk of infection. A positive response to peptide 22 in an ELISPOT assay revealed that if measured at the start of malaria season, a correlation is witnessed with parasitemia burden. Of those that showed a positive result only 8% would contract malaria in contrast to those with a negative result, which would have a 36%probability (59). Therefore, a specific ELISPOT response against peptide 22 is a correlate of protection against natural *P.falciparum*

infection, indicating the importance of cell-mediated immunity against this pathogen. In general, the DNA platform induces strong cell-mediated immune responses which to correlate with protection in a variety of diseases.

5.3 – Immune Response Induced by DNA

Application of the DNA platform has been widely used in various diseases, extensively in the HIV research field. In one study, the env, and rev gene from a HIV isolate was incorporated into a DNA backbone and injected into healthy individuals to monitor the immune response induced (60). Peripheral mononuclear blood cells (PBMCs) were isolated from individuals vaccinated with 1000ug DNA vaccine, where in vivo stimulation with the rev and env protein resulted in the production of IFNγ. The primary response however was $CD4^+$T cell biased with a lower response, though $CD8^+$ T cells were stimulated at high doses of the vaccine.

Predominately, DNA immunizations results in a Th1, or cell-mediated, response. This is characterized by the induction of $CD4^+$ T helper cells, which selectively secrete of IL-2, and $CD8^+$ T IFNγ secreting cells following intramuscular injection (23). Stimulation of $CD8^+$ T cells involves direct transfection of APCs by the DNA plasmid, expression of the viral antigen, and presentation to $CD8^+$ T cytotoxic cells (21).Recent technologies such as electroporation have been able to trigger better humoral responses leading to better balanced immunity although always predominantly cell-mediated(58).

5.4 – DNA as Vaccine against EBOV

In the filovirus field, DNA vaccine has shown promise in the mouse and guinea pig model. Vaccination in the mouse model with plasmids expressing GP or NP elicited both humoral and cell-mediated responses, resulting in protection based on the dose of DNA vaccine administered (61). In the guinea pig model, vaccination with plasmids expressing GP stimulated a humoral and cell-mediated response which resulted in full protection (62). The most successful application of the DNA platform however involves the use of a prime-boost strategy involving a DNA prime followed by a rAd5 boost(16). A DNA vaccine encompassing three plasmids expressing EBOV NP, EBOV GP, and SEBOV GP was evaluated in the first filovirus Phase I clinical trial. The combination of the vaccine at varying doses demonstrated no adverse reactions in the individuals, and an antibody as well as a $CD4^+$ T cell response was detected in all the participants (63). Antibody titers were reported to be comparable to NHPs immunized with a similar vaccination regime.

Table 4.4 – DNA, AAV, VSV and Ad Platform Advantages and Disadvantages

Platform	Advantages	Disadvantages	Vaccine Type Predominance
DNA	• Construct from gene sequence without working with virulent pathogen • Mammalian post-TL modifications • No pre-existing immunity • Stable at room temperature • Rapid construction • Encode multiple immunogenic epitopes	• Low immmunogenecity • Multiple immunizations • Transfection of cells less efficient than virus • May need additional technologies such as delivery device, formulation, or	Cell-mediated

		heterologous boost	
Ad	• High transduction efficiency • Insert up to 8kb • High viral titre • Infects replicating and differentiated cells • Uses a human virus as vector and human cells as host (human proteins have identical post-translational modifications as native proteins)	• Pre-existing immunity • Large doses (>10^{10}) • Concerns with inflammatory response to vector • Anti-vector immunity	Cell-Mediated
AAV	• No illness or pathology associated with infection in humans • Does not integrate into the genome, but forms circular, episomal forms which are predicted to cause long term gene expression • infecting replicating and non-replicating cells • Many serotypes with different tissue tropism • Constructed so no gene from original virus present in recombinant particle	• Pre-existing immunity • Integration considered a possible risk (evidence not clear)	Humoral
VSV	• It has been engineered to serve as a vector for recombinant gene delivery • Produced high titres • Relatively stable • Multi-route delivery • the lack of recombination into the host genome • minimal pathogenicity in humans	• Replication competent • safety concerns due to neural tissue receptor	Humoral

6.0 – Prime-Boost Vaccination Regimens

6.1 – Introduction to Prime-Boost Vaccinations

The theory behind prime-boost vaccinations is to prime the immune system to a specific antigen, and then subsequently boost the immunity previously established with another or the identical vaccine platform expressing the same antigen as the prime. The strategy of using this method is to obtain elevated levels of immunity in comparison to each individual platform or using the same vector for both the prime and boost (64). This synergistic increase of the immune response can be measured by heightened cellular immunity though secretion of effector cytokines, humoral secretion of selective antibodies, and increased efficacy against a pathogen.

In general, the first exposure to the antigen imprints on the immune response. This is particularly strong for T cells, and is therefore utilized in prime-boost vaccinations to establish high functioning T memory cells against the specific antigen(64). Avidity of the boosted T cell response is higher, which leads to increased efficacy against a particular pathogen. Stimulation of $CD4^+$ memory T cells from the prime can further aid in the expansion of B cells that are specific for a different epitope presented by the boost vaccination(65). This phenomenon is based on when the antigen is initially presented to the T helper cell via MHCII presentation from the prime, the epitope recognized by the B cells does not have to be identical to the one presented to the T helper cell. In this way, once the memory T cell is stimulated by its prime epitope, it can aid in expansion of the B cells that express the different epitopes from the boost (65). The T cell epitope that is

recognized can both be from the prime and boost vaccinations, whereas the B cell epitope can be specific to the boost.

6.2 – Initial Application of Prime-Boost Strategies

Initial prospects of applying the heterologous prime-boost vaccination strategy in the HIV field were based on the following rationale: recombinant envelope glycoproteins induce robust nAb responses but not CTLs, in contrast, recombinant vaccinia virus expressing the same antigen could stimulate potent CTL cells but fell short on production of protective Abs. As a result, combination of both of these vaccines may complement the respective shortcomings of the individual vaccine alone (66). The key strategy for heterologous prime-boost vaccinations is to induce both the humoral and cell-mediated arms of the immune response with the use of two different vaccine platforms (67). A balance between both arms working synergistically is vital to protection against viral infections and other pathogens. The application of this rationale in the HIV field is important as an ideal vaccine requires incorporation of both B and T cells for protection.

Humoral responses prevent the integration of HIV by the ability to induce sterilizing Abs and T cell responses aid in controlling infection, therefore both immune responses are essential when combating such an aggressive virus (65). Production of a soluble antigen generally causes the induction of a $CD4^+$ T cell and B cell response. However, $CD8^+$ T cell responses are weak in comparison since endogenous expression of the antigen is required. In contrast, DNA and live virus vectors instruct endogenous expression of the host cell, leading to robust $CD4^+$ T cell, $CD8^+$ T cell and B cell responses.

6.3 – Definition of Homologous vs. Heterologous Vaccinations

It has been well established in various prime-boost strategies that use different vaccine platformsresults in superior immune responses and survival compared to strategies using the same vaccine platform for the prime as well as subsequent boost (64,66,67). In the literature, generally the definition of a heterologous vaccination is the application of two different vaccine vectors, while a homologous combination is the use of one platform for both the prime and boost. However, in the context of this studya different definition of heterologous vs. homologous prime-boost vaccination is explored compared to the conventional classification. A homologous prime-boost vaccination regime refers to the use two platforms that elicit a similar immune response, either humoral or cell-mediated, while a heterologous strategy utilizes two platforms that individually induce different arms of the immune response.

6.4 – Advantages of Heterologous Combinations

The use of the same vector in a boost strategy to direct antigen expression can lead to an anti-vector immune response which may block the efficacy of the boost. Prior immunity to the vector during a homologous prime-boost administration leads to deficient antigen presentation(64). This is easily overcome through the use of a different vaccine platform to overcome vector-specific immunity by protecting the boost from vector-specific antibodies and the host cells from a cytotoxic attack (65). Another advantage of using two vectors is the production of a heterologous lymphocyte population that can target different epitopes of the immunogen (66).

While homologous prime-boost strategies have demonstrated incremental increases in the immune responses directed to a limited amount of epitopes, a heterologous strategy may improve not only the magnitude of the response, but enhance the functionality of the immune responses as well (68). This is a consequence of how the individual vaccines in the heterologous combination are presented to the immune system after their administration. This may result in a more robust combination of cellular and humoral immune response compared to the one elicited by each vaccine alone or prime-boost vaccinations using only one platform.

The cell-mediated response, for example, is dependent on the quality and magnitude of the immune response to a given vaccine. Generation of high levels of T cell memory have been linked to prime-boost strategies in several animal models. In an HIV setting, when a prime-boost regimen is applied using the HIV gag protein followed by a rAd containing the same antigen, the quality of the $CD4^+$ and $CD8^+$ T cell response remained consistent for over a year (69). The phenomenon of a prolonged qualitative cellular immune response has been demonstrated in several disease models, including Leshmania and smallpox (68).

6.5 – Factors that Affect Prime-Boost Vaccinations

There are several factors that must be considered when deciding the desired prime-boost vaccination regimen. Some examples are: vaccine vectors, dose, time interval between the prime and boost, virus tropism, pre-existing immunity, order of administration, and desired immune response (63, 70). The platforms chosen are correlated to the immune

response preferred against the specific pathogen depending on what is considered the correlate of protection. It has been previously demonstrated how the choice of vaccine platform results on which arm of the immune response is stimulated.Primarily, the important factor in a prime-boost strategy is to strike a balance between the vectors involved.

The main purpose of the prime is to stimulate naïve immune cells, although an increased prime dose does not necessarily coordinate with increased immunogenicity. A higher dose can dampen the effect of the boost and prevent establishment of memory, while lower doses may stimulate a different subset of immune cells in comparison to a high dosage of the same vaccine (67). Increased antigen presentation can cause adverse effects on the function of $CD8^+$ IFNγ memory cells compared to those CTL cells subjected to a lower dosage of the expressed antigen (70). This affects the outcome of the immune response with regards to establishment of memory as well as effector functions. The dose of the vaccine also affects the immune system and the direction of the immune response, especially with regards to establishment of memory cells. Therefore antigen dosage must be considered when aiming to induce stable memory cells in a vaccine strategy.

The difference in serotypes found within the same virus play an integral role in stimulating polyfunctional subsets of $CD4^+$ and $CD8^+$ T cells. In a study that investigated the immune response elicited by rare adenovirus serotypes, it was discovered that using a prime-boost strategy with these vectors resulted in a population of T cells distinctive from the immune response elicited from rAd5, a vector associated with pre-existing immunity(21). Priming with rAd 35, 26 and 48 vaccines followed by a subsequent boost

with rAd5 managed to circumvent the pre-existing immunity and result in a more robust immune response. Analysis of the immune response revealed a bias towards $CD8^+$ T cells with secretion of IFN and TNF, when vaccinated with the rAd5 vaccine. When compared to the rare rAd26, the vector induced lower $CD8^+$ T cells, but rather a more balanced $CD4^+$ and $CD8^+$ population secreting elevated Il-2, IFN and TNF cytokines (30).

7.0 – Prime-Boost Vaccinations in Different Disease Models

7.1 – Prime-Boost Strategies in Tuberculosis

There are several disease models that have successfully implemented prime-boost strategies using various vaccine platforms consisting of the same antigen, resulting in promising results that have progressed to the stage of Phase I clinical trials. Research in the field of HIV, tuberculosis, and influenza are just a sample of diseases that implement prime-boost strategies. In the field of tuberculosis it is generally accepted that the correlate of protection involves secretion of IFNγ by T cells (71). In general, the T cell response elicited by the only currently approved vaccine, Bacillus Calmette–Guérin (BCG), confers protection against non-pulmonary rather than against the higher mortality, pulmonary form. One of the most promising vaccines is a low-serotype adenovirus vector, rAd35, which expresses a combination of three TB proteins. When administered as a boost in combination with the BCG vaccine in a mouse model, elevated T cell immunity was observed when compared to the BCG vaccine alone (38). The promising results led to progression into Phase I clinical trials, which demonstrated its safety. This was followed by administration to adults in South Africa that had previously

been given the BCG vaccine at least 5 years beforehand. The results indicated levels of IFNγ secreting T cells higher than any other levels seen in humans during a clinical trial (38).

7.2 – Prime-Boost Strategies in Malaria

Research into a vaccine against malaria has led to the implementation of several heterologous vaccine developments. Leading to approximately 1 million deaths alone in 2006, the most serious human parasite is *Plasmodium falciparum*(67). The life cycle of the parasite leads to different antigens being expressed in various parts of the body, leading to development of a vaccine difficult. Generally, vaccines that target the pre-erythrocyte phase via antibody and T cell effector functions clear the infected cells, while preventing further infection of the target tissues. The modified vaccine ankara (MVA) platform has been evaluated in heterologous prime-boost strategies, in combination with the most advanced malaria candidate, AS02. Unfortunately, it failed to produce robust T cell responses and efficacy in clinical trials (72). Further evaluation using a DNA prime followed by an AS02 boost also proved ineffective in human trials, emphasizing the importance of selecting suitable vectors in prime-boost strategies. One of the most promising vaccination regimens in clinical trials involves the use of a chimp adenovirus vector, AdCh63, in combination with MVA. Pre-clinical trials indicated increased immune responses when used in a prime-boost strategy compared to the individual vaccines alone (67). In NHP studies, MVA could be utilized up to three times to boost the

AdCh63 vaccine, resulting in heightened cytokine secretion by T cells and high antibody titers maintained for a long period following vaccination(73).

7.3 – Prime-Boost Strategies in HIV

The prime-boost strategy has been adapted by many in the field of HIV with the use of a variety of vaccine platforms. Many variations of this method have been applied, a DNA prime and adenovirus boost (74) and an adenovirus prime and a protein boost (75)are just a couple of examples that have been researched. Successful candidates, such as the DNA prime and Ad5 boost regimen has even progressed to Phase II clinical trials. The clinical HVTN204 trial involved a cocktail of 6 DNA plasmids encompassing the prime (envA, envB, envC, gagB, polB, nefB) and the boost consisted of four rAd5 vectors encoding matching antigens (76). Evaluation of the vaccination combinations were conducted in sero-negative HIV patients from the United States, South Africa, the Caribbean, and Latin America. The study demonstrated that the vaccination combinations were safe and that the primary immune response elicited was a T-cell mediated response. This occurred in about 70% of all vaccine recipients, predominantly against the gag and env proteins. Pre-existing immunity against rAd5 did dampen the T cell response, though it did not play a major role in cytokine secretion by $CD8^+$ and $CD4^+$ T cells. The humoral antibody response was increased with the boost, especially on the multi-clade env proteins. In general, the both humoral and cell-mediated responses were elevated with the rAd5 boost following the DNA prime.

7.4 – Prime-Boost Strategies in EBOV

There are few examples of prime-boost vaccination strategies in the Ebolavirus field. The first utilization of a heterologous vaccination strategy was by Sullivan, *et al* in 2000, where a DNA prime and rAd5 boost was implemented in NHP. The animals were vaccinated three times with DNA plasmids expressing GP from EBOV, SEBOV and ICEBOV, followed by a rAd boost expressing EBOV GP three months later. This vaccination regime resulted in full protection and was the first study to demonstrate full protection in NHPs against a lethal challenge of EBOV(16). The DNA/rAd5 combination resulted in broad immunity that stimulated both T and B cells, increasing the immune responseby one order of magnitudecompared to DNA or rAd5 vaccine alone (77).

Further assessment of this strategy has been implemented for protection against emerging Ebolavirus species, such as the BEBOV outbreak in 2007. In this study, the DNA/rAd5 vaccine expressing the GPs of both EBOV and SEBOV provided 100% protection against a challenge with BEBOV (78). Despite the amino acid sequence of approximately 43% similarity, cross-protective immunity was observed against a heterologous lethal challenge. While the mechanism providing cross-protection is not clear, it is speculated that the prime-boost strategy plays an integral role in stimulating more robust immune responses compared to each vaccine alone.

Due to the success generated from the DNA/rAd5 platform, several other heterologous combinations have been tested with both DNA and Ad. The success of a DNA prime followed by a recombinant baculovirus boost encoding MARV GP encouraged evaluation of this combination against EBOV(84). Though antibody levels were higher

in comparison to the controls, the DNA/baculovirus vaccination only conferred partial protection against an EBOV challenge. These results indicate that success of a vaccine approach against one pathogen does not necessarily translate against others. Implementing a single platform, a novel DNA consensus sequence encoding the EBOV GP was tested with a mixture of two other DNA plasmids encoding SEBOV and MARV GPs(62) against a EBOV or MARV challenge. Prime-boost vaccinations were administered via intradermal injection followed by electroporation, which resulted in increased IgG and neutralizing antibodies titers in all animals that were vaccinated against all GPs. Full efficacy was also observed against lethal EBOV orMARV challenges, indicating success of prime-boost strategies involving one platform.

One of the reasons the DNA/rAd5 vaccination was successful was it circumvented the pre-existing immunity of rAd5. Another alternative to overcome pre-existing immunity is through the use of different adenoviral serotypes such as Ad26 and Ad35, which have low seropervalance in humans. The use of these vectors has shown to induce robust humoral and cell-mediated immune responses in NHPs against EBOV GP even with previous exposure to Ad5 (79). Partial protection is observed with rAd26 EBOV-GP is administered alone, while complete protection is conferred when the Ad26 vaccine is boosted with Ad35. These results indicate the synergistic effect of a prime-boost strategy, as well the advantages of using two different serotypes within the same platform.

The difficulty with designing vaccines against Ebolavirus is the application of vaccinations which use the same antigen limitingthe immune system to a more specific response to one strain at a time. This can result in a significant delay between reporting of

an outbreak and getting the vaccine into the areas where it is needed. Therefore, other vaccination strategies that can stimulate broad spectrum and robust immune responsescould recognize a wider range of virus strains.The lack of an approved antiviral treatment or vaccine and its potential use as a bioterrorism weapon has prompted research on the development of vaccines as a means of protection against EBOV(47,78). Investigating the immune responses of heterologous vaccinations against Zaire ebolavirus will provide a more comprehensive understanding of how vaccinations can work synergistically to induce strong, long-term immune responses against Ebola, a strategy that may have merit against other viruses.

8.0 – Overview of Objectives and Statement of Hypothesis

Similar to vaccine development against other pathogens, optimization of individual platforms has been the main focus in Ebolavirus vaccine research whilethe effects of using combinations of vaccines, such as DNA prime and a viral vaccine boost, are in its initial phases of investigation. As a consequence, heterologous prime-boost regiments are in its primary stages of exploration.

The purpose of this project is to analyze immune responses and efficacy induced by various heterologous or homologous prime-boost vaccination strategies against *Zaire ebolavirus* in the mouse model. The four vaccine platforms evaluated prime-boost regimens were DNA, AAV, VSV, and Ad5. Each of the vaccine platforms are categorized based on the dominant immune response elicited. As previously demonstrated, DNA and Ad5 stimulate robust cell-mediated responses in comparison to VSV and AAV that induce strong humoral immunity.

Both arms of the immune response work synergistically to induce a protective response, and as a result a combination of vaccine vectors that individually induce reciprocating immune responses may be beneficial in inducing a more robust immune response.

It was hypothesized that: Heterologous vaccine regimes will enhance the breadth of immune response elicited both in a qualitative and quantitative manner.

The objective of the experiments conducted in this thesis was to:

1) Evaluate the effect of different prime-boost vaccination regimes on humoral and cell mediated immunity.

2) Correlate the immune responses observed to protection against mouse adapted*Zaire ebolavirus*.

This was accomplished through the insertion of an optimized EBOV-GP into the four vaccine platforms and carrying out homologous or heterologous vaccination regimens in mice. A protective immune response was based on a combination of cell-mediated and humoral immunological assays as well as survival studies.

9.0 – Material and Methods

9.1 – Construction of Novel EBOV glycoprotein (EBOV-GPc)

The pEBOV DNA platform encodes a full length glycoprotein of EBOV proteins constructed by amino acid consensus (Vector NTI, Invitrogen) for published (GenBank), clinically relevant strains. Non- consensus amino acid residues for the pEBOV were weighted towards Etoumbi and Mbanza strains, which cause greater than 75% lethality in humans.

The synthesis of the EBOV-GP sequence was optimized for expression in humans through codon and RNA optimization (GenScript, Pescataway, NJ). This sequence was then subcloned into a modified pVAX1 mammalian expression vector.

The antigen used in the heterologous vs. homologous prime-boost vaccinations was an optimized EBOV-GP previously constructed in the lab.

9.2 – Insertion of Restriction Sites to EBOV-GPc

Primers were constructed to PCR amplify EBOV-GPc from the pEBOV plasmid for insertion of the required restrictions sites into the three viral platforms used in this study: AAV, VSV, Ad5. Primers AAVF (5'-ATATATAAGCGGCCGCCATGGGGGTCACTGGGAT-3') and AAVR (5'-GCGGATATTGATTGGATCCCATGCTCAGAACACGAACTTA-3') were used to introduce restriction sites NotI and BamHI required for insertion into the required AAV vector. Similarly, primers Ad5F (5'-ATATGCTAGCGCCACCATGGGG-3') and Ad5R (5'-ATATTCTAGATTATCAGAACACGAACTTACAAATACAGAACAGAGC-3')

were used to introduce restriction sites NheI and XbaI for insertion into a modified pshuttle and pCAGα vector, and finally for incorporation into the Adeno-X system. Lastly, VSVF (5'AACTCCAGGCCACCATGGGGGTCACTG-3') and VSVR 5'-AAGCTAGCTTATCAGAACACGAACTTACAAATACAGAACAGAG-3') were used to introduce restriction sites XhoI and NheI for insertion into the required VSV vector. Below is the reaction mixture and for introduction of the required restriction sites for each vector:

Table 9.2a – Outline of Reagents used for PCR Amplification of EBOV-GP for Introduction of Restriction Sites

Reagent	**Amount**
Nuclease Free water	variable
10x *Pfu* buffer (contains $MgSO_4$)	5uL
dNTP mix (2mM)	1.0uM
Forward Primer	1.0uM
Reverse Primer	1.0uM
pEBOV plasmid	3ug
Pfu polymerase	2.5u
Total Volume	50uL

The conditions used to PCR amplify the EBOV-GPc with the required restriction sites were as follows:

Table 9.2b – Conditions for PCR Amplification of EBOV-GP for Introduction of Restriction Sites

Step	**Temperaure (^oC)**	**Time**	**Number of Cycles**
Initial denaturation	98	2 mins	1
Denaturation	95	30s	35
Annealing	Tm	30s	
Extension	72	2 mins	
Final Extension	72	7 min	1

The denaturation temperature for each set of primers was primer specific. For VSV the Tm was a gradual increase from 64^{o}C to 68^{o}C, for AAV it was 54^{o}C to 70^{o}C, and for Ad it was 54^{o}C to 70^{o}C. The difference between the denaturation temperatures was based on the GC content of each pair of primers.

9.3 – Cloning of EBOV-GPc with Restriction Sites

All PCR amplified products were then isolated by loading a 1% agarose gel at 100V for approximately for 1 hour in TAE buffer (Life Technologies, Carlsbad, CA, USA). A 2 log DNA marker ladder was run alongside. After imaging the gel, the corresponding bands were excised using a QIAquick® Gel Extraction Kit. To add the necessary A's to clone into the PCR® 2.1-TOPO® vector, the gel extracted products were incubated with dATPs along with Taq polymerasefor 30mins at 72^{o}C. Insertion of the fragments into the PCR® 2.1-TOPO® vectors was achieved through the TOPO® TA cloning® kit (Invitrogen). This mixture was then transformed into One Shot® TOP10 Chemically Competent *E. coli* (Invitrogen) as outlined in the consumer's recommended protocol. Lysogeny broth (LB) plates supplemented with 100ug/mL of ampicillin and X-gal for screening purposes. The LB plates were inoculated with the transformed cells and incubated overnight at 37^{o}C.

Isolated white colonies were picked and incubated in 5mL of LB broth supplemented with ampicillin and 0.5% NaCl in a shaking incubator at 37^{o}C overnight. The cell cultures were spun down at 4,000 RPM and the resulting pellets were DNA extracted using a QIAprep spin miniprep kit, following the consumer's recommended protocol.

The extracted DNA was then screened by a double restriction digest based on the inserted restriction sites added on. The reaction mixture was as follows:

Table 9.3 – Reaction Mixture for Restriction Digestions Using Two Enzymes

Reagent	**Volume (1x)**
Plasmid (1ug/uL)	5uL
Restriction Enzyme #1	1uL
Restriction Enzyme #2	1uL
Buffer (10x)	2uL
BSA supplement (10X)	2uL
Nuclease free water	9uL
Total	20uL

The resulting digests were then ran on a 1% agarose gel using the same methods as mentioned above, and visualized to ensure that the insert was properly incorporated into the PCR® 2.1-TOPO® vector. This was based on the appearance of a double band corresponding to the size of the digested vector as well as the EBOV-GPc gene (~2kb). The colonies whose DNA corresponded the proper sized fragments when digested were selected for further clarification through sequencing. This was to ensure the EBOV-GPc sequence was not mutated during the cloning process.

The remaining DNA extract of the selected colony was transformed into MAX efficiency® STBL2^{TM} competent cells through a quick transformation as outlined in the consumer guidelines (Life Technologies). These cells were then incubated on LB plates supplemented with ampicillin using the same protocol as mentioned previously. Asubsequent colony was picked to inoculate a culture of 100ml LB broth supplemented with ampicillin that was grown overnight at a 37^{o}C shaking incubator. A QIAGEN midi prep kit was then used to extract the DNA from the grown cultures, using the

recommended consumer's protocol. Following extraction, each of the PCR amplified inserts were sequenced at the DNA core facilities at the NML using external and internal primers of the EBOV-GP gene. Once the EBOV-GP insert and the proper restriction sites for insertion into the AAV, VSV and Ad5 vectors was confirmed using SeqMan alignment, these plasmids were used to introduce the EBOV-GP gene into the individual viral platforms.

Due to the difficulty of cloning in the EBOV-GP insert into the required AdenoX system, another modified optimized EBOV glycoprotein (EBOV-GP) was used as the antigen for both the homologous and heterologous prime-boost vaccination regimes.

10.0 – Insertion of EBOV-GPc into AAV Platform

10.1 – Cloning of EBOV-GPc into AAV Vector

The AAV viral platform involves the insertion of the gene of interest into the AAV-2 "cis" plasmid between two ITR regions, as referred to in the introduction. The previously cloned EBOV-GP gene containing the restriction sites, NotI at the 3' end and BamHI at the 5' end in the PCR® 2.1-TOPO® was used to introduce this gene into the AAV platform. Isolation of the EBOV-GP sequence involves restriction digestion of both TOPO vector and the AAV-2 cis plasmid, and running the digested reaction mixtures on a 1% low melt agarose gel to separate the insert from the vector. A scalpel was used to cut out the ~2kb band visualized on a Safe Imager ^{TM} that corresponded to the EBOV-GP insert, and the ~4.6kB that corresponded to the digested AAV-2 vector. An in gel ligation was then performed on the excised fragments. This involved the incubation of the

extracted gel at -80^{o}C for 5 minutes, then the transfer of the Eppendorf tubes containing the gel extractions to a water bath set at 72^{o}C for ten minutes. Several vector to ligation ratio mixtures were set up to cover a range of ligation conditions. The ratios were as follows:

Table 10.1 – Various Ratios of AAV Vector and EBOV-GP for Ligation Mixtures

Vector (uL)	**EBOV-GP Insert (uL)**
10 (control)	
7	3
8	2
9	1

These ratios were thoroughly mixed, centrifuged, and incubated at 37^{o}C for 1 minute. A T4 DNA ligase (Invitrogen) reaction mixture was then added to each of the ligation ratios. The T4 ligase reaction mixture contained: 2uL ligase, 4 uL ligase buffer, and 2uL water per reaction and was incubated on ice prior to addition. After the addition of the ligation reaction mixture, each of the ratios was thoroughly vortexed and spun down on a table-top centrifuge. The four reaction mixtures were then incubated in 16^{o}C water bath overnight.

The incubated ligation mixtures were then transferred from the 16^{o}C to at 37^{o}C water bath for 30 minutes one hour before they were transformed. Following incubation the ligation mixtures were transformed into One Shot® TOP10 Chemically Competent *E. coli* cells using the same protocol previously described. The resulting cells were screened on LB plates supplemented with ampicillin as a selectable marker for the AAV-2 vector.

A similar process involving the screening of these colonies via restriction digestion and sequencing was performed on approximately 10 isolated colonies picked from the transformed cells, as previously explained. Once the screening process was completed, the AAV-2 vector containing the EBOV-GPc insert in the proper orientation was selected to be amplified via Qiagen plasmid mega kit using the recommended consumer guidelines. This involved a quick transformation into STBL2 cells, inoculation of a LB plate supplemented with ampicillin, isolation of a single colony, and growth of a 1L culture to amplify the AAV-2 plasmid with the EBOV-GPc insert.

10.2 – Cell Culture and DNA Transfections

HEK 293T cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Sigma-Aldrich, Oakville, Ontario) supplemented with 10% Fetal Bovine Serum (FBS, Wisent, St. Bruno, Quebec), 1% penicillin (Gibco, Carlsbad, California), 1% L-glutamine (Gibco), and 1% NaPyr (Gibco). The cells were cultured in incubators maintained at 37^{o}C, containing 5% CO_2 humidity. The cells were maintained in 150mm x 25mm tissue culture dishes (Corning, NY, USA) and split when 70-80% confluent by the removal of media followed by addition of 5mL of 0.25% (Invitrogen) for cell detachment. All cell culturing was carried out in a BSL2 safety cabinet to ensure sterility.

AAV particles were created using a triple transfection protocol involving the use of three plasmids. This cis plasmid is the AAV-2 plasmid containing the desired transgene inserted between the ITRs as well as the AAV-2*rep* gene, as previously described and produced. A *trans* plasmid, encoding a unique AAV porcine cap gene previously isolated

in our lab, PO6, was used based on preliminary studies showing higher cellular immune responses in Balb/c mice (data not published). The third plasmid is the *helper* plasmid, pAd- DeltaF6, which contains the adenoviral genes (E2A, E4 and VA RNA) whose products drive the generation of AAV particles.

A seeding concentration of $6x10^6$ HEK 293T cells were used to inoculate 50 plates 24 hours before transfection in order to achieve a confluency of approximately 60-80%. Triple tranfection was carried out using the CalPhos™ Mammalian Tranfection Kit (Clontech, Mountain View, CA) as described in the user's manual. The solutions used for the transfection/ plates were as follows:

Solution A:

650ug trans plasmid (AAVrep + CAP)

650ug cis plasmid (AAVITR + transgene)

2.4mg helper plasmid (pAd- DeltaF6)

12mL sterile water

2mL Calphos solution

Solution B:

16mL HEPES solution

One hour before transfection, both the HEPES solution and the sterile water provided were incubated in a 37°C water bath. After Solution A and Solution B were made, Solution A was added in a drop-wise fashion to Solution B while Solution B was being

votexed at 1600 rpm. The combined solution was incubated at room temperature for 30 minutes, after which the solution became slightly cloudy. Approximately 3.1mL of the cloudy solution was added to each plate of HEK 293T cells. The plates were then incubated at 37^{o}C incubator supplemented with 5% CO_2. The next morning, media was aspirated and 25mL of new media was added. 72 hours after transfection, the plates were scraped using a cell scraper and harvested. Cells were then spun down in 500mL centrifuge tubes at 4000rpm for 30 minutes. The supernatant was carefully aspirated, the resulting pellet resuspended in 20mL of Resuspension Buffer I, aliquoted into a 50mL falcon tube, and stored at -80^{o}C.

10.3 – Purification of AAV-EBOV-GPc

The lysate was then transferred from -80^{o}C freezer into the 37^{o}C water bath for 15minutes. It was intermittently freeze/thawed three times with dry ice in ethanol and the 37^{o}C water bath. Dnase (Invitrogen) at a concentration of 5000U/mL was added to the prep, inverted gently, and then incubated at 37^{o}C for 20 minutes, with the inversion of the 50mL falcon every 5 minutes during incubation. The lysate was then spun at 3000rpm for 15 minutes at 4^{o}C in the table-top centrifuge and the supernatant transferred to a new 50mL falcon tube. This process was repeated one more time to the newly transferred supernatant. The final volume of the supernatant was then brought up to 24mL with resuspension buffer I. Salt concentration was increased with the addition of 4.5mL of NaCl to the supernatant. Following this, 1.5mL of 10% octyl-β-glucopyranoside was added to the supernatant and the tube was gently mixed by inversion.

10.4 – Purification of AAV – EBOVGPc by Caesium Chloride Gradient

All viral work was done aseptically in a BSL2 safety hood. For each preparation, two 2-tier gradientswere prepared consisting of 7.5mL of 1.5g/mL caesium chloride and 15mL of 1.3g/mL caesium chloride in centrifuge tubes (Beckman). The gradient was created by first adding 15mL of the 1.3g/mL caesium chloride and slowly dispensing the 1.5g/mL caesium chloride at the bottom of the tube. On top of each of the tubes, 15mL of the sample was added slowly to the side of the tube to avoid disturbance of the gradients. The tubes were then balanced on a scale using the 1.3g/mL caesium chloride solution and loaded onto the Sw32Ti (Beckman) rotor along with blanks to ensure balance.

Using a Beckman ultracentrifuge, the gradients were spun at 25700 RPM at 4^{o}C for a minimum of 20 hours. After the spin, the tubes were gently transferred to a stand holder, and securely fashioned to ensure that no motion occurred during collection. Fractions were then collected by sanitizing the bottom of the tube with an alcohol wipe and then gently piercing the bottom of the tube at a right angle with an 18 ½ gauge needle (Beckton Dickson). A 15mL falcon tube was used to discard the first 4mL, followed by 2.0mL Eppendorf tubes to collect 1.7mL fractions. Any remaining solution in the tube was discarded. The fractions were evaluated for AAV virus particles based on real-time polymerase chain reaction (RT-PCR) and refractive index.

10.5 – Isolation of AAV-EBOV-GPc from Fractions

RT- PCR involved the detection of the number of genome copies present in each of the fractions. The fractions were first diluted 1:100 in ddH_2O (5uL sample and 495uL

ddH_2O) in sterile 1.5mL Eppendorf tubes. From this dilution, 10uL aliquotswere taken and transferred to 1.5mL Eppendorf tubes containing 90uL of DnaseI which was at a concentration of 40U. The final dilution of the individual fractions was 1:200. These diluted fractions were then incubated in a 37^{o}C water bath for 30 minutes and then transferred to a thermocycler set at 99^{o}C for 5 minutes. The samples were then cooled down in an ice bucket for 5 minutes. During this time, the light cycler 480II (Roche) was turned on 15 minutes prior to reading.

To quantify the number of genome copies using the light cycler, each of the diluted fractions were mixed with the following reaction mixture and added onto a 96-well plate reader. The following reaction mixture indicated represents the amount added of each reagent per diluted fraction. The RT-PCR reaction mixture and all of its components were incubated on ice.

Table 10.5 – Reagents for Quantification of AAV through Real-Time PCR

Reagent	**Volume/Reaction**	**Final Concentration**
(C6)-TaqMan® Universal MM 200 reaction (Applied Biosystems)	12.5uL	1x
Primer BGHFor(100uM) (tctagttgccagccatctgttgt)	0.27uL	1uM
Primer BGHRev(100uM) (tgggagtggcaccttcca)	0.27uL	1uM
Probe (2uM) BGH probe (6-FAM-tcccccgtgccttccttgacc-TAMARA)	2.5uL	0.2uM

An aliquot of 10uL of each 1:200 diluted fraction was added to 15.4uL of the reaction mixture on the 96 well plate. The default RT-PCR cycling conditions were as follows: 2 minutes at 50^{o}C, 10 minutes at 95^{o}C followed by 40 cycles of 15 seconds at 95^{o}C and 1 minute at 60^{o}C.

Refractive indices of the 1.7mL fractions was read using a densitometer. It was first calibrated using ddH_2O which corresponds to a refractive index of ~1.333 as an initial starting point. Aliquots of 6uL per fraction was placed on the plate of the densitometer, and read based on the coloured portion of the field of view intersecting with the crossland in the middle of the field. The switch on the lefthand side was pulled to read the corresponding refractive index. The range of refractive index for AAV is between 1.3740-1.3600. This refractive index data was correlated with the RT-PCR data to select the fractions used to concentrate the AAV virus through a second caesium chloride gradient.

The fractions that fell within these guidelines were quickly spun down at 10,000 RPM to ensure residual virus was not on the Eppendorflids and then pooled together in a 50mL falcon tube. The volume of the amount collected was measured and the final volume brought up to 18.5mL using filtered sterilized 1.41g/mL caesium chloride. Once mixed, 8.9mL was aliquoted into two optiseal tubes (Beckman). Along with balances, these tubes were loaded in to a Type90Ti rotor and spun at 62100 RPM at 4^{o}C for 20 hours minimum. Following the spin, the fractions were collected in the same manner as the first spin, though no initial portion of the gradient was discarded, and 0.5mL fractions were collected in 1.5mL Eppendorf tubes. The following fractions were analyzed using the same refractive index and qPCR techniques mentioned prior. However, the range for the

fractions that are considered positive for the refractive index method was between 1.3750-1.3660. After the proper fractions containing the AAV virus particles with the EBOV-GP insert were selected, it was purified.

The purification steps involved the transfer of the chosen fractions into an Amicon filter device (Millipore) where a 50mL aliquot of the Final Formulation Buffer was placed in a 50mL falcon tube and used to bring the volume in the Amicon filter device to the top. This solution was then thoroughly mixed by a pipette, loaded in the bench top sorvall, and spun at 2000-4000 RPM for 2 minutes at 4^{o}C. During the spins, the Amicon filter tube was consistently topped up with the aliquoted Final Formulation buffer to ensure that the liquid did not dip below the top of the membrane which held the virus. The 50mL buffer was spun through until approximately 1.8mL remained. This was then pipetted up and down using a 1000uL pipette and a 200uL pipette to dislodge the AAV virus attached to the membrane of the filter. After detachment, 250uL aliquotes of the purified virus were pipetted into 2.0mL screw capped tubes and frozen at -80^{o}C.

10.6 – Quantification of AAV-EBOVGPc by Real-Time PCR

A standard ladder was created using the purified transgene plasmid to titer the virus. The transgene plasmid containing the EBOV-GP insert was linearized by taking a 7ul aliquot and digesting it using 1uL of NotI at 37^{o}C for 1 hour. After, the linearized plasmid was purified using the QIAquick PCR purification kit and eluted in a final volume of 25uL. The final concentration was determined by the nanodrop ND-1000 (Nanodrop)as outlined

in the user's guidelines. Determination of the copy number/uL of linearized plasmid was based on molar concentration and was calculated using the following equations:

A) Conversion of the concentration of the plasmid by the nanodrop into g/uL

B) Determination of the number of basepairs in the EBOV-GP insert(n)

   a. n=grams of linearized plasmid/molecular weight of EBOV-GP

C) Number of molecules/uL = [(n)(Avagadro's number)]

$$= (n)(6.02 \times 10^{23})$$

Based on the number obtained in equation C, the linearized plasmid was diluted using ddH_2O to make a standard ladder from 5×10^6-5.0 copies/uL, diluting down in 2.5×10^5 increments. The ladder was then stored at -80^{o}C in 10uL aliquots until needed.

The RT-PCR protocol previously used was applied to the purified virus. The diluted virus was plated on the 96 well plate along with the standard ladder using the same PCR cycling conditions in order to quantify the amount of AAV particles in the purified fractions. After the run, the cycle threshold (CT) values were compared with the ladder to create a linear standard curve where genome copies was plotted on the y axis and CT value wason the x axis. Using the line of best fit equation obtained from the graph, the CT value of the purified fractions was used to calculate the number of genome copies. The equation to calculate AAV particles/mL in the purified samples is:

GC x 10 (uL DNA loaded) x 100 (dilution of sample) x 2 (RT-PCR only read ss, convert to ddDNA x 1000 (ul/mL conversion)

11.0 – Insertion of EBOV-GPc into VSV Platform

11.1 – Cloning of EBOV-GPc into VSV Vector

As mentioned prior, the insertion of the gene of interest into the rVSV vectorwas achieved through replacement of the VSV glycoprotein. The previously mentioned TOPO TA vector containing the EBOV-GP insert with the appropriate XhoI and NheI sites was utilized. Restriction digestion of the TOPO TA vector and the rVSV vector using the aforementioned enzymes, separation by gel electrophoresis, isolation of corresponding bands, and ligation was carried out using the aforementioned techniques mentioned for insertion of EBOV-GPc into the AAV platform.

11.2 – Transfection of DNA into Cell Culture

Once the above was achieved, transfection for viral particle production was carried out using a mixture of HEK 293T and Vero cells in a 50:50 ratio using DMEM media supplemented with 3% FBS, for a total of approximately 2.5mL per well. The cells were split 1:6 the day prior to transfection to achieve ~70% confluency. The following day, the media was changed 2 hours prior to transfection with plain DMEM. Different ratios of each of the helper plasmids described previously were mixed in order to increase the chance of a successful rescue. The ratios used were as follows:

Table 11.2 – Ratio of Helper Plasmids and Lipofectamine Reagent used for Transfection of Vero cells

Tube #	$VSVXN_2$ (1ug/uL)	T7 (1ug/uL)	N (0.5ug/uL)	L (0.25ug/uL)	P (1 ug/uL)	LP2000
1	2	2	0.5	0.35	1.25	14
2	2	2.5	0.5	0.35	1.25	14
3	2.5	2.5	0.625	0.75	1.56	14
4	2	2.5	0.5	0.6	1.25	14
5	No LP + no plasmids					
6	LP + no plasmids					

The following ratios were divided into two tubes: Tube A and Tube B. Tube A contained all the helper plasmids as well as 90uL of serum free Opti MEM media, while Tube B contained the Lipofectamine suspended in 90 uL of Opti MEM media. Once the necessary reagents were added to the set of tubes, they were incubated at room temperature for 5 minutes. Afterwards, the contents of Tube B were added to Tube A, which was then incubated for 15 minutes at room temperature. Following incubation, 720mL of Opti MEM was added to newly combined solution in Tube A to bring the final volume up to 1mL. Media was removed from the cells that were split the day before and then washed with 2mL of fresh Opti MEM media.

The media was then aspirated from the wells, and the Tube A mixtures added individually to each well. The 6 well plate was then incubated at 37^oC for 24 hours. The next day 1mL of DMEM supplemented with 3% FBS and 1% L-glutamine was added to each of the wells, bringing the total volume of each well to 2 mL. This plate was then

incubated at 37^{o}C for 48 hours. After 72 hours, a blind passage was performed where 500uL from each of the previously transfected wells were carefully applied down the side of a 6-well plate containing previously aspirated VeroE6 cells at 90% confluency. Following 2 hours of incubation at 37^{o}C, 1.5mL of DMEM supplemented with 3% FBS and 1% L-glutamine was added to the VeroE6 cells. The 6-well plate was checked daily for signs of cells undergoing CPE. When CPE was finally detected, the wells containing CPE were harvested by pipetting up and down to dislodge any residual cells stuck. Once collected in a 15mL falcon tube containing 10% FBS, the tube was spun down for 7 minutes at 2000 RPM. The supernatant was then collected, aliquoted into 2mL cryovial tubes and stored at -80^{o}C.

11.3 – Quantification of VSV EBOV-GPc by $TCID_{50}$

Vero cells were plated on a flat bottom 96 well plate in DMEM supplemented with 10% FBS. The previously harvested virus was serially diluted 1:10 in DMEM supplemented with 2% FBS in Eppendorf tubes. The media was then carefully aspirated off the Vero cells and 100uL of the diluted virus was added on. The plates were then incubated at 37^{o}C for 2 days and monitored for CPE.CPE threshold is defined as cells rounding off due to the virus causing a pathological change in 50% of Vero cells. The dilution at which CPE occurred was documented and $TCID_{50}$ was calculated used the Karber formula.

The 8 replicates of the 10x serially diluted virus (n=10) were counted the dilution at which CPE was observed:

10^{-1} 8/8 = 1
10^{-2} 8/8 = 1
10^{-3} 8/8 = 0.6
10^{-4} 8/8 = 0.2
10^{-5} 8/8 = 0
10^{-6} 2/8 = 0
10^{-7} 0/8 = 0

The resulting $TCID_{50}$ is calculated by the equation:

log TCID50 = L-d(S - 0.5)

L= log of lowest dilution used in the test (ie. showing 100%CPE)

d = difference between log dilution steps

S = sum of proportion of positive tests (positive = show CPE)

In this example:

L= -5 d=1
log TCID50 = - 5 - (1)(8/8 + 2/8 + 0/8)

log TCID50 = -5-1(2/3)

log $TCID_{50}$ = |-17/3|

$TCID_{50} = 10^{17/3}$

$TCID_{50} = 4.64x10^{5}$/mL

12.0 – Confirmation of Protein Expression by Western Blot

T150 flasks containing 70% confluent HEK293T cells were infected with each of the viruses. The cells were then harvested 72 hours after inoculation, spun down at 4000rpm for 30 minutes, and the pellets collected for analysis by Western blot. Standard Western blot techniques were used to visualize the proteins extracted from the pellets collected. Protein samples were separated using electrophoresis on a 10% SDS-PAGE (sodium dodecyl sulphate-polyacrylamide gel), then wet transferred onto a PVDF membrane (GE Healthcare) membrane at 4^{o}C. The primary antibody used was a mouse adapted anti-Ebola glycoprotein (GP) monoclonal antibody produced in our lab and the secondary a goat-anti mouse antibody conjugated to horseradish peroxidise. Protein bands were then visualised using a SuperSignal West Pico Chemilumenescent Substrate kit.

13.0 – Mouse Models and Vaccination Routes

The mouse models used for this study were B10.Br mice [(MHC H-2K), The Jackson Laboratory, ME)] and Balb/c mice (Charles River, Wilmington, MA). Both were used in this study to evaluate protection, as well as humoral and cell-mediated responses to the Zaire ebolavirus DNA optimized glycoprotein (EBOV-GP) immunogen.

The viral vaccinations were administered intramuscularly (left posterior hind limb) using a 28 gauge syringe. The DNA construct suspended in water was administered into the quadriceps muscle through shallow intramuscular injection followed by electroporation. Electroporation was utilized solely for delivery of the DNA plasmid. This

involvedinsertion of a three-pronged electrode approximately 2mm into the quadriceps muscle which will deliver two electrical pulses, as previously described (64).

14.0 – Evaluation of Individual Platforms

A group of 10 mice were vaccinated with individual platforms that would convey partial protection. The schedule was as follows:

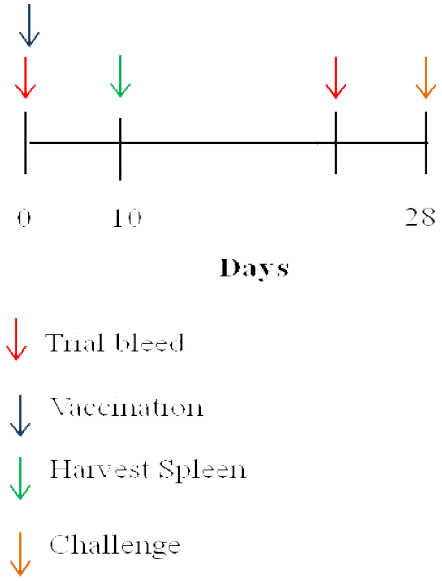


Figure 14.0 – Outline of Schedule for B10Br Mice Vaccinated with Various Doses of DNA, AAV, VSV, Ad Expressing EBOV-GP

The dose administered for each respective platform was as follows:

Table 14.0 – Doses of DNA, AAV, VSV, and Ad Expressing EBOV-GP used to Vaccinate B10Br Mice

Vaccine Platform	Concentration	Resuspension (50uL)
DNA	0.1ug, 1ug, 10ug	PBS
AAV	$1x10^9$ GC, $1x10^{10}$ GC, $1x10^{11}$ GC	PBS
VSV	1 pfu, 10 pfu, 100pfu	DMEM with 2% FBS
Ad5	$1x10^3$ pfu, $5x10^3$ pfu	PBS

15.0 – Homologous and Heterologous Prime-Boost Regimens

Vaccination combinations for individual platform, homologous and heterologous regiments are found in the table below:

Table 15.0 – Outline of Homologous and Heterologous Prime-Boost Vaccination Regimens

Vaccine Type	Prime-Boost
Cell-Mediated	DNA, Adenovirus
	Adenovirus, DNA
Humoral	AAV, VSV
	VSV,AAV
Humoral, Cell-Mediated	AAV, Adenovirus
	AAV,DNA
	VSV, Adenovirus
	VSV, DNA
Cell-Mediated, Humoral	Adenovirus, AAV
	DNA, AAV
	Adenovirus, VSV
	DNA,VSV
Individual Platforms	DNA, DNA
	AAV, AAV
	VSV, VSV
	Ad, Ad
Control	PBS, PBS

In this context, heterologous and homologous vaccinations are defined based on which platform elicits a stronger humoral or cell-mediated response based on previous research.

These combinations will be further compared to homologous vaccinations, where both the prime and boost immunization involves the use of a single platform.

15.1 – Schedule for Prime-Boost Regimens in Balb/c Mice

Initially the heterologous vaccination combinations were used solely for a survival study in Balb/c mice, bypassing any cell-mediated assays. The mice were trial bled through the saphenous vein for subsequent humoral assays in order to assess a part of the immune response. The experimental outline was as follows:

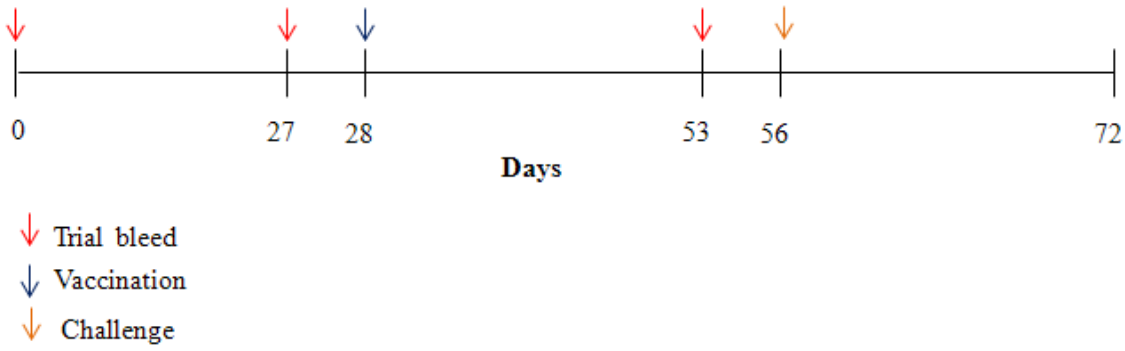


Figure 15.1 – Schedule for Balb/c Mice Immunized with Heterologous and Homologous Prime-Boost Vaccination Regimens

15.2 – Schedule for Prime-Boost Regimens in B10Br Mice

The following experimental outline was used to evaluate the heterologous and homologous prime-boost vaccination regimes on the basis of both survival and immunological importance in B10Br mice:

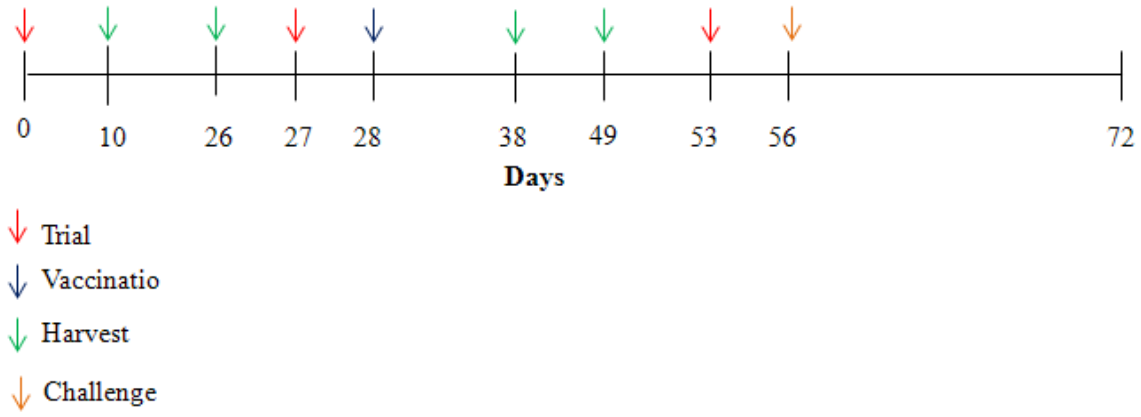


Figure 15.2 – Schedule for B10Br Mice Immunized with Heterologous and Homologous Prime-Boost Vaccination Regimens

16.0 – Immunological Assays

16.1 – Harvesting of Spleens for Characterizing Immune Responses

Three mice from days 21 and 49 mice were sacrificed and their spleens placed in a 60mm petri dish containing a wire mesh submerged in 5mL of L-15 media. Splenocytes were isolated by mechanical disruption of the spleen using a plunger from a sterile 3mL syringe. The resulting product was filtered through a 40 μm cell strainer (BD Falcon) and collected in a 50mL Falcon tube. The collected splenocytes were spun at 485g for 7 minutes at room temperature and the resulting pellet resuspended in 10 mL of L-15. Cells were then treated for 5 min with ACK lysis buffer (Lonza, Switzerland) for lysis of red blood cells and trypan blue added to enumerate live splenocytes with a haemocytometer. The resulting cells were then diluted in RPMI 1640 medium (Mediatech Inc., Manassas, VA) supplemented with 10% FBS, 1X PenStrep, 1X L-glutamine, 1X HEPES, 1X sodium pyruvate, and 1X β-ME (Invitrogen)to a concentration of $5x10^5$ cells/well for ELISPOT analysis.

16.2 – Measurement of Cell-Mediated Response by ELISPOT

An ELISPOT assay was conducted as recommended by the consumer's guidelines (BD bioscience). Briefly, ELISPOT 96-well plates (Millipore, Billerica, MA) were coated with anti-mouse IFNγ capture antibody and incubated for 24h at 4°C (R&D Systems, Minneapolis, MN). The following day, plates were washed with PBS and then blocked for 2h with blocking buffer (10% FBS in RPMI media). Splenocytes (5×10^5 cells/well) were plated and stimulated overnight at 37°C in 5% CO_2 and in the presence of RPMI 1640 (negative control), SEB at a concentration of 1ug/uL (positive control), or the respective peptide pools at a final concentration of 2.5ug/mL. The respective peptide pools consisted of 11 15mers, which spanned the entire length of the EBOV-GP gene and contained overlapping segments which were 9 amino acids in length. After 18 - 24 hours of stimulation, the cells were washed in PBS + 0.05% Tween and incubated for 24h at 4°C with biotinylated anti-mouse IFN-γAb (R&D Systems, Minneapolis, MN). The plates were washed in PBS, and 100uL of streptavidin–alkaline phosphatase (MabTech, Sweden) was added to each well and incubated for 1 h at room temperature. The plates were washed again in PBS + 0.05% Tween, and 100uL of ACE Chromagen (BD biosciences) was added to each well for 15 – 30 min. Finally, the plateswere rinsed with distilled water and dried overnight at room temperature. Spots were counted with an automated ELISPOT reader (Cellular Technology Ltd., Shaker Heights, OH).

16.3 – Measurement of Humoral Response by IgG ELISA

To determine sera antibody titers from immunized mice, 96-well high protein binding ELISA plates (Corning) were coated with 30ng of purified EBOV-GP. The plates were then incubated for 18 h at 4^{o} C, washed with PBS + 0.1% Tween-20, and 30 µl/sample of diluted sera were tested in triplicate at dilutions 1:100, 1:400, 1:1,600, and 1:6,400 in PBS supplemented with 5% skim milk and 0.5% Tween-20. Following an incubation at 37^{o}C for1 h in a moist container, the plates were washed 6x with PBS + 0.1% Tween-20 and then 100 µL of goat anti-mouse IgG-conjugated HRP antibody (Cedarlane) was added (1:3,000 dilution). The plates were then incubated for another 37^{o}C for 1 h in a moist container. After washing 6x with PBS + 0.1% Tween, 30 µl of the ABST (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) and peroxidise substrate (Cedarlane) was added to visualize Ab binding. Again in a moist container, the plate was incubated for 30 min at 37^{o}C and then later read at 405 nm using a plate reader. Positive binding results were characterized by being > 3SD when subtracting the positive control from the negative control serum.

16.4 – Measurement of Humoral Response by Neutralizing Antibody Assay

The EBOV neutralization assay was performed as previously described by Richardson, *et al* (55). Briefly, sera collected from immunized mice were heat inactivated at 56°C for 45 minutes and serially diluted two fold for eight dilutions starting at 1:20in DMEM supplemented with 2% FBS. The diluted sera was mixed with equal volume of EBOV expressing the EGFP reporter gene (EBOV-EGFP) at a concentration of 60 transducing

units/well, according to EGFP expression, and then incubated at 37ºC for 90 minutes. The mixture was then transferred onto ~70% confluent VeroE6 cells in 96-well flat-bottomed plates and incubated for 1 hour at room temperature. Control wells were infected with equal amounts of the EBOV-EGFP virus without addition of serum. After 1 hour, 100uL of DMEM supplemented with 20% FBS was then added to each well and plates were incubated at 37ºC in 5% CO_2 for 48h. EBOV-GFP positive cells were counted in each well and sample dilutions showing >50% reduction in the number of green cells compared to controls scored positive for nAbs. All infectious work was performed in the BSL4 laboratory of NML,PHAC.

17.0 – Survival Studies against MA-EBOV

Mice that were previously vaccinated were weighed and then transferred into the level 4 laboratory in order to be challenged. The dose administered was a concentration of $1000LD_{50}$ of MA-EBOV by intraperitoneal injection. Following challenge, the animals were weighed every day for 12-16 days and monitored for disease progression using an approved score sheet. All procedures were executed according the guidelines outlined by the Institutional Animal Care Committee at the National Microbiology Laboratory (NML) of the Public Health Agency of Canada (PHAC) according to the guidelines of the Canadian Council on Animal Care. All infectious work was performed in the 'Biosafety Level 4' (BSL4) facility at NML, PHAC.

18.0 – Results

18.1 – Production of AAV, VSV and Ad Vectors Expressing EBOV-GP

Cloning techniques were applied for the insertion of the EBOV-GP gene into each of the individual platforms. Introduction of the EBOV-GP gene into the AAV platform involved insertion into the cis plasmid containing two ITR regions. The VSV platform on the other hand involved inserting the gene of interest into the G gene that was deleted from the VSV backbone. Confirmation of proper insertion into the AAV and VSV was determined by restriction digest as well as sequencing of the EBOV-GP gene to ensure that no mutations had occurred.

Previously isolated and characterized in our lab was a porcine AAV virus encoding a unique cap gene, PO6. Successful production of the AAV PO6 EBOV-GP virus was obtained through a triple transfection system using HEK293T cells. The three plasmids involved were the trans plasmid encoding the rep and cap genes, along with the EBOV-GP inserted into the cis plasmid, and the Ad gene encoding the helper plasmid required to drive replication. Introduction of the EBOV-GP gene into the rVSV system involved transfection of VERO and HEK293T cells with a combination of five helper plasmids. Confirmation of successful transfection was through CPE.

Once the EBOV-GP gene was inserted into each of the individual platforms, verification of protein expression was determined through western blot analysis of protein isolates that were collected from each of the four vaccine platforms. As shown in the figure below, the Western blot confirmed EBOV-GP protein expression from each of the vaccine platforms.

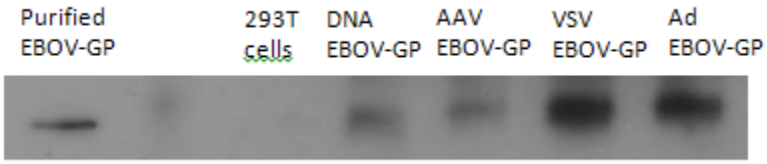


Figure 18.1 – Lysate was collected from transfected HEK293T cells and separated by 10% SDS-PAGE, then transferred to a PVDF membrane. Anti-EBOV-GP monoclonal antibodies were used as the primary antibody and a goat-anti mouse HRP conjugated antibody was used as the secondary.

18.2 – Characterization of DNA, AAV, VSV, and Ad vectors *in Vitro*

18.2.1 – Vaccination of B10Br Mice *in Vitro*

B10Br mice were vaccinated IM, except for the DNA vaccine which was administered IM/EP. Various concentrations of each platform were used to determine the optimal concentration to be utilized in the prime-boost vaccination studies. The DNA platform concentrations were 0.1ug, 1ug, and 10ug; AAV were $1x10^9$ GC, $1x10^{10}$ GC, and $1x10^{11}$ GC; VSV were 1pfu, 10 pfu, and 100 pfu; while Ad was $1x10^3$ pfu, and $5x10^3$ pfu. The concentrations wereselected based on the partial protection conferred against MA-EBOV in order to observe increases or decreases in protection.

18.2.2 – Cell Mediated Responses to Vaccine Platforms

T cell response assays were conducted to determine which platforms are potent inducers of cell-mediated immunity. Mice were sacrificed and their splenocytes isolated on day 10 post-immunization. ELISPOT analysis was performed to determine the population of IFNγ secreting cells upon re-stimulation with 36 overlapping peptide pools encompassing

the EBOV-GP protein. T cell responses were visualized through the detection of spots through the secretion of IFNγ from the cells.

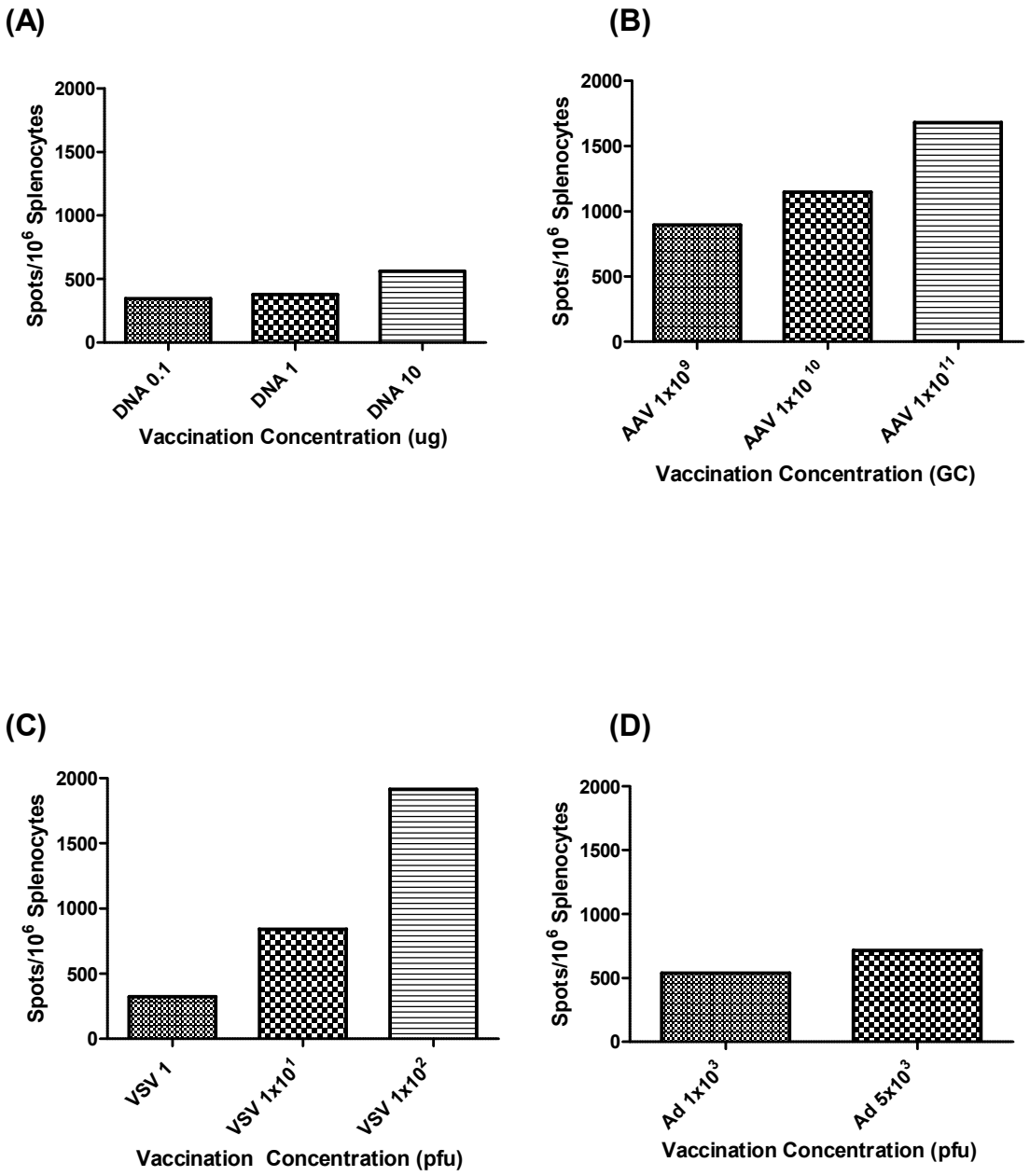


Figure 18.2.2 – Pooled peptide pool responses for groups of 3 B10Br mice vaccinated IM or IM/EP with varying concentrations (A) DNA, (B) AAV, (C) VSV, and (D) Ad expression EBOV-GP. ELISPOTs were performed on splenocytes harvested day 10 post-vaccination and stimulated with 36 different pools of 15mer peptides

corresponding to the EBOV-GP. Responses were visualized based on spots formed by IFNγ secreting T cells.

Both AAV and VSV appear to be good inducers of IFNγ secreting cells in comparison to higher concentrations of DNA and Ad. However, the T cell responses followed a general trend, where an increase in vaccination concentration correlated to an increase of IFNγ secreting T cells.

18.2.3 – Humoral Responses to Vaccine Platforms

To understand the role humoral immunity played in each of the platforms, antibody responses were also evaluated. Serum was collected from the B10Br mice via a saphenous bleed on day 27 post-vaccination and heat inactivated to ensure no complement activation occurred. IgG tiers were determined based on a direct ELISA using horseradish peroxidise and streptavidin signalling for detection. A high protein binding 96-well plate was coated with purified EBOV-GP and serially diluted heat activated serum was added to the plates. The concentration of EBOV-GP specific antibody titers was then determined based on the resulting absorbance read at 405nm. Positive results were based on >3 SD when subtracting the experimental serum from the control serum.

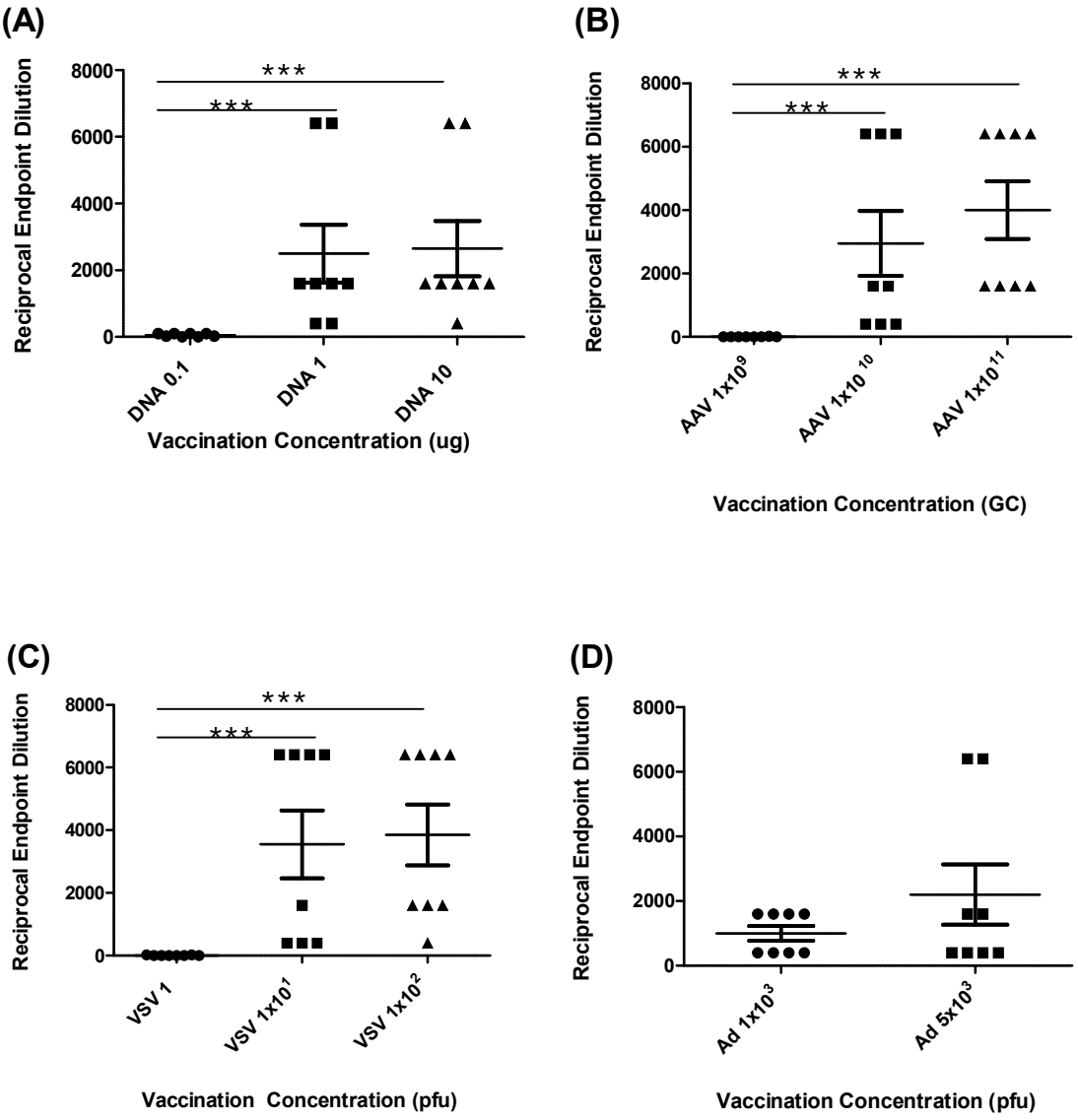
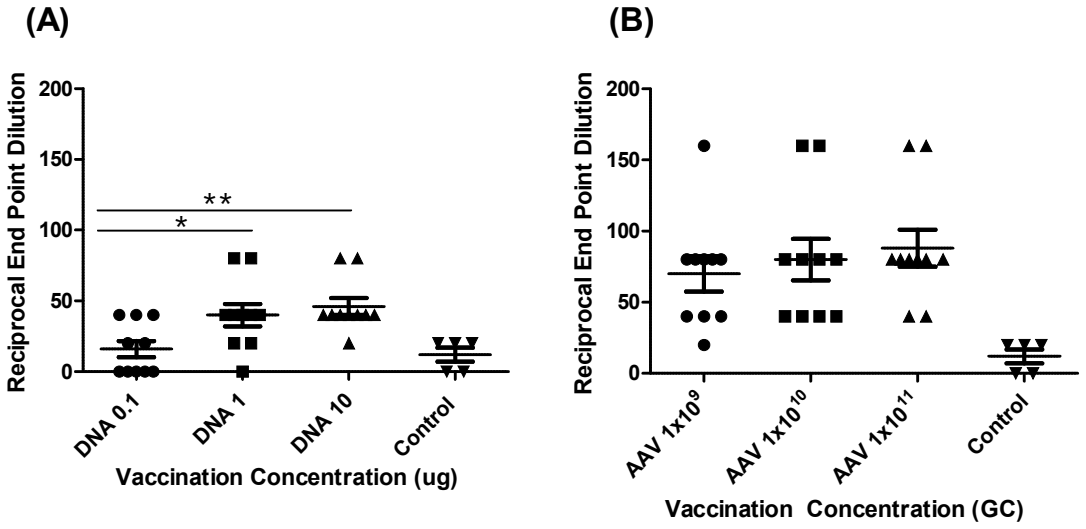


Fig 18.2.3a –Serum collected from groups of 8 B10Br mice vaccinated with varying concentrations of (A) DNA, (B) AAV, (C) VSV, and (D) Ad expressing EBOV-GP day 27 post-immunization. IgG direct ELISA was performed on serially diluted serum based on a horseradish peroxidise reporter system.

Similar to the ELISPOT data, a general trend is observed where an increase in vaccination dose results in an increase in EBOV-GP specific IgG titers.

Further evaluation of the humoral response was conducted using an EBOV-GFP construct to test the presence of nAbs in the collected sera. A mixture of EBOV-GFP and serial diluted sera were added to VERO cells. Entry of the EBOV-GFP virus was detected using a UV light and the reciprocal endpoint dilution was calculated for each serum sample. A lower concentration of fluorescent cells correlated with higher concentrations of nAbs and vice versa. This is due to the ability of nAbs specific against EBOV, therefore, the greater the amount of nAbs present, the more bound to the virus and prevented entry. Sample dilutions showing >50% reduction in the number of florescent cells compared to control sera were scored positive for nAb.



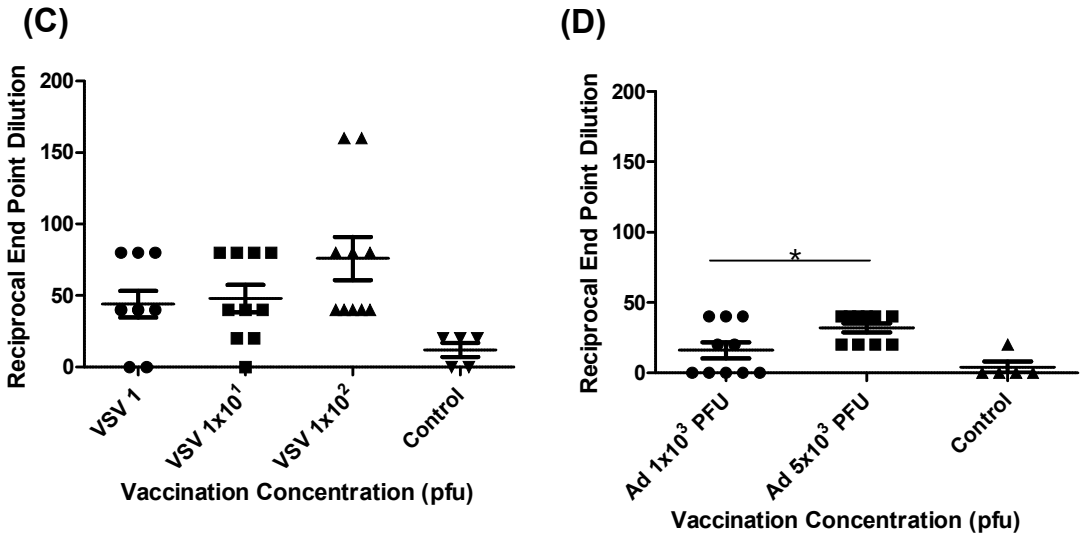
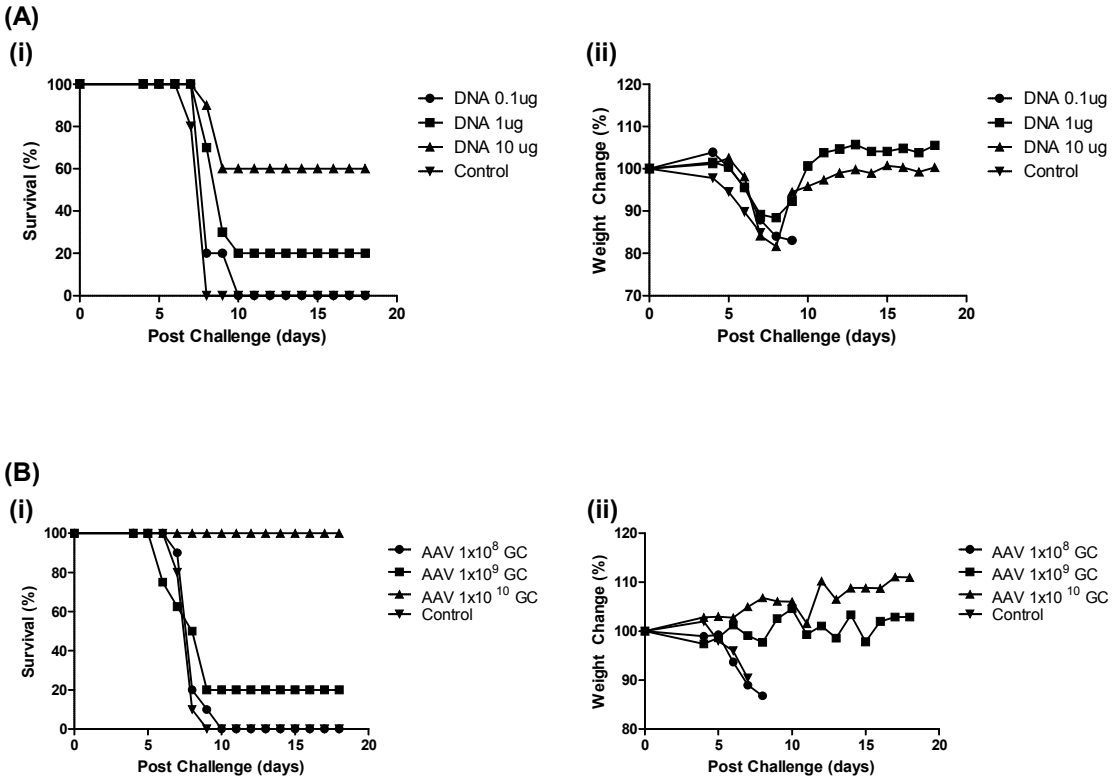


Fig 18.2.3b – Serum collected from B10Br mice vaccinated with varying concentrations of (A) DNA, (B) AAV, (C) VSV, and (D) Ad expressing EBOV-GP day 27 post-immunization. nAb assays were performed on serially diluted serum based on a EBOV-GFP entry into VERO E6 cells.

The same trend was observed as with the previous IgG ELISA and ELISPOT results for the DNA and Ad platforms. However, there does not appear to be any difference in the nAb titers for the AAV and VSV platforms at the doses evaluated.

18.2.4 – Survival against MA-EBOV Challenge

The efficacy of the B10Br mice vaccinated with various doses of the DNA, AAV, VSV and Ad platforms were determined through a lethal challenge of MA-EBOV. Groups of 8 B10Br mice for each vaccination dose were intraperitoneal injected with 1000$LD_{50}$of MA-EBOV and were monitored for weight loss as well as scored for signs of disease. All surviving mice that survived showed no signs of the disease and minimal weight change while all the control mice succumbed to the disease around day 6 post-infection.


(A)
(i)
Survival (%)
Post Challenge (days)
DNA 0.1ug
DNA 1ug
DNA 10 ug
Control
(ii)
Weight Change (%)
Post Challenge (days)
DNA 0.1ug
DNA 1ug
DNA 10 ug
Control
(B)
(i)
Survival (%)
Post Challenge (days)
AAV 1x10^8 GC
AAV 1x10^9 GC
AAV 1x10^10 GC
Control
(ii)
Weight Change (%)
Post Challenge (days)
AAV 1x10^8 GC
AAV 1x10^9 GC
AAV 1x10^10 GC
Control

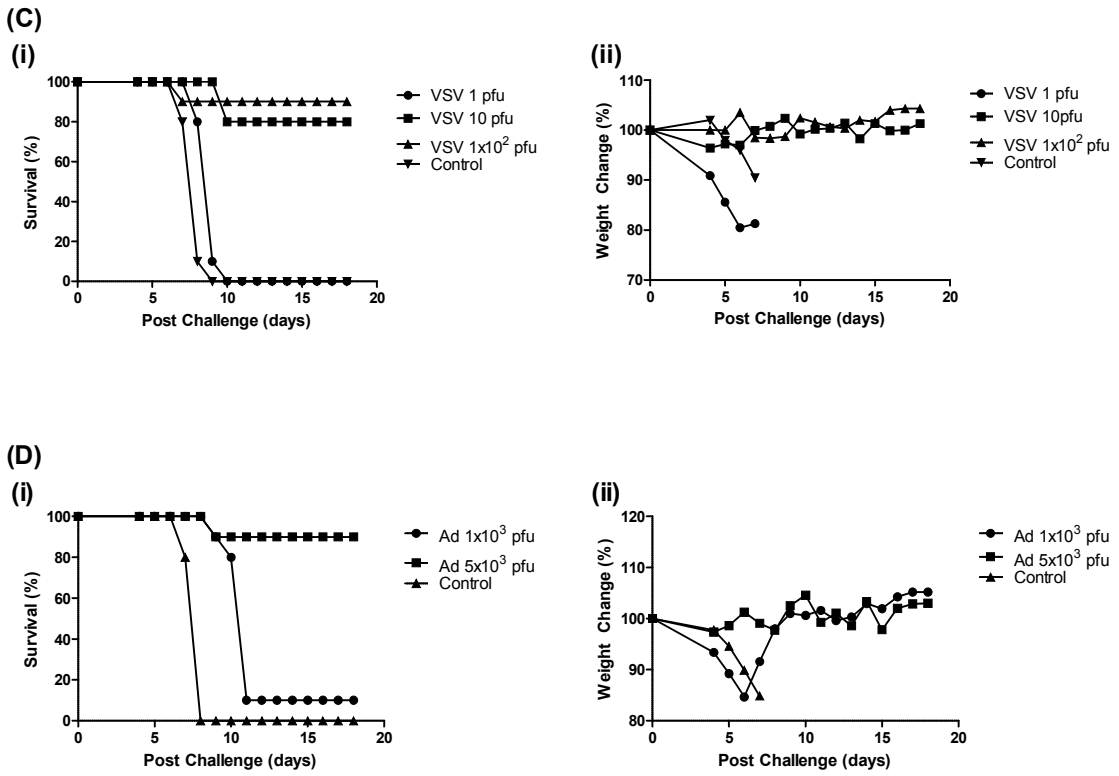


Fig 18.2.4 – Groups of 10 B10Br mice vaccinated IM/EP or IM with different doses of (A) DNA, (B) AAV, (C) VSV, or (D) Ad expressing EBOV-GP. Groups of 5 B10Br mice were vaccinated with PBS as control. Mice were challenged with 1000LD_{50} MA-EBOV by IP injection. Mice were observed for (i) survival and (ii) weight loss.

18.3 – Individual Prime-Boost Vaccination Regimens in B10Br Mice

18.3.1 –Prime-Boost Vaccination of B10Br Mice in Vitro

The selected dose used in heterologous and homologous prime-boost vaccination regimens were DNA was 1ug, AAV was $1x10^9$GC, VSV was $1x10^2$pfu, and Ad was $1x10^3$pfu. The concentrations for each platform were based on the percentage of survival. Groups of 8 B10Br mice were primed and boosted with the selected platform dosage 4 weeks apart either IM or IM/EP.

18.3.2 –Cell-Mediated Responses to Prime-Boost Vaccination

Determination of an increase or decrease in IFNγ secreting T cells, ELISPOTs was conducted on splenocytes harvested 21 days post-prime and post-boost vaccinations. The harvested splenocytes were stimulated with 36 pools of 15mer peptides spanning the EBOV-GP protein. Following stimulation, positive cells were counted based on spots formed based on IFNγ secretion.

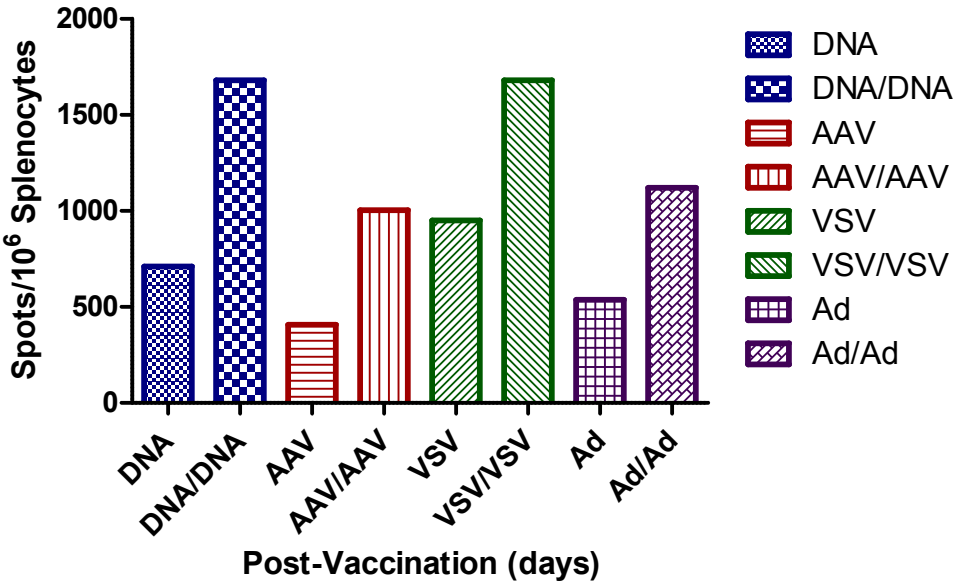


Fig 18.3.2 –Peptide pool responses for groups of 3 B10Br mice vaccinated IM or IM/EP. The mice were primed and boosted with either DNA at 1ug, AAV at $1x10^{9}$GC, VSV at $1x10^{1}$ pfu, or Ad at $1x10^{3}$pfu. ELISPOTs were performed on splenocytes harvested day 10, 38 post-vaccination and stimulated with 36 different pools of 15mer peptides corresponding to the EBOV-GP. Responses were visualized based on spots formed by IFNγ secreting T cells.

Irrespective of platform, there was an increase in IFNγ secreting T cells when boosted with the same vaccine in comparison to mice that had received only one vaccination

18.3.3 – Humoral Responses to Prime-Boost Vaccination

Serum was collected by saphenous bleed from the 8 groups of B10Br mice on day 27 post-prime immunization and day 27 post-boost immunization. IgG tiers were determined based on a direct ELISA that used horseradish peroxidase as a reporter. Heat inactivated serum was serially diluted and was added to 96 well plates coated with purified EBOV-GP. The plates were read at an absorbance of 405nm, a positive result based on whether the experimental serum subtracted from the control serum was >3SD.

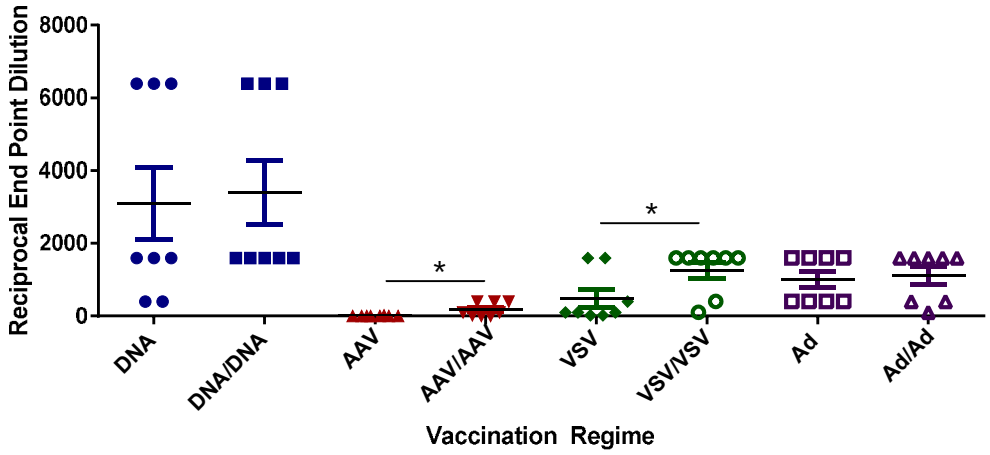


Fig 18.3.3a – Serum collected from groups of 8 B10Br mice prime-boost vaccinated with either 1ug DNA, $1x10^9$ GC AAV, $1x10^1$ VSV, or $1x10^3$pfu Ad expressing EBOV-GP on day 27 post-prime and day 27 post-boost vaccination. IgG direct ELISA was performed on serially diluted serum based on a horseradish peroxidise reporter system.

There were significant specific IgG Abs to EBOV following a single immunization with DNA and Ad compared to the naïve sera which did not significantly increase after a second immunizations. In contrast, with AAV and VSV a single immunization did not

induce significant Ab titers. However, following a second immunization both AAV (p=0.036) and VSV EBOV-GP (p=0.034) generated significant IgG titers.

The humoral response of the prime-boost vaccinations involving the use of a single platform was evaluated for the presence of nAbs based on an EBOV-GFP construct. The serum that was collected day 27 post-prime and post-boost vaccinations were serially diluted and mixed with the EBOV-GFP virus. This mixture was then added onto a 96-well plate containing VERO cells and read under a UV light to test the presence of GFP. The reciprocal end point dilution was calculated for each experimental serum based on the degree of fluorescence counted. High concentration of fluorescence correlated with a lower titer of nAbs. Samples demonstrating a reduction of >50% in the number of florescent cells compared to the control sera were scored positive for nAbs.

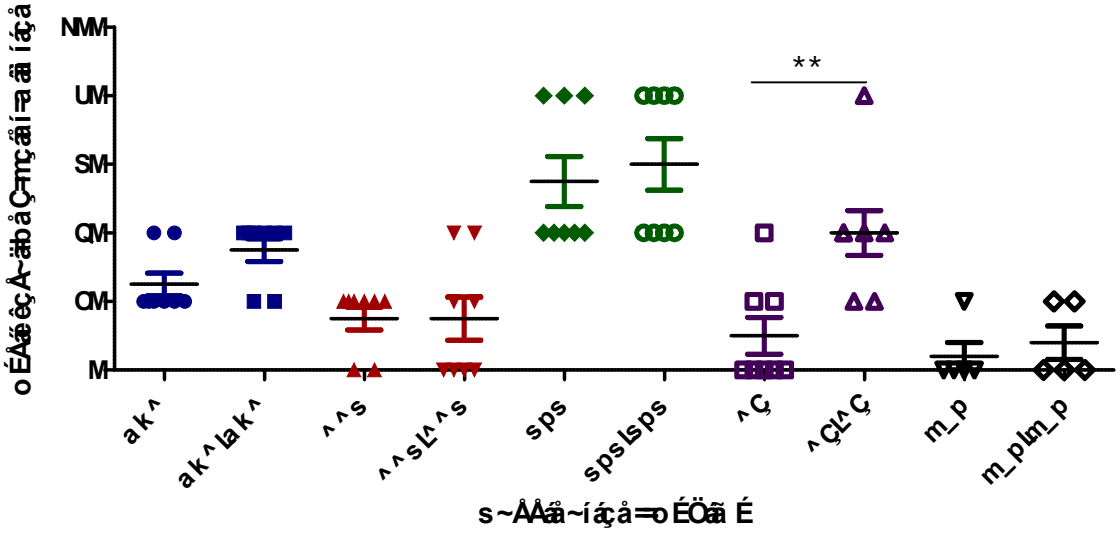
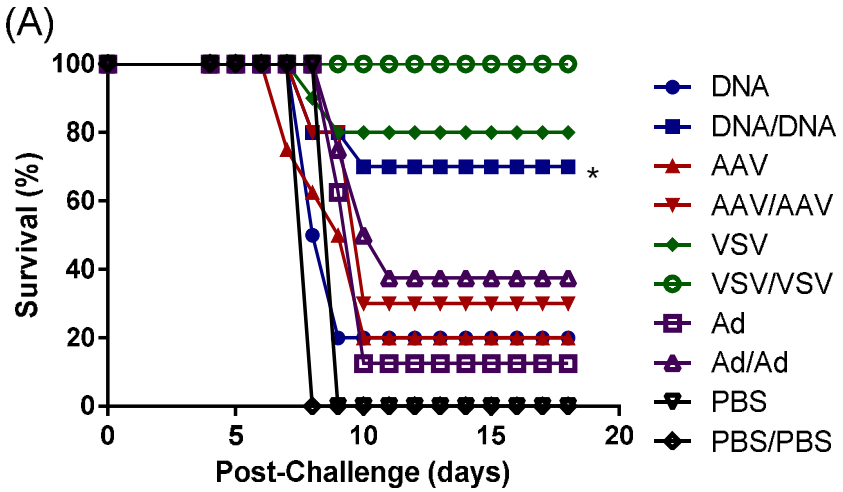


Fig 18.3.3b – Serum collected from groups of 8 B10Br mice prime-boost vaccinated with either 1ug DNA, $1x10^9$ GC AAV, $1x10^1$pfu VSV, or $1x10^3$pfu Ad expressing EBOV-GP on day 27 post-prime and day 27 post-boost vaccination. nAb assays were performed on serially diluted serum based on a EBOV-GFP entry into VERO E6 cells.

In contrast to the IgG ELISA data, there was no significant increase in nAbs with an AAV or VSV prime-boost vaccination strategy. The Ad platform is the only prime-boost strategy that resulted in a significant increase.

18.3.4 – Survival of Prime-Boost Vaccination against MA-EBOV

Protection against MA-EBOV was evaluated for each prime-boost vaccination involving the use of one platform. B10Br mice were primed and boosted with either 1ug DNA, $1x10^9$ GC AAV, $1x10^1$pfu VSV, or $1x10^3$pfu Ad expressing EBOV-GP, then challenged with a lethal dose of MA-EBOV. All mice were monitored for weight loss and scored for signs of disease. Control mice vaccinated with PBS succumbed to the disease around day 6 post-infection. Mice that survived show no symptoms of disease and marginal weight loss.



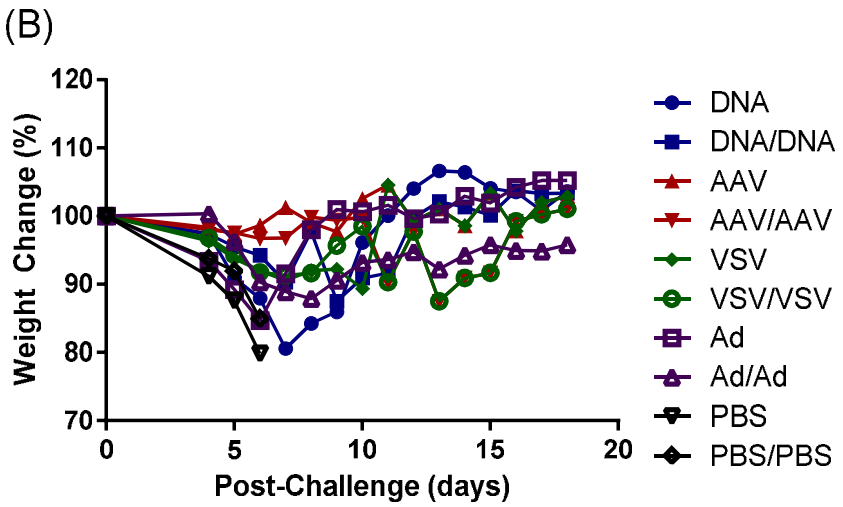


Fig 18.3.4 – Groups of 8 B10Br mice prime-boost vaccinated IM or IM/EP with vaccinated with either1ug DNA, $1x10^{9}$ GC AAV, $1x10^{1}$pfu VSV, or $1x10^{3}$pfu Ad expressing EBOV-GP 4 weeks apart. Groups of 5 B10Br mice were vaccinated with PBS as control. Mice were challenged with $1000LD_{50}$ MA-EBOV by IP injection. Mice were observed for (A) survival and (B) weight loss.

There was a general increase in survival of the mice that had received the prime-boost vaccination regimen in comparison to the mice that had only received one immunization of each platform. However, the utilization of the DNA platform was the only prime-boost regimen that statistically increased survival (p=0.012) against MA-EBOV in comparison to a single immunization.

18.4 – Homologous and Heterologous Regimens in B10Br Mice

18.4.1 – Homologous and Heterologous Vaccination of B10Br Mice

Groups of 8 B10Br mice were vaccinated with a heterologous or homologous vaccination 4 weeks apart with different vaccine platforms. The prime or boost dosage for each

platform was 1ug of DNA, $1x10^9$ GC of AAV, $1x10^1$pfu of VSV, and $1x10^3$pfu of Ad. Definition of a heterologous vs. a homologous prime-boost vaccination is based on the dominant immune response that is induced by the individual platform. A heterologous combination would therefore involve two vaccine vectors that induce a robust cellular immune response, while the other induced a strong humoral response (ie. AAV which induces a strong humoral response paired with DNA, which induces robust cell-mediated responses). In contrast, a homologous prime-boost vaccination is defined by two vectors that stimulate either a strong cellular immune response or humoral response (ie. Ad and DNA vectors which both induce strong cell-mediated responses).

18.4.2. – Cell-Mediated Responses of Regimens in B10Br Mice

T cell response was evaluated to determine whether the homologous or heterologous vaccinations work synergistically to increase the amount of IFNγ secreting cells. Splenocytes were harvested on days 10,26,38, and 49 post-prime vaccination and stimulated with 36 pools of peptides overlapping the EBOV-GP protein. Positives were counted by spot formation based on IFNγ secretion following stimulation.

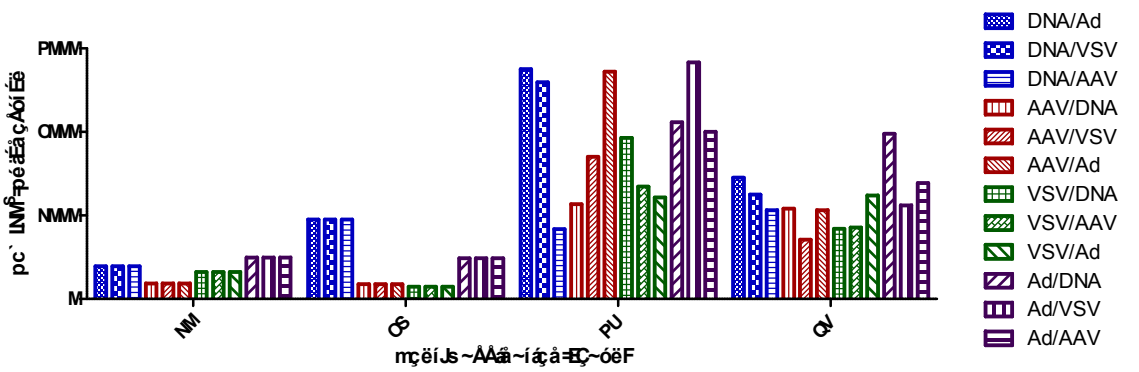
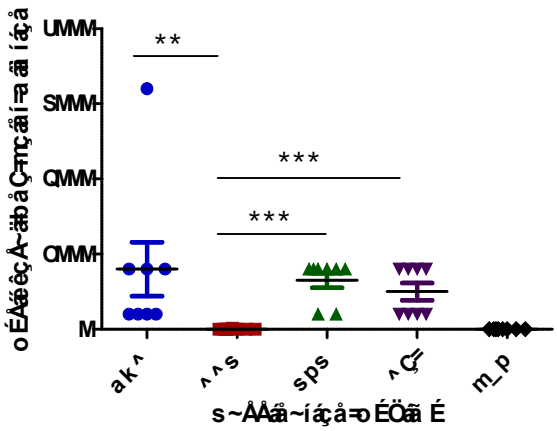


Fig 18.4.2 – Peptide pool responses for groups of 3 B10Br mice vaccinated IM or IM/EP. The mice were primed and boosted with either DNA at 1ug, AAV at $1x10^{9}$GC, VSV at $1x10^{1}$pfu, or Ad at $1x10^{3}$pfu. ELISPOTs were performed on splenocytes harvested day 10,26,38, and 49 post-vaccination and stimulated with 36 different pools of 15mer peptides corresponding to the EBOV-GP. Responses were visualized based on spots formed by IFNγ secreting T cells.

18.4.3 – Humoral Responses of Regimens in B10Br Mice

Serum was collected from 8 groups of B10Br mice by saphenous bleed on day 27 and 53 post-prime immunization. After heat inactivation of the serum, it was serially diluted and added onto 96 well plates coated with EBOV-GP. The plates were read at an absorbance of 405nm, a positive result based on whether the vaccinated serum was >3SD than the control serum.

(A)



(B)

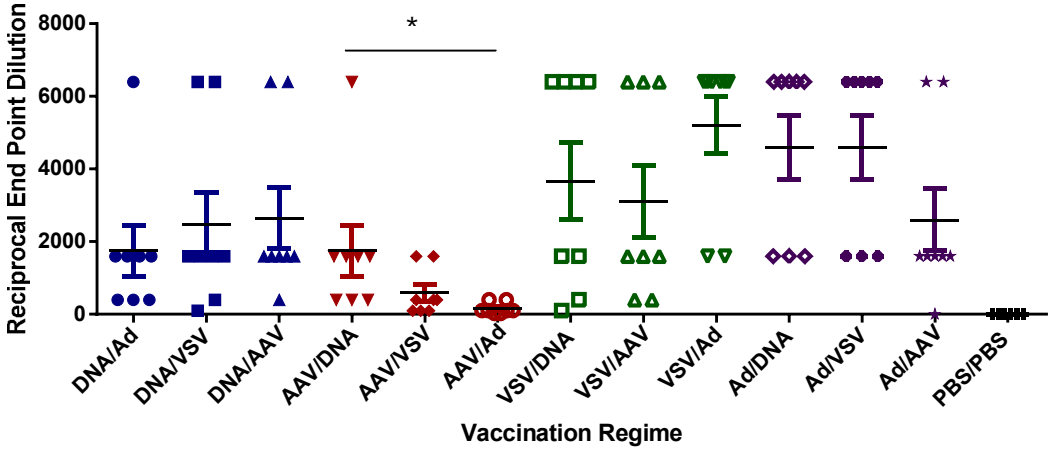
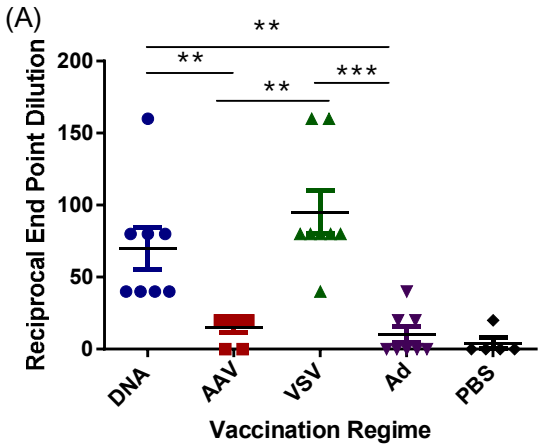


Fig 18.4.3a – Serum collected from groups of 8 B10Br mice heterologous or homologous prime-boost vaccinated with either 1ug DNA, $1x10^9$ GC AAV, $1x10^1$pfu VSV, or $1x10^3$pfu Ad expressing EBOV-GP on (A) day 27 post-prime and (B) day 27 post-boost vaccination. IgG direct ELISA was performed on serially diluted serum based on a horseradish peroxidise reporter system.

There is a general increase in IgG titers when boosted with a second platform, regardless of vaccine selected as the boost. DNA, VSV and Ad vaccines appear to produce high IgG concentrations against EBOV-GP alone, and maintain high IgG concentrations when

utilized as a prime. Whereas, AAV and VSV appear to stimulate lower concentrations of IgG individually their levels are increased when boosted with DNA.

The presence of nAbs in the sera collected was determined by an EBOV-GFP construct to further assess the effects of homologous and heterologous prime-boost vaccinations on humoral response. Diluted sera was mixed with EBOV-GFP and then introduced to wells containing VERO cells. Entry of the EBOV-GFP virus was detected under UV light, where excessive florescence was correlated to lower concentrations of nAbs in the experimental serum. The threshold for positive nAbs calculated based on a >50% reduction in florescent cells compared to the more concentrated sera dilution.



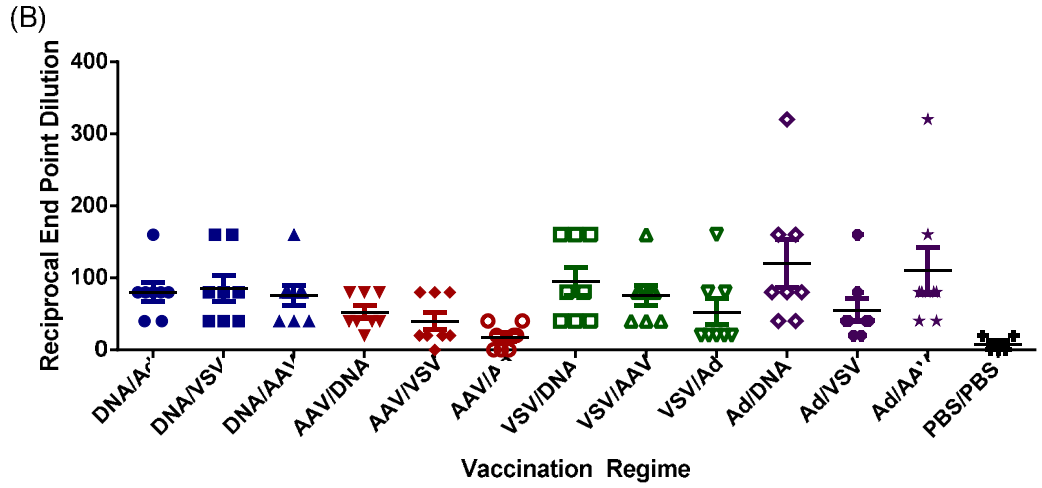


Fig 18.4.3b - Serum collected from groups of 8 B10Br mice heterologous or homologous prime-boost vaccinated with either 1ug DNA, $1x10^9$ GC AAV, $1x10^1$pfu VSV, or $1x10^3$pfu Ad expressing EBOV-GP on (A) day 27 post-prime and (B) day 27 post-boost vaccination. nAb assays were performed on serially diluted serum based on a EBOV-GFP entry into VERO E6 cells.

18.4.4 – Survival of Regiments against MA-EBOV in B10Br Mice

Determination of survival against MA-EBOV was evaluated for the homologous and heterologous prime-boost regimens in B10Br mice. Mice were primed and boosted with either1ug DNA, $1x10^9$ GC AAV, $1x10^1$pfu VSV, or $1x10^3$pfu Ad expressing EBOV-GP. Following challenge all mice were monitored for weight loss and signs of disease. The mice that survived displayed no disease symptoms and minimal weight loss. Control mice succumbed to the disease approximately 6 days post-infection.

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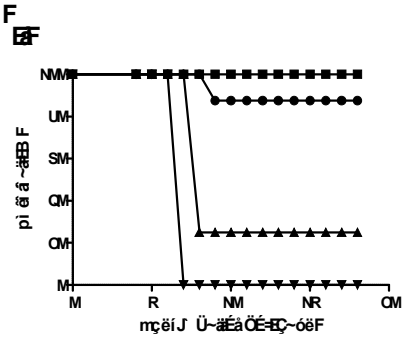
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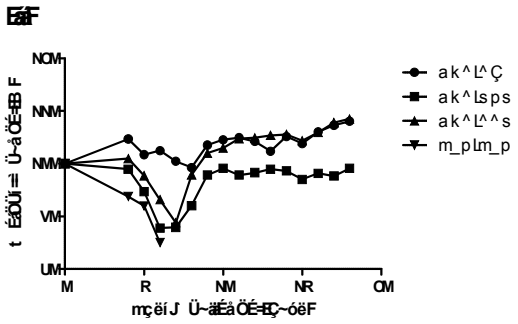
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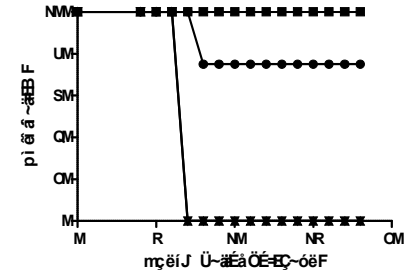
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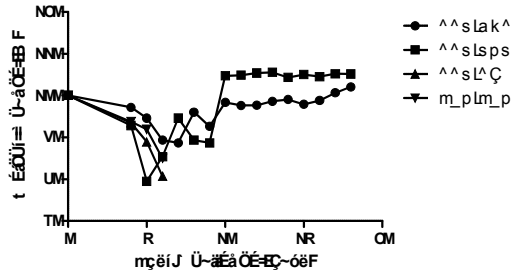
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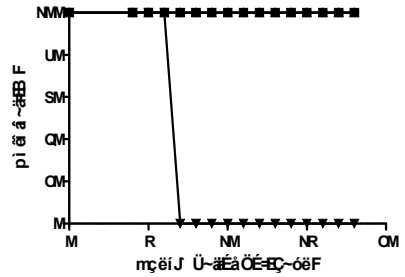
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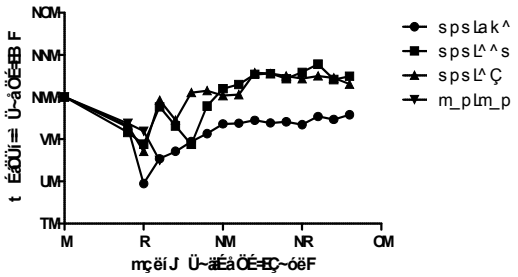
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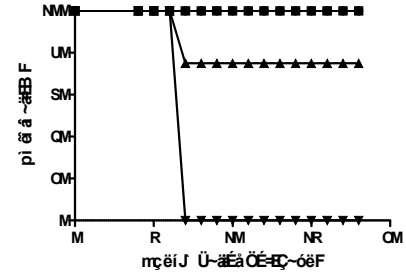
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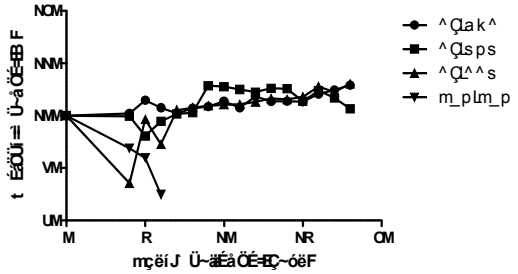
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Fig 18.4.4 – Groups of 8 B10Br mice homologous or heterologous prime-boost vaccinated 4 weeks apart IM or IM/EP primed with either (A) 1ug DNA, (B) $1x10^9$ GC AAV, (C) $1x10^1$pfu VSV, or (D) $1x10^3$pfu Ad expressing EBOV-GP 4. Groups of 5 B10Br mice were vaccinated with PBS as control. Mice were challenged with $1000LD_{50}$ MA-EBOV by IP injection. Mice were observed for (i) survival and (ii) weight loss

Those mice that had been primed with VSV displayed 100% efficacy against MA-EBOV, while there was variation among the other three platforms when utilized as a prime. In general an increase in survival in comparison to an individual vaccination alone was observed.

Table 18.4.4 – Summary of survival for individual platform, homologous, and heterologous prime-boost vaccinations in B10Br mice challenged with MA-EBOV.

Percent Survival Prime in B10Br mice (%)		Percent Survival (%)		Efficacy (% possible enhancement)
DNA	20	DNA/DNA	70	62.5
		DNA/Ad	87.5	84.4
		DNA/VSV	100	100
		DNA/AAV	25	6.25
AAV	20	AAV/AAV	30	12.5
		AAV/DNA	75	68.75
		AAV/VSV	100	100
		AAV/Ad	0	0
VSV	80	VSV/VSV	100	100
		VSV/DNA	100	100
		VSV/AAV	100	100
		VSV/Ad	100	100
Ad	12.5	Ad/Ad	37.5	28.6
		Ad/DNA	100	100
		Ad/VSV	100	100
		Ad/AAV	75	71.4

18.5 – Homologous and Heterologous Regimens in Balb/c Mice

18.5.1 – Homologous and Heterologous Vaccination of Balb/c Mice

To compare the humoral immune response and efficacy of two different mice strains, Balb/c mice in groups of 8 were immunized with either a homologous or heterologous combination of vaccines 4 weeks apart. The prime or boost dosage for each platform was 1ug of DNA, $1x10^9$ GC of AAV, $1x10^1$pfu of VSV, and $1x10^3$pfu of Ad, while the control Balb/c mice received PBS. A heterologous vaccination is defined as utilizing two vectors that elicit opposite arms of the immune response (ie. humoral **vs.** cell-mediated), while a homologous vaccination employs two platforms that induce a similar immune response (ie. humoral **or** cell-mediated).

18.5.2 – Humoral Responses of Regimens in Balb/c Mice

Day 27 and 53 post-prime vaccination, serum was collected via saphenous bleed from groups of 8 immunized Balb/c mice. The serum was then serially diluted and added onto 96 well plates that were coated with purified EBOV-GP. Absorbance was measured at 405nm and a positive outcome was the result of the vaccinated serum containing an absorbance reading >3 SD than the control serum.

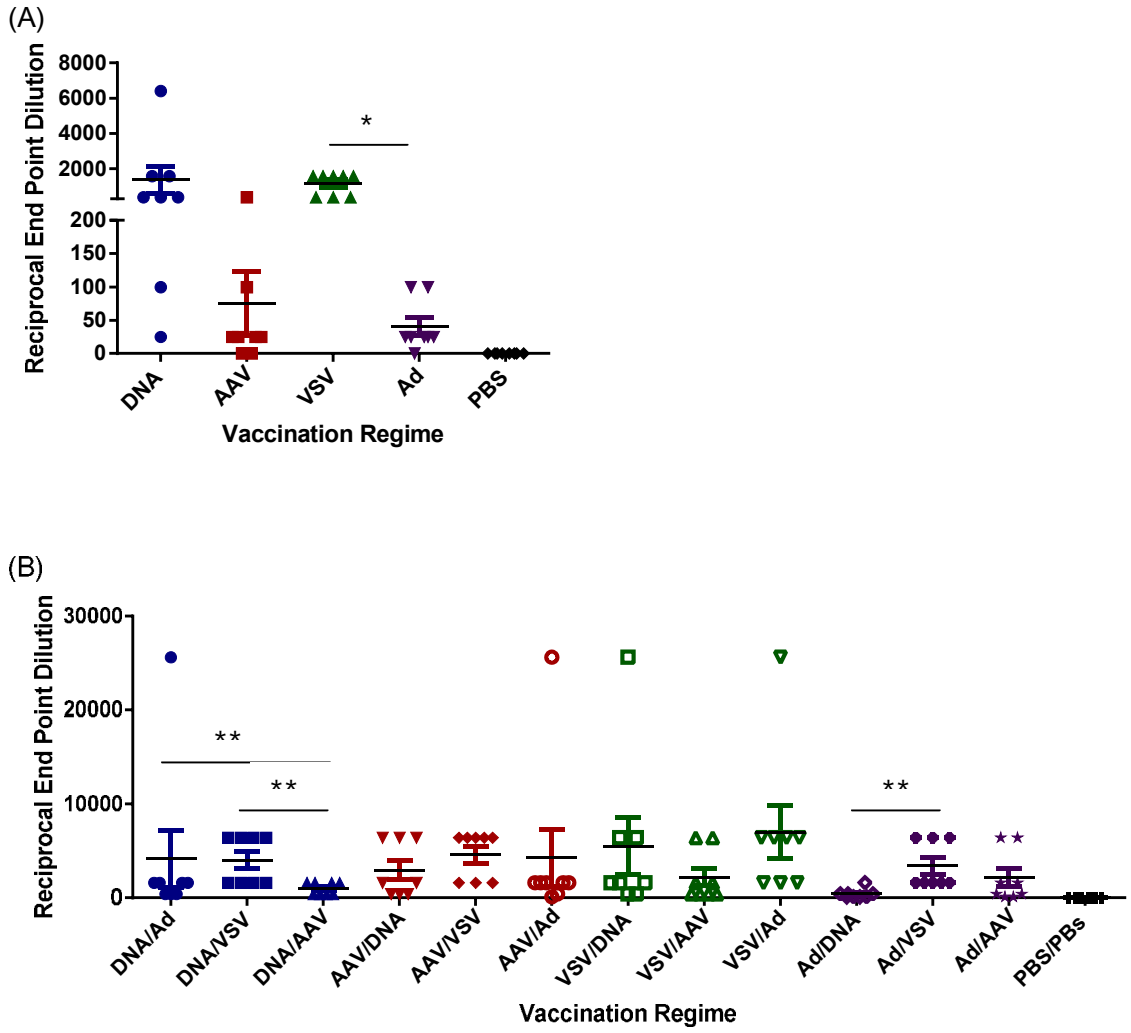

Fig 18.5.2a – Serum collected from groups of 8 Balb/c mice heterologous or homologous prime-boost vaccinated with either 1ug DNA, $1x10^9$ GC AAV, $1x10^1$pfu VSV, or $1x10^3$pfu Ad expressing EBOV-GP on (A) day 27 post-prime and (B) day 27 post-boost vaccination. IgG direct ELISA was performed on serially diluted serum based on a horseradish peroxidise reporter system.

Further evaluation of the humoral response was accomplished by a nAb assay though the use of a EBOV-GFP construct. Sera serially diluted were added with EBOV-GFP virus onto VERO cells. Entry of EBOV-GFP into the cells was based on florescence observed under a UV light. Inhibition of viral entry indicated the presence of nAbs, resulting in a

decrease of florescence. A positive nAb titer was based on a >50% reduction in florescent cells compared to the more concentrated serial dilution.

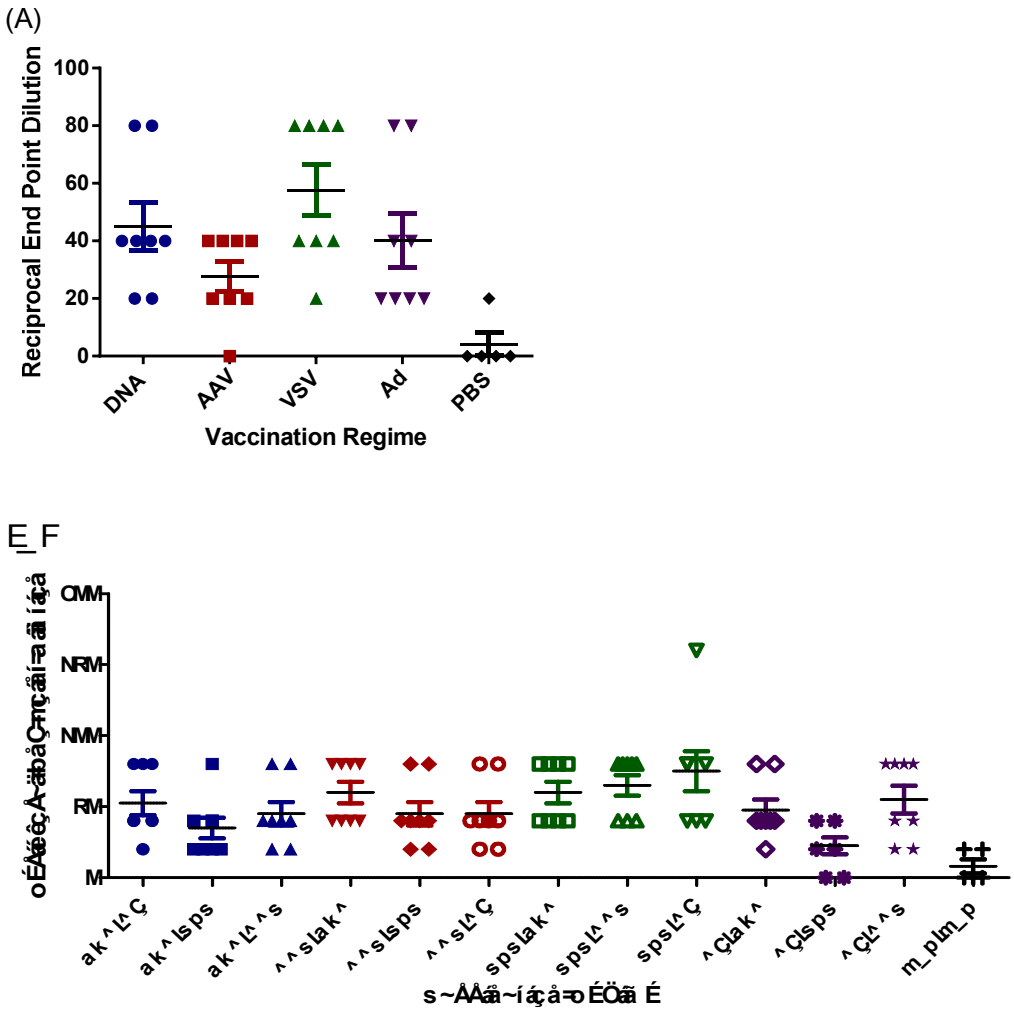

Fig 18.5.2b – Serum collected from groups of 8 Balb/c mice heterologous or homologous prime-boost vaccinated with either 1ug DNA, 1x10^{9} GC AAV, 1x10^{1}pfu VSV, or 1x10^{3}pfu Ad expressing EBOV-GP on (A) day 27 post-prime and (B) day 27 post-boost vaccination. nAb assays were performed on serially diluted serum based on a EBOV-GFP entry into VERO E6 cells.

In general, the nAb titers for all prime-boost vaccination regimens are similar. A similar or increase in titer is observed for the majority in comparison to a single immunization of each platform.

18.5.3 – Survival of Regimens against MA-EBOV in Balb/c Mice

To determine whether there is a difference in survival when the prime-boost regimens are applied to a different mice strain, Balb/c mice were immunized with the various vaccine combinations and challenged with a lethal dose of MA-EBOV. Mice were primed and then boosted with either 1ug DNA, $1x10^{9}$ GC AAV, $1x10^{1}$pfu VSV, or $1x10^{3}$pfu Ad expressing EBOV-GP. The Balb/c mice were monitored for weight loss and signs of disease post-infection. The mice that survived displayed no disease symptoms and minimal weight loss. Control mice succumbed to the disease approximately 6 days post-infection.

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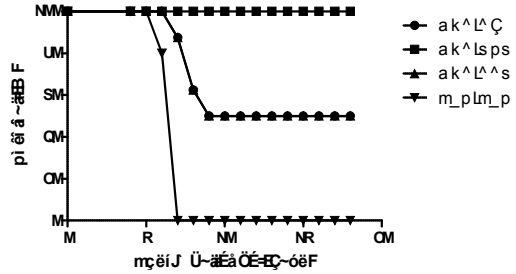
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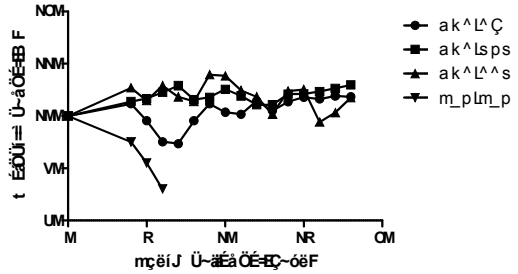
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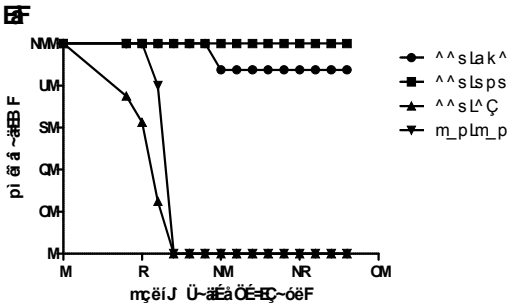
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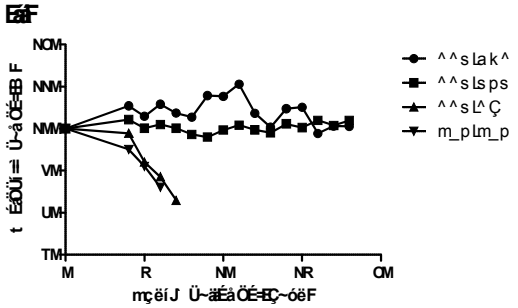
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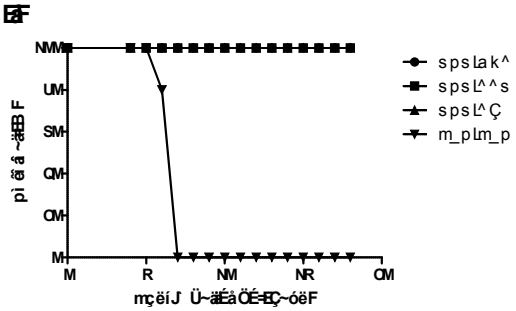
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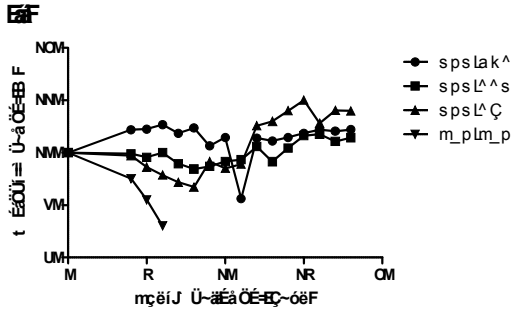
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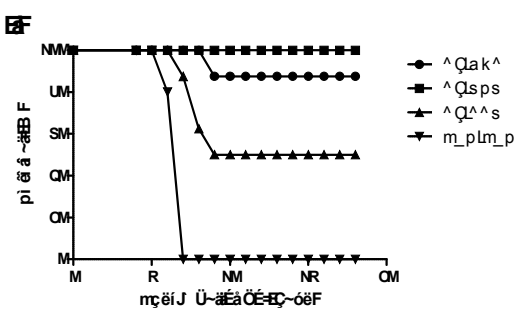
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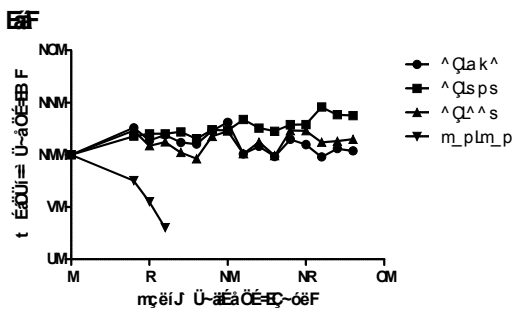
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Fig 18.5.2 – Groups of 8 Balb/c mice homologous or heterologous prime-boost vaccinated 4 weeks apart IM or IM/EP primed with either (A) 1ug DNA, (B) $1x10^9$ GC AAV, (C) $1x10^1$pfu VSV, or (D) $1x10^3$pfu Ad expressing EBOV-GP 4. Groups of 5 B10Br mice were vaccinated with PBS as control. Mice were challenged with $1000LD_{50}$ MA-EBOV by IP injection. Mice were observed for (i) survival and (ii) weight loss

Balb/c mice prime with VSV demonstrated 100% efficacy against MA-EBOV, while the survival varied amongst the other three platforms when utilized as a prime. Overall, there was an increase in survival with the prime-boost vaccinations in comparison to a single administration of each platform.

Table 18.5.2 – Summary of Heterologous and Homologous Prime-Boost Vaccination Regimens in Balb/c Mice Challenged with MA-EBOV

Percent Survival Prime in Balb/c mice (%)		Percent Survival Boost (%)		Efficacy (% possible enhancement)
DNA	20	DNA/DNA	70	87.5
		DNA/Ad	50	62.5
		DNA/VSV	100	100
		DNA/AAV	50	62.5
AAV	20	AAV/AAV	30	37.5
		AAV/DNA	87.5	84.3
		AAV/VSV	100	100
		AAV/Ad	0	0
VSV	80	VSV/VSV	100	100
		VSV/DNA	100	100
		VSV/AAV	100	100
		VSV/Ad	100	100
Ad	12.5	Ad/Ad	37.5	28.6
		Ad/DNA	87.5	85.7
		Ad/VSV	100	100
		Ad/AAV	50	42.9

19.0 – Discussion

19.1 – Characterization of DNA, AAV, VSV, and Ad Expressing EBOV-GP

In the EBOV field, all platforms utilized in this study have been applied in the past as potential vaccine strategies against EBOV, with the exemption of AAV. Thefocus on individual platform development has resulted in heterologous prime-boost regimens to be overlooked. This study is the first to define heterologous prime-boost vaccinations based on the dominant immune response induced. Past research on the individual platforms in different disease models has permitted categorizing DNA (26, 57) and Ad (49, 51) as potent inducers of cell-mediated immunity, in contrast to VSV (21, 33) and AAV (19, 20) which induce strong humoral responses. This strategic characterization of the platforms allows insight on how cell-mediated and humoral immunity maylead to an increase or decrease in survival against MA-EBOV.

19.1.1 – Cell-Mediated and Humoral Immunity of Platforms

Successful expression of EBOV-GP was clarified by Western blot, where all four platforms were comparable to the positive control. Following confirmation, immunological characterizations of the individual platforms were conducted to evaluate cell-mediated and humoral immunity. ELISPOT assaysassessed cell-mediated immunity based on spot development of IFNγ secreting cells harvested from splenocytes. All platforms exhibited higher amounts of IFNγ secreting cellswith an increase in vaccine dose. Higher concentrations of VSV and AAV platformsresulted inelevated spot formation development in comparison to DNA and Ad platforms. The minimal amount

of T cells inducedby the DNA platform can be attributed to the 10x fold increase in dosage in comparison to the viral strategies which received a 1 log difference.

The humoral immune response for all vaccine platforms mirrored the trend illustrated by the ELISPOT data, where an increase in vaccine dosage correlated to elevated humoral immunity. One exception was the Ad platform, where a greater dose of the vaccine did not yield greater IgG or nAbs titers. The AAV and VSV strategieswererobust stimulators of the humoral immune response compared to the DNA and Ad platform.

Overall, there appears to be a general increase of both cellular, as well as humoral immunity, with the AAV and VSV vectors as opposeto the DNA and Ad platforms. Humoral responses revealed that AAV and VSV generate strong antibody responses, in agreement to previous findings (30,41,41). Surprisingly, AAV and VSV elicited greater cell-mediated immunity in comparison to the DNA and Ad platforms, thoughthe latter are recognized as robust T cell inducers (23,52). However, comparison based on dosage is difficult since there are various factors, such as route of administration and antigen presentation, which play integral roles in stimulation of the immune response. Investigation of the functionality of the activated T-cells could also play an important role in a more global evaluation.

19.1.2 – Survival of Platforms Expressing EBOV-GP against MA-EBOV

Selection of an appropriate dose to employ in homologous and heterologous prime-boost vaccinations was determined through a survival study. In general,an increase in survival against MA-EBOV was associated with a higher vaccine dose regardless of which

platform expressing EBOV-GP was administered. Initially it was challenging to select a proper dose for the viral platforms so anincrease or decrease in efficacy could be observed in a prime-boost setting. The most difficult platform was the VSV construct, where a log increase in dose increased survival from 0% to almost 100% efficacy against MA-EBOV. This can be attributed to the VSV vector beingreplication competent. The final doses that were chosen to be utilized in the prime-boost strategies were: DNA at 1ug at 20%, AAV at $1x10^9$GC at 20%, VSV at $1x10^1$ pfu at 80%, and Ad at $1x10^3$ pfu at 12.5%. These doses conferred partial protection and therefore a range where increases or decreases in efficacy could be observed with the different conditions investigated in this study.

19.2 – Individual Platform Prime-Boost Regimens

19.2.1 – Humoral Immune Response of Individual Platform Regimens

At the concentrations chosen, B10Br mice were primed and boosted with the same vaccine platform 4 weeks apart to assess whether an increase in cell-mediated or humoral immunitywould be achieved compared to a single vaccination. Based on the ELISPOT data, all platforms exhibited a general increase in IFNγ secreting T cells compared to a single vaccination. Application of the VSV and DNA vectors in a prime-boost regimen expressed the highest boost in IFNγ response. DNA is known to elicit strong cell-mediated responses therefore this observation is on conjunction with established research (63). The elevated level of IFNγ secretion by VSV can be associated with the 80% survival following one immunization in the previous study.

Significantly induced humoral immunity of both AAV and VSV prime-boost regimenswere demonstrated byelevated IgG titers,in contrast to DNA and Ad whose subsequent boosts had minimal effect. With regards to nAb titer, the only significant increase was observed with the Ad platform. This solidifies the conclusion that AAV and VSV are potent stimulators of humoral immunity. However, the humoral responsesfor mice that received a single immunization are comparable to those vaccinated with a prime-boost regimen. The minimal induction of immunityobserved by prime-boost regimens with a single platform is consistent with whatwas previously established (65,67).

19.2.2. – Survival of Individual Platform Regimens against MA-EBOV

To determine whether an individual platform employed both as the prime and boost vaccination would increase efficacy, mice were subjected to a lethal challenge of MA-EBOV. Elevated survival rates were found for all vaccine platforms subjected to a prime-boost strategy in comparison to those that had received a single vaccination. The marginal 10-25% increase in survival against MA-EBOV for all viral platforms can be a consequence of anti-vector immunity, which may affect the efficacy of the boost due to deficient antigen presentation (64).In contrast, the DNA vaccine demonstrated a 50% increase in survival when implemented as a prime-boost strategy. Since the DNA vaccine does not rely on a viral delivery system, there is no development of an anti-viral immune response. Thereforethe immunity developedis focused on the antigen of interest,which leads to elevated efficacy (23,62).

19.3. – Homologous and Heterologous Vaccinations Regimens

19.3.1. – Defining Heterologous vs. Homologous Regimens

In the context of this study, homologous vs. heterologous prime-boost vaccinations are categorized according to the predominant immune response induced by each individual platform. Based on previous research, DNA (23) and Ad (53) are strong inducers of cell-mediated immunity, while AAV (42) and VSV (30) elicit robust humoral responses. Therefore a homologous prime-boost immunization involves two vaccine platforms that inducethe same immune response (ie. a DNA prime and Ad boost, which individually evoke strong cell-mediated responses). In contrast, a heterologous prime-boost combination utilizes two platforms that individually induce opposing arms of the immune response (ie. an AAV prime followed by a DNA boost, where AAV elicits a potent humoral response while the latter evokes strong cell-mediated immunity).

19.3.2. – Cell-Mediated Responses of Regimens in B10Br Mice

Enumeration of IFNγ secreting cells was established on day 10, 26, 38, and 49 post-prime vaccinations to determine whether the homologous or heterologous prime-boost combinations collectively induce cellular immunity. Regardless of the prime administered, all mice boosted had higher IFNγ secretion in contrast to the prime alone. This is in compliance with previous prime-boost research indicting elevated immune responses following a prime-boost regimen (69, 72). Following prime administration, the

day 10 time point indicated that both DNA and Ad had higher levels of IFNγ in comparison to VSV and AAV. Following 10 days post-boost vaccination, there was an increase in cell-mediated immunity for all prime-boost vaccinations. However, successive time points for the DNA EBOV-GP vaccine revealed a continual enlargement of T cell IFNγ secretion in contrast to the viral platforms which plateau at day 26. If IFNγ expression is subdivided based on the prime administered, peak expression for the all groups received the Ad boost. The exception was the VSV group, where DNA proved to be the superior boost vaccination. The optimal boost for the Ad prime was DNA.

If categorized based on the prime vaccination, IFNγ expression by heterologous prime-boost vaccinations (AAV/Ad, VSV/DNA, and Ad/VSV)were superior to homologous regimens (DNA/Ad). The DNA/Adstrategy appeared to collectively enhance cell-mediated immunity, similar to an a NHP study, where a DNA/Ad boost resulted in strong $CD8^+$ T cell responses in comparison to the animals only administered with DNA(78).

19.3.3 – Humoral Responses of Regimens in Balb/c and B10Br Mice

All viral platforms on day 27 post-prime and post-boost immunization resulted in elevated IgG titers for both B10Br and Balb/c mouse models compared to the mice that received a single vaccination. Analysis according to the prime administered demonstrated robust titers for heterologous strategies in both mouse models when measured against homologous combinations. For B10Br mice, VSV and Ad provide the ideal prime for IgG Ab production against EBOV-GP in contrast to Balb/c mice where DNA and VSV prime immunizations obtained higher IgG titers.

Marginal stimulation of humoral immunity was observed in the VSV and Ad prime vaccinations in comparison to DNA and AAV in B10Br mice. This minimal induction may aid in setting the foundation of the immune response since a prime vaccination that induces a strong immune response may dampen the effect of the boost and prevent memory establishment (65). This phenomenon canexplain the small increase in IgG titers in mice that received the DNA primein contrast to thesubstantial boost in IgG Abs observed for regimens receiving the VSV or Ad prime.

The optimal boost for B10Br mice was the DNA platform. Since it is administered as asecondary vaccination, the DNA platform can focus on tuning the previously induced immune response against EBOV-GP and not against anti-viral immunity that may have developed from the prime (21). Unlike the B10Br data, the best boost in Balb/c mice appears to be either Ad or VSV. This is accordance to previously published data where viral boosts have stimulated stronger humoral immune responses (67) .

The nAbs assay did not yield any noticeable differences between homologous or heterologous prime-boost regimens for all platforms in both B10Br and Balb/c mouse models. Additionally, when grouped according to the prime administered, there appears to be no distinguishablechange between the secondary vaccination regimes. The nAb assay in general has been shown to be a poor indicator of survival against EBOV (35), therefore it is not surprising that the inconclusive results from the nAbs assay were obtained.

19.3.4 – Survival of Regimens in Both Mouse Models against MA-EBOV

Regardless of mouse model, there was an increase in survival against MA-EBOV when the mice received a homologous or heterologous immunization in comparison to thosethat received a single administration. The only exception was AAV/Ad, which decreased efficacy compared to a single vaccination with AAV EBOV-GP. Elevation in survival for all prime-boost regimens is observed in many disease models (65,67), due to the increase in immunity following a boost vaccination. Speculations based on VSV as a prime or boost iscomplicated with the initial percentage of survival for a single vaccinationconveying 80% protection. As a result, a 20% increase in survival was the highest increase that can be observed.

Irrespective of VSV, the most substantial elevation in survival was a DNA boost following all of the prime vaccinations in both B10Br and Balb/c mice. The greatest increase in efficacy for all prime-boost regimens involved Ad as a prime, with an increase of 100% when boosted with either DNA or VSV for B10Br mice. Similar results were found with Balb/c mice, where an Ad/DNA combination conferred85.7% protection. Surprisingly, the DNA platform served as the second best prime vaccination candidate for both B10Br and Balb/c mice, contrary to several studies indicating that DNA is best utilized as a prime in comparison to a boost (25, 30, 81). The use ofAd as a prime has been exploredusing several Ad serotypes with lower prevalence. When a boostwas administeredwith a different Ad serotype than the prime, a more robust immune response was elicited (80).

Efficacy of homologous vs. heterologous prime-boost vaccinations based on the prime administered revealed that the most successful combinations involve homologous vaccination regimens (DNA/Ad, Ad/DNA) compared to heterologous combinations

(AAV/DNA). Since the individual platforms in the homologous vaccination regimens both induce strong cell-mediated responses, it suggests that T cells may play an integral role in protection against MA-EBOV (13,14).

In general, due to the constraints applied from the animal rule and logistics of the experiment, it is difficult to generate statistically significant data on the amount of mice used in these studies. An increase in the number of mice utilized would generate more conclusive results and an overall better illustration of which prime-boost combination correlated to greater efficacy.

19.3.5 – Correlation of Protection against MA-EBOV

Based on the prime vaccination administered, DNA/Ad, AAV/DNA, and Ad/DNA, demonstrated the highest efficacy against MA-EBOV. Due to the high dose of VSV administered as a prime, as well as survival of all prime-boost vaccinations, it is difficult to correlate increase survival with immune responses elicited based on the VSV platform.

The DNA/Ad combination which achieved the highest efficacy correlated with the largest increase of IFNγ secreting cells, while the lowest survival combination (DNA/AAV) correlated to the lowest concentration of activated T cells.

However, the opposite phenomenon is observed for those that utilized AAV as a prime. In this scenario, the AAV/Ad vaccination had a detrimental effect on survival,although the combination achieved the highest amount of T cell activation. When Ad was used as prime, no distinction could be drawn based on survival and IFNγ secreting cells.

In B10Br and Balb/c there appears to be conflicting results with IgG titers based on the prime administered. Although, it can be concluded that the nAb assays could not be correlated with survival since no statistical significance was observed between groups administered with the same prime. The highest IgG titer for groups that received a DNA prime in B10Br mice was DNA/AAV. However this prime-boost regimen received the lowest IgG titer in Balb/c mice. The opposite circumstance was seen in Balb/c mice, where the DNA/Ad group obtained the highest IgG titer, however, there was no discernible difference with the other DNA prime groups in B10Br mice. In contrast, AAV prime vaccination regimens appeared to correlate with IgG titers, where the highest IgG titer, AAV/DNA, corresponded to one of the greatest increase in efficacy in B10Br mice and Balb/c. Groups that were administered an Ad prime had a correlation between survival and IgG titers for both B10Br mice and Balb/c mice.

In B10Br mice, the highest level of IFNγ secretion by $CD8^+$ T cells was associated with the heterologous Ad/DNA combination which correlated to the highest increase in efficacy. It has been demonstrated that the use of an Ad vaccine against EBOV in NHPs has been correlated to cytotoxic $CD8^+$ Tcells, although this has later been challenged by a study showing that total IgG correlated more precisely with survival against a lethal exposure to EBOV(35). Therefore, potential reasoning behind increased survival can be attributed to the Ad/DNA strategysufficiently stimulating the cellular immune responseto provide protection against MA-EBOV.

The highest IgG titers in this study with B10Br mice were achieved by the Ad/DNA, VSV/Ad and Ad/VSV prime-boost combinations, correlating to full protection against

MA-EBOV. This is in compliance to previously established research stating that IgG Ab titers have a strong correlation to survival against EBOV infection (15,35).

With regards to the Balb/c mouse model, the highest IgG titers were attributed to the VSV/Ad heterologous combination which resulted in full efficacy against MA-EBOV. Since the VSV vaccine is replication competent, the high IgG Ab titers can be accredited to the persistent exposure of the immune system to the EBOV-GP protein, resulting in robust stimulation of the humoral response.

However, the Ad/DNA combination had lower IgG Ab production and obtained one of the greatest increases in efficacy in Balb/c mice. Through cell-mediated immunity was not evaluated in this mouse model, it could be speculated that a sufficient increase in cell-mediated immunity by the Ad/DNA combination resulted in the increase in efficacy since the individual platforms are known to be strong stimulators of the cellular immune response.

For both B10Br mice and Balb/c mice, the only combination that resulted in a decrease in efficacy was the AAV/Ad combination. The AAV/Ad combination in B10Br mice was one of the lower inducers of IFNγ secretion in conjunction with negligible IgG titers. This is in compliance with the Balb/c data where the AAV/Ad group also had an insignificant increase in IgG titers except for one outlier. This anomaly emphasizes that the selection of platform and the order of delivery play an important role in prime-boost strategies, a well-renowned example of this being the respiratory syncytial virus (RSV) where a formalin-inactivated RSV vaccine used in children resulted in a detrimental immune response that produced pathogenic antibodies (81).

Other factors such as selection of mouse model, choice of vaccine platform, and route of administration may have played an integral role in influencing the immune response observed and therefore must be taken into account. With an aggressive virus such as EBOV, both cell-mediated and humoral immunity have importantfunctions in providing protection and play important roles in establishing a sustainable immune network.

20.0 – Conclusion and Future Directions

Testing heterologous vs. homologous vaccinations based on the predominant immune response elicited by each platform is a novel approach that is explored in this study. Initial studies performed were to confirm previous research characterizing DNA and Ad (41,52) as prominent inducers of cell-mediated immunity, as well as AAV and VSV (30,41) as strong elicitors of humoral immunity. As demonstrated by the increase in IFNγ via ELISPOT, DNA and Ad as prime vaccinations are strong inducers of cell-mediated immunity. Humoral responses were prominent in both AAV and VSV platforms based on IgG titres, confirming what is found in the literature.

Individual platform prime-boost vaccinations using these four vectors were immunologically characterized to determine whether the use of acombination strategy would be advantageous. In general, there was an increase in both cell-mediated and humoral immunity, though only a marginal increase in survival was observed for all viral platforms. This phenomenon can be attributed to anti-vector immune responses which affect the efficacy of the boost and lead to poor antigen presentation (64). The DNA platform demonstrated higher efficacy when used in a prime-boost regimen. (23,62).

The experimental findings suggest that heterologous prime-boost vaccination regimens induce more robust cell-mediated and humoral immune responses in compared to homologous regimens. This is based on elevated IFNγ secreting T cells as well as IgG titers found in the heterologous vaccination strategies. However, the homologous prime-boost strategies were superior in increasing efficacy against MA-EBOV. There was a

general increase in both cell-mediated and humoral immune responses for all homologous and heterologous prime-boost strategies, though conflicting results did not allow selection of a correlate of protection against MA-EBOV. However, this discrepancy can be associated with several factors, such as vaccine administration route and choice of mouse model. Regardless of homologous or heterologous prime-boost vaccinations, all were generally superior to vaccination regimens involving only one immunization, as shown in many disease models in the literature (65,78). Selective characterization of prime-boost regimens that elicit detrimental or synergistic immunity against EBOV may provide further insight into the mechanisms that aid in protection.

Additional studies could be implemented in determining whether heterologous cell-mediated or humoral responses induce protective immunity in the MA-EBOV model. One such experiment is to adoptively transfer B cells and T cells from individually boosted, as well as selected homologous and heterologous vaccination regimens into SCID mice, which are unable to generate a proper adaptive immune response. Once vaccinated, these mice could be challenged with MA-EBOV and the resulting survival studies could determine which immune response is crucial for which platforms, and whether this is translated to homologous or heterologous vaccination strategies.Further studies could also be conducted in guinea pigs and the gold standard NHP model to determine whether the phenomenon observed in micewill translateto higher animal models. Characterization of the immune responses from this study can further aid in selection of prime-boost regimens for other disease models where cell-mediated or humoral immunity has been associated with efficacy. However, immune parameters

correlating with protection against Ebola virus in rodents and nonhuman primates was recently published pointing at IgG levels as the most reliable predictor of survival (36).

In conclusion, the characterization of the immune responses elicited by heterologous vaccinations against MA-EBOV in relation to survivalallows for a more comprehensive understanding of how vaccinations can work synergisticallycreating arobust and long-lasting protective immune response. Heterologous prime/boost vaccination strategies hold promises for future development of immunisation regiments, notably to protect against challenging infectious agents.

21.0 – Bibliography

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